Development of a reliable diagnostic test for canine hypothyroidism: Differentiating between dogs with primary hypothyroidism and dogs with non-thyroidal illness based on plasma concentrations of growth hormone and thyroid stimulating hormone (TSH) with a TSH-releasing hormone stimulation test

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Abstract

Introduction

Plasma concentrations of total thyroxin (TT4) and total triiodothyronine (TT3) are below the reference values in most dogs with hypothyroidism, but these values can also be low in dogs with normal thyroid function due to drugs or illness, which is called non-thyroidal illness (NTI). To diagnose hypothyroidism, an additional plasma thyroid stimulating hormone (TSH) concentration measurement is necessary, because a clearly elevated plasma TSH concentration is diagnostic for hypothyroidism. However, 30% of dogs with hypothyroidism have a plasma TSH concentration within the reference range and can therefore not be distinguished from dogs with NTI. Other methods previously investigated to differentiate between hypothyroid dogs and dogs with NTI are lacking sufficient specificity, are expensive, not widely available or too invasive. There is a need for a widely available, inexpensive and accurate test that can differentiate between hypothyroid dogs and dogs with NTI. Recent findings suggest that the plasma growth hormone (GH) concentration may be used in the differentiation between dogs with hypothyroidism and NTI.

Aim

The aim of this study is to evaluate whether plasma concentrations of GH and TSH following a TRH stimulation test can differentiate between dogs with NTI and dogs with hypothyroidism that have plasma TSH concentrations within the reference range, using thyroid scintigraphy as the gold standard.

Methods

Dogs with clinical signs of hypothyroidism, a plasma TT4 concentration below the reference interval (19-46 nmol/l), and a plasma TSH concentration within the reference interval (<0.60 µg/l) were eligible to participate in the study. Thyroid scintigraphy was performed to classify dogs as having hypothyroidism or NTI. All dogs underwent a TRH stimulation test in which plasma concentrations of TSH and GH were measured twice before administration of TRH (t=-15 and t=0) and 30 (t=30) and 45 (t=45) minutes after TRH was administered intravenously in a dose of 10 µg/kg. The plasma TSH and GH concentrations before and after TRH stimulation are presented as: 1) the absolute values at each time point, 2) the GH/TSH ratio at each time point and 3) the increase or decrease after the administration of TRH compared to the basal concentrations (both as absolute values and as percentages). Differences in each of these outcomes between hypothyroid dogs and NTI dogs were tested using the appropriate statistical tests. If outcome values did not overlap between the two groups, cut-off values were calculated by receiver operating characteristics (ROC) analysis.

Results

For the final analysis, 21 dogs were included of which 11 dogs were classified as hypothyroid and 10 dogs as NTI. There were no differences in baseline characteristics between the groups except for gender; 91% was male in the hypothyroid group versus only 40% in the NTI group (p=0.013). The plasma TSH concentration did not change in the hypothyroid dogs after administration of TRH (p=0.68), whereas it significantly increased in the NTI dogs. The mean plasma TSH concentration in NTI dogs was 0.19 µg/l (±0.18) at baseline, 0.72 µg/l (±0.43) at t=30 (p<0.001) and 0.61 µg/l (±0.39) at t=45 (p=0.001). The absolute plasma TSH concentration at t=30 (p=0.034), the increment in TSH concentration at t=30 (p<0.001) and t=45 (p<0.001) and the percentage increase in TSH concentration at t=30 (p<0.001) and t=45 (p<0.001) were all significantly higher in the NTI dogs than in the hypothyroid dogs. In contrast, the plasma GH concentration increased significantly in the hypothyroid dogs after TRH administration, whereas it did not change in the NTI dogs (p=0.13). In the hypothyroid dogs, the median plasma GH

concentration increased from 3.2 µg/l (range 2.0 – 12.5) at baseline to 6.1 µg/l (range 2.0 – 54.4, p=0.009) at t=30 and to 6.6 µg/l (range 2.4 – 100, p=0.006) at t=45. The baseline plasma GH concentrations ($p<0.001$), the absolute plasma GH concentration at t=30 ($p<0.001$) and t=45 ($p<0.001$), the increment in plasma GH concentration at t=30 (p=0.008) and t=45 (p=0.001) and the percentage increase in GH concentration at t=45 (p=0.002) were all significantly higher in the hypothyroid dogs than in the NTI dogs. The GH/TSH ratio at t=30 (p=0.003) and t=45 (p=0.013), the increment in GH/TSH at t=30 (p=0.002) and t=45 ($p<0.001$) and the percentage increase in GH/TSH at t=30 ($p<0.001$) and t=45 ($p<0.001$) were significantly higher in the hypothyroid dogs than in the NTI dogs. There was no overlap between hypothyroid dogs and NTI dogs for the percentage increase of TSH at t=45, the absolute concentration of GH at t=30 and t=45, the GH/TSH ratio at t=45 and the percentage increase of the GH/TSH ratio at t=45. The values in which there was no overlap thus resulted in an area under the ROC curve of 1.0, corresponding to a 100% sensitivity and 100% specificity to reliably identify dogs with hypothyroidism in which the plasma TSH concentration is not clearly increased and distinguish these dogs from dogs with NTI.

Conclusion

Circulating concentrations of TSH and GH after a TRH stimulation test can differentiate between hypothyroid dogs and NTI dogs that have clinical signs of hypothyroidism, a low basal TT4 concentration and a basal TSH concentration within the reference interval. This is a promising test which might be used in primary veterinary practice as it seems to be a reliable, inexpensive and widely available test.

Introduction

Aetiology of hypothyroidism

Canine hypothyroidism is one of the most common endocrine disorders in dogs. Hypothyroidism may be acquired or congenital, the latter being rare. Two forms of acquired hypothyroidism exist: primary and central. Central hypothyroidism only accounts for <5% of the cases of acquired hypothyroidism, leaving primary hypothyroidism to be the most common form. Primary hypothyroidism results from a progressive destruction of the thyroid glands, in most cases either due to lymphocytic thyroiditis, which is autoimmune mediated, or idiopathic atrophy [1-7].

Signalment

Hypothyroidism is mainly a condition of young-adult and middle aged dogs [3,7]. Whether certain breeds are more frequently affected by hypothyroidism than others remains inconclusive. Some studies report a higher incidence in Dobermans, Golden Retrievers, Dachshunds, Shetland Sheepdogs, Irish Setters, Pomeranians, Miniature Schnauzers, Cocker Spaniels and Airedales [8,9], while others fail to demonstrate a difference between breeds [5]. Conflicting results exist about whether or not neutered status is a risk factor for hypothyroidism [5,8]. Sex is probably not associated with hypothyroidism [3,5].

Clinical signs of hypothyroidism

Clinical signs can be vague, non-specific and subtle in onset. Symptoms result from the effects of thyroid hormone deficiency on general metabolism as well as on specific organs [10]. There may be concurrent effects of growth hormone excess like maxillary prognathia with widening of the interdental spaces [3,11]. Most dogs with hypothyroidism present with a combination of signs related to a decrease in metabolic rate and dermatologic changes. Examples of effects on general metabolism include obesity or weight gain, lethargy, little willingness to exercise and cold intolerance. There may be different and variable dermatologic abnormalities such as alopecia, a poor quality coat, excessive shedding, hyperpigmentation, a dry scaly skin, seborrhea, thickening of the skin (myxoedema) and pyoderma. Myxoedema, caused by accumulation of hyaluronic acid, occurs particularly in the eyelids, cheeks and forehead and leads to a typical “tragic” facial expression as shown in Figure 1. Less common clinical manifestations include neurological abnormalities, effects on the cardiovascular system, effects on the female reproductive system, ophthalmological disorders and hematologic abnormalities such as a mild anemia [3,5-8,10]. The duration for the onset of apparent clinical signs varies considerably. It has been reported that lethargy may be noticed within a few months’ time, but skin changes may take up to a year [3,12].

FIGURE 1. A 4-YEAR-OLD MALE GOLDEN RETRIEVER WITH PRIMARY HYPOTHYROIDISM. LEFT: THE DOG HAS A LETHARGIC APPEARANCE, A TRAGIC FACIAL EXPRESSION (DUE TO MYXOEDEMA), HYPERPIGMENTATION AND ALOPECIA OF THE DORSAL ASPECT OF THE NOSE. MIDDLE: ALOPECIA AND A THIN COAT OF THE TAIL. RIGHT: A STIFF GAIT HAS CAUSED ABNORMAL WEARING OF THE NAILS.

Non-thyroidal illness

Drugs and illness can alter thyroid hormone secretion, transfer, distribution and metabolism. Glucocorticoids, phenobarbital, sulphonamides and nonsteroidal anti-inflammatory drugs have been shown to influence thyroid function. The terms non-thyroidal illness (NTI) and sick euthyroid syndrome (SES) are used to describe dogs with a normal thyroid function, but lowered plasma levels of thyroid hormones due to systemic disease or administration of drugs. Dogs with NTI can have the same clinical signs as dogs with hypothyroidism [13,14].

Hypothalamic-pituitary-thyroid axis

Thyroid stimulating hormone (TSH, thyrotropin) is a pituitary hormone and plays an important role in the regulation of the thyroid function [3]. TSH is released in a pulsatile manner, but the fluctuations in plasma TSH are small, especially in euthyroid dogs [15]. As shown in Figure 2 TSH secretion itself is regulated by the hypothalamic TSH-releasing hormone (TRH), somatostatin (SS) and the thyroid hormones [3,15].

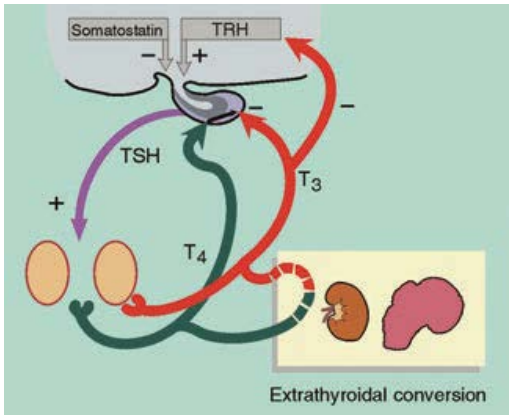

FIGURE 2. THE HYPOTHALAMIC-PITUITARY-THYROID AXIS. HYPOTHALAMIC TRH REACHES THE THYROTROPHIC CELLS IN THE ANTERIOR LOBE OF THE PITUITARY VIA THE LOCAL PORTAL VESSELS AND ENHANCES TSH SECRETION. THYROID HORMONES, PARTICULARLY SYSTEMICALLY AND LOCALLY PRODUCED T3, EXERT NEGATIVE FEEDBACK AT PITUITARY AND HYPOTHALAMIC LEVELS. ADAPTED FROM: *CLINICAL ENDOCRINOLOGY OF DOGS AND CATS: AN ILLUSTRATED TEXT. 2010, RIJNBERK*

Growth hormone

Growth hormone (GH) is produced in the anterior lobe of the pituitary and is secreted in a pulsatile fashion [16]. As can be seen in Figure 3, the release of GH is stimulated by GH-releasing hormone (GHRH), whereas GH is inhibited by SS, both originating from the hypothalamus. Furthermore, GH secretion is modulated by insulin-like growth factor 1 (IGF-1), ghrelin and GH itself. [3,16,17] .

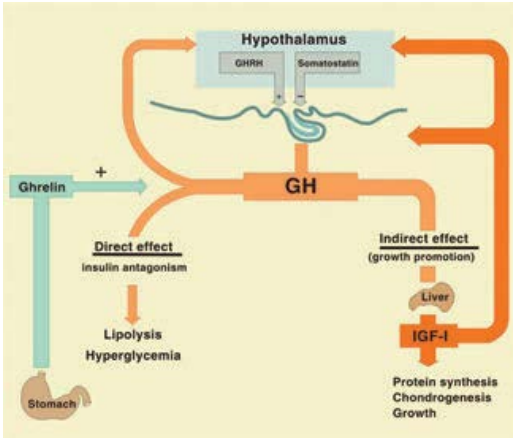

FIGURE 3. THE SECRETION OF GH IS UNDER INHIBITORY (SOMATOSTATIN) AND STIMULATORY (GHRH) HYPOTHALAMIC CONTROL AND IS ALSO MODULATED BY A LONG-LOOP FEEDBACK CONTROL BY IGF-I, A PEPTIDE PRIMARILY FORMED IN THE LIVER UNDER THE INFLUENCE OF GH. GH ITSELF EXERTS A SHORT-LOOP NEGATIVE FEEDBACK BY ACTIVATING SOMATOSTATIN NEURONS. THE GASTRIC PEPTIDE GHRELIN IS THE NATURAL LIGAND FOR THE GH SECRETAGOGUE RECEPTOR THAT STIMULATES GH SECRETION AT THE PITUITARY LEVEL. GH HAS DIRECT CATABOLIC EFFECTS AND INDIRECT OR SLOW ANABOLIC ACTIONS. ADAPTED FROM: *CLINICAL ENDOCRINOLOGY OF DOGS AND CATS: AN ILLUSTRATED TEXT. 2010, RIJNBERK & KOOISTRA* [3].

Diagnosis of hypothyroidism

The diagnosis of primary hypothyroidism poses a challenge due to the lack of discriminative ability of current tests between dogs with primary hypothyroidism and dogs with NTI. In most dogs with primary hypothyroidism, total triiodothyronine (TT3) and total thyroxine (TT4) concentrations are below the reference range. However, many dogs with NTI can also have low TT4 and TT3 concentrations [7,18,19]. The serum free thyroxine (fT4) concentration is more specific than the TT4 concentration (if determined with equilibrium dialysis), but may also be low in dogs with NTI as well as in some healthy dogs. The sole finding of a low TT4 or fT4 concentration is therefore of limited diagnostic value [7,13,20]. The present diagnosis of primary hypothyroidism in clinical practice is therefore often based on a low plasma TT4 concentration in combination with an elevated plasma thyroid stimulating hormone (TSH) concentration. However, 30% of dogs with primary hypothyroidism have a TSH concentration within the reference range and thus cannot be distinguished from dogs with NTI [21,22].

Methods that have been proposed to differentiate between primary hypothyroidism and NTI are a TSH stimulation test and a TSH-releasing hormone (TRH) stimulation test. The first test is relatively expensive as bovine TSH is no longer commercially available and only recombinant human TSH is available nowadays, but it is a reliable method. In contrast, the TRH stimulation test with subsequent measurement of plasma TT4 concentration lacks specificity [7,23].

Moreover, radiological investigations have been suggested to diagnose primary hypothyroidism. Ultrasonographic examination of the thyroid gland has been described, but does not provide sufficient reliable information for a diagnosis [7]. Likewise, computed tomography (CT) and magnetic resonance imaging (MRI) are improbable to ever play a significant role in the assessment of primary hypothyroidism due to their expensive nature. Interestingly, quantitative measurement of radioactive pertechnetate ($^{99m}TcO_4^-$) uptake in the thyroid glands in dogs has a very high discriminatory power with regard to the differentiation between dogs with primary hypothyroidism and those with NTI, and has been suggested as the new "gold standard". However, scintigraphy is not a primary veterinary practice test and can only be performed in a few specialized institutions [7,13,24].

Other diagnostic tests include biopsy and histological examination of the thyroid gland. Even though this is a sensitive method to identify thyroid disease, it is too invasive as a routine diagnostic method [2]. Lastly, antibodies against thyroglobulin have been investigated. In some cases they can help to differentiate between dogs with primary hypothyroidism and NTI, but unfortunately not in all cases and hence an unsuitable routine method [25].

Preliminary studies

Considering the current diagnostic tools to differentiate between primary hypothyroidism and NTI, it can be summarised that most of the above mentioned biochemical tests lack specificity and more accurate tests are relatively expensive, not widely available, or too invasive. Interestingly, previous research has shown that the circulating TSH concentration did not increase after TRH administration in dogs with prolonged primary hypothyroidism [11]. In contrast, the TSH concentration in euthyroid dogs did increase after TRH administration [26]. Another finding in the search for a more accurate and widely available test is that primary hypothyroidism in dogs has been associated with an increased release of growth hormone (GH) [11,26]. Basal plasma GH concentration was higher in dogs with primary hypothyroidism than in euthyroid dogs [11,16,27]. Moreover, GH concentration increased significantly after administration of TRH in hypothyroid dogs, whereas it did not increase in euthyroid dogs [26]. Measurements of circulating TSH and GH concentrations in a TRH stimulation test are therefore promising tests to differentiate between dogs with primary hypothyroidism and dogs with NTI. However, only limited data is available on these measurements in euthyroid, hypothyroid and NTI dogs. No data is available on hypothyroid dogs with

TSH concentrations within the reference range. It is therefore unknown if plasma TSH and GH concentrations after a TRH test can differentiate between hypothyroid and NTI dogs who have low T4 values and basal TSH concentration within the reference range.

Aim of the study

The aim of this study is to evaluate whether plasma concentrations of GH and TSH in a TRH stimulation test can differentiate between dogs with NTI and dogs with hypothyroidism that have a plasma TSH concentration within the reference range, using thyroid scintigraphy as the gold standard.

Hypothesis

It is hypothesised that dogs with primary hypothyroidism have higher levels of plasma GH and lower levels of plasma TSH following a TRH stimulation test than dogs with NTI.

Material and methods

Dogs

All cases with plasma TT4 and plasma TSH concentration measurements conducted at Utrecht University between November 2014 and April 2016 were evaluated. The veterinarians of dogs with a plasma TT4 concentration below the reference interval (19-46 nmol/l) and a plasma TSH concentration within the reference interval (<0.60 µg/l) were contacted by telephone. The clinicians were interviewed about the clinical signs and concurrent or previous drug therapy of the dogs. If the dog had clinical signs consistent with hypothyroidism and did not receive any medication which could influence the test results, the owner was invited to participate in the study. All owners from the dogs that joined the study signed an informed consent.

Tests and sample collection

Thyroid scintigraphy

Radioactive pertechnetate ($^{99m}TcO_4^-$) was administered through an IV catheter in the cephalic vein. After 45 minutes a diagnostic thyroid scintigraphy was obtained with the Integrated ORBITER Gamma Camera System with Open Icon Workstation, equipped with a high resolution parallel-hole collimator (Siemens Medical Systems). Based on $^{99m}TcO_4^-$ uptake in the thyroid glands, the dogs were diagnosed with either hypothyroidism (no or very limited uptake, Figure 4) or NTI (normal uptake, Figure 5).

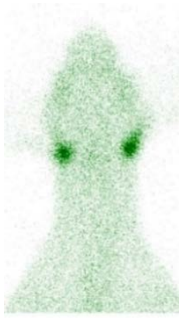

FIGURE 4. A TYPICAL SCINTISCAN OF A HYPOTHYROID DOG. THERE IS NO $^{99M}TcO_4^-$ UPTAKE IN THE THYROID GLANDS. NOTE THE UPTAKE IN THE SALIVARY GLANDS.

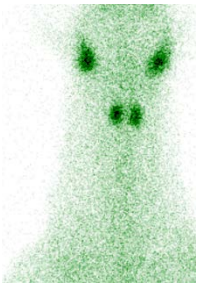

FIGURE 5. A TYPICAL SCINTISCAN OF A DOG WITH NTI. THERE IS BOTH $^{99M}TcO_4^-$UPTAKE IN THE THYROID GLANDS AND SALIVARY GLANDS.

TRH stimulation test

Blood samples for TSH and GH measurements were collected 15 minutes before (t=-15), just before (t=0) and 30 (t=30) and 45 (t=45) minutes after intravenous (cephalic vein) administration of 10 µg/kg body weight TRH (TRH, Ferring Arzneimittel GmbH, Protirelin 0.2 mg, Kiel, Germany). The samples were collected by jugular venipuncture and immediately transferred into chilled heparin-coated tubes for plasma TSH concentration analysis and chilled EDTA-coated tubes for plasma GH analysis. Subsequently the samples were centrifuged at 4°C and plasma samples were stored at -70°C until analysis.

Hormone measurements

Plasma TSH concentrations were determined by a homologous solid-phase, two-site chemiluminescent enzyme immunometric assay (Immulite 2000 canine TSH®, Siemens), in accordance with the instruction of the manufacturer. The intra-assay coefficients of variation were 5.0 % and 4.0 % at TSH

concentrations of 0.20 µg/l and 0.50 µg/l, respectively. The inter-assay coefficient of variation was 6.3 % at a TSH concentration of 0.16 µg/l [15,27]. The detection limit of the TSH assay was 0.03 µg/l.
Plasma GH concentrations were measured by a homologous radioimmunoassay as described previously by Eigenmann et al. [27,28]. The intra and inter-assay coefficients of variation were 3.8 and 7.2%, respectively, and the sensitivity was 0.3 µg/l [27,28].

Power Calculation

A power calculation was performed with a coefficient of variation of 20%, a power of 80%, and an alpha of 0.05. This showed that 10 dogs were needed in each group to obtain a significant difference of at least 30% in plasma concentrations between the groups. The minimum increase of 30% was chosen based on previous studies.

Data analysis

The normality of the data was assessed with the Kolmogorov-Smirnov test (Table 1, Appendix). Differences in baseline characteristics between the two groups were tested using an independent samples T-test for normally distributed continuous data (age and weight), while dichotomous variables (such as gender and neutering status) were tested with a chi-squared test. Baseline plasma TSH and GH concentrations were determined by the average of the samples collected at t=-15 and t= 0. The plasma TSH and GH concentrations before and after the TRH stimulation test are presented as: 1) the absolute values at each time point, 2) the GH/TSH ratio at each time point and 3) the increase or decrease after administration of TRH compared to baseline values (both as absolute values and percentages). Normally distributed values are presented as the mean ± standard deviation (SD) and non-normally distributed values are presented as the median and range. Differences in above mentioned outcomes between the hypothyroid group and the NTI group were tested with an independent samples T-test when the values were normally distributed. The Mann-Whitney U test was used for these comparisons when the data were non-normally distributed. Differences between the various time points (baseline, t=30 and t=45) within the groups were analysed using an one-way ANOVA test when normally distributed and with a Kruskal Wallis test when non-normally distributed. When significant differences were found, further analyses were performed using a paired T-test when normally distributed and with a Wilcoxon signed rank test when non-normally distributed to determine which groups differed significantly. Finally, if outcome values did not overlap between the two groups, cut-off values were calculated by receiver operating characteristics (ROC) analysis, with hypothyroid dogs as the reference group. Only cut-off values with 100% sensitivity and 100% specificity are given. Differences were considered statistically significant if the p-value was <0.05. The analyses were performed in SPSS version 20.0.

Results

Study population

A total of 23 dogs were included for this study. Based on thyroid scintigraphy, 11 dogs were proven hypothyroid, 11 dogs were diagnosed with NTI and 1 dog had an unclear scintigraphy result. This dog showed a decreased uptake of $^{99m}TcO_4^-$, but the thyroid glands were still clearly visible and no conclusive diagnosis could be made from the scintigraphy. Therefore, the dog was excluded from the analysis. Also a dog that was originally classified as NTI was excluded from the study, because five months after the study the dog still had clinical symptoms (lethargic, weight gain, stiff gait) consistent with hypothyroidism and treatment with l-thyroxine had a very positive effect, suggesting that this dog was wrongly classified. Therefore in total 11 dogs were classified as dogs with hypothyroidism and 10 as dogs with NTI.

Consistent with the inclusion criteria, all dogs had clinical symptoms of hypothyroidism. Changes in coat or skin, such as alopecia, hyperpigmentation, excessive shedding and seborrhea (n=16); lethargy (little willingness to exercise and sleeping more during the day) (n=18); and weight gain (n=14) were the most common presenting signs. In addition, locomotion problems (lameness, stiff gait) (n=9); myxoedema (tragic facial expression) (n=7); excessive snoring (n=5) and preference for warm places (n=3) were noticed. Some dogs had concurrent clinical symptoms which are not typical for hypothyroidism, such as coughing (n=1), diarrhoea (n=1), polyuria and polydipsia (n=2) and pollakiuria (n=1). At presentation the time from onset of the symptoms ranged from 3 weeks to 2 years (Table 2, Appendix).

Baseline characteristics

There was a wide variety of breeds. The mean ± SD of the baseline characteristics of the included dogs were as follows: age 6.9 ± 2.3 years, body weight 33.3 ±14.8 kg, 67% male and 33% female, 48% not neutered and 52% neutered. For age, body weight and neutering status there were no differences between the hypothyroid dogs and NTI dogs. The age was respectively 6.7 years versus 7.1 years (p=0.72), body weight 34.8 kg versus 31.7 kg (p=0.64) and percentage of dogs neutered 46% versus 60% (p=0.51). There were more male dogs in the hypothyroid group (91%) than in the NTI group (40%) (p=0.013). The mean plasma TSH concentration of the included dogs was 0.31 ± 0.16 µg/l. This value was significantly higher (p=0.002) in the hypothyroid dogs (0.40 ± 0.13 µg/l) than in the NTI dogs (0.21 ± 0.12 µg/l). All plasma TT4 concentrations were ≤17 µg/l, but no mean or median values or comparison between the two groups could be made, as about half of the values were not provided as a continuous variable but were below the detection limit of the assay (< 2 µg/l or < 9 µg/l).

Plasma TSH concentrations in the TRH stimulation test

The mean (±SD) baseline plasma TSH concentrations in the TRH stimulation test of all dogs was 0.25 ± 0.17 µg/l. There was no statistically significant difference (p=0.13) in baseline plasma TSH concentrations between the hypothyroid group (0.30 ± 0.14 µg/l) and the NTI group (0.19 ± 0.18 µg/l).

The mean plasma TSH concentration in the hypothyroid group was not statistically different (p=0.68) between baseline, t=30 and t=45. The mean plasma TSH concentration at t=30 was 0.37 ± 0.17 µg/l and at t=45 0.36 ± 0.21 µg/l.

In the NTI group, the mean plasma TSH concentrations were significantly different (p=0.008) between baseline, t=30 and t=45. The mean (±SD) plasma TSH concentrations were 0.72 µg/l (± 0.43) at t=30 and 0.61 µg/l (± 0.39) at t=45, which were both significantly higher than the mean baseline value before TRH was administrated (0.19 ± 0.18 µg/l), p<0.001 and p=0.001, respectively. There was also a significant difference (p=0.007) between the t=30 and t=45 values.

The mean plasma TSH concentration at t=30 was significantly lower in the hypothyroid group than in the NTI group (p=0.034), but no difference between the groups was found at t=45 (p=0.095) (Figure 6).

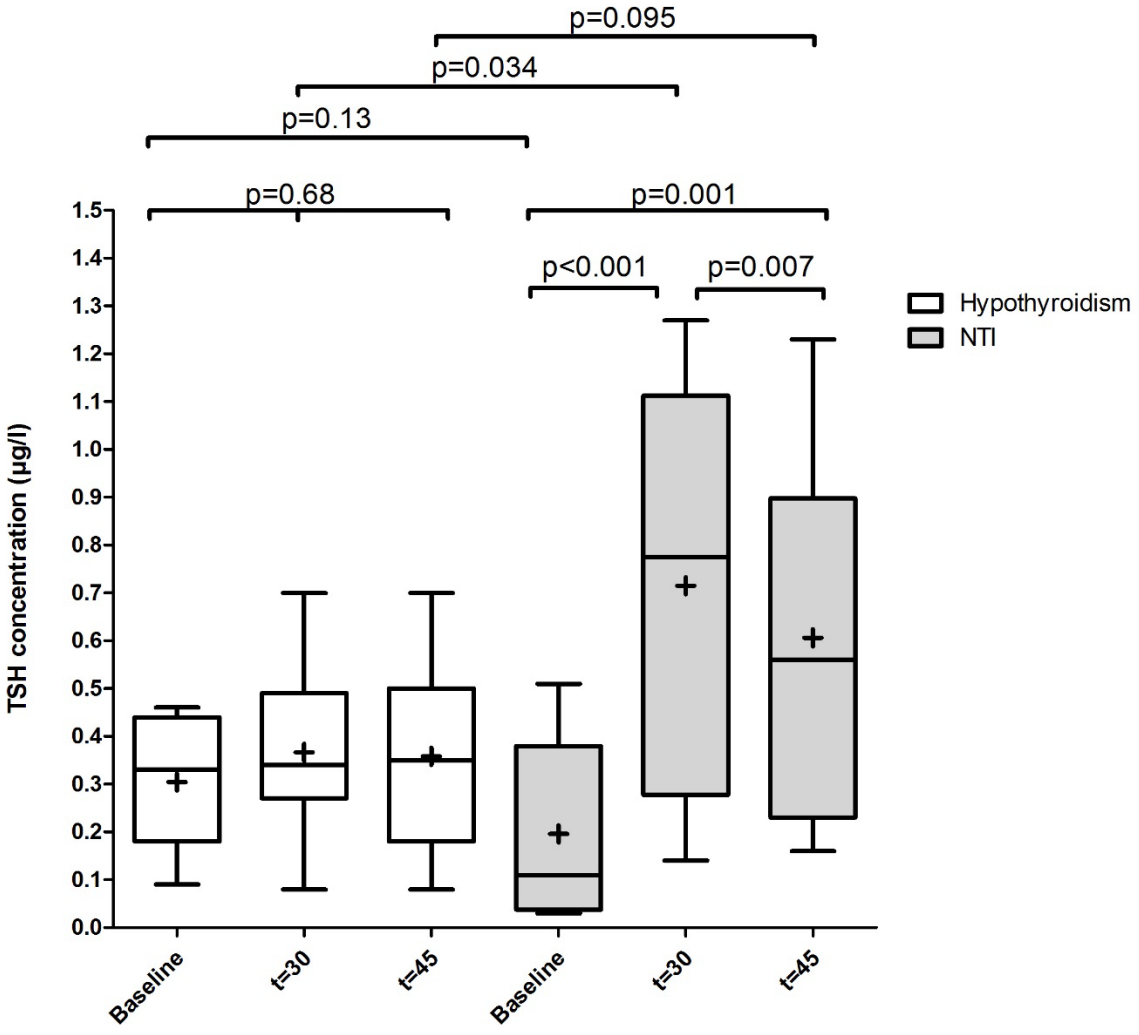

FIGURE 6. A BOXPLOT SHOWING THE PLASMA TSH CONCENTRATIONS AT BASELINE, T=30 AND T=45 FOR BOTH GROUPS, THE WHISKERS INDICATE THE MINIMUM AND MAXIMUM VALUES AND THE '+'SIGN INDICATES THE MEAN. FOR THE HYPOTHYROID GROUP ONLY ONE P-VALUE IS PROVIDED AS THE ONE-WAY ANOVA TEST DID NOT SHOW A DIFFERENCE BETWEEN THE THREE TIME POINTS (P=0.677) AND THEREFORE NO FURTHER ANALYSIS BETWEEN THE TIME POINTS WAS PERFORMED.

The median increment in plasma TSH concentration at 30 and 45 minutes after TRH was administered was significantly different between the hypothyroid group and NTI group. The plasma TSH concentration at 30 minutes rose with only 0.03 µg/l (range -0.01 – 0.24) in the hypothyroid group as compared to 0.49 µg/l (range 0.11 – 1.02) in the NTI group (p<0.001). The same trend was observed at 45 minutes after TRH administration, with a respective TSH increment of 0.01 µg/l (range -0.02 – 0.24) vs 0.33 µg/l (range 0.13 – 0.87) (p<0.001) (Figure 7).

The median plasma TSH concentration increase expressed as percentages was significantly different between the hypothyroid dogs and NTI dogs at t=30 (p<0.001) and t=45 (p<0.001) (Figure 8). In

hypothyroid dogs the median percentage increase between baseline values and t=30 was 10% (range -6 – 161%) and between baseline values and t=45 4% (range -6 – 52%). In NTI dogs the plasma TSH concentration increased with 351% (range 96 – 1457%) at t=30 and 311% (range 62 – 1167%) at t=45.

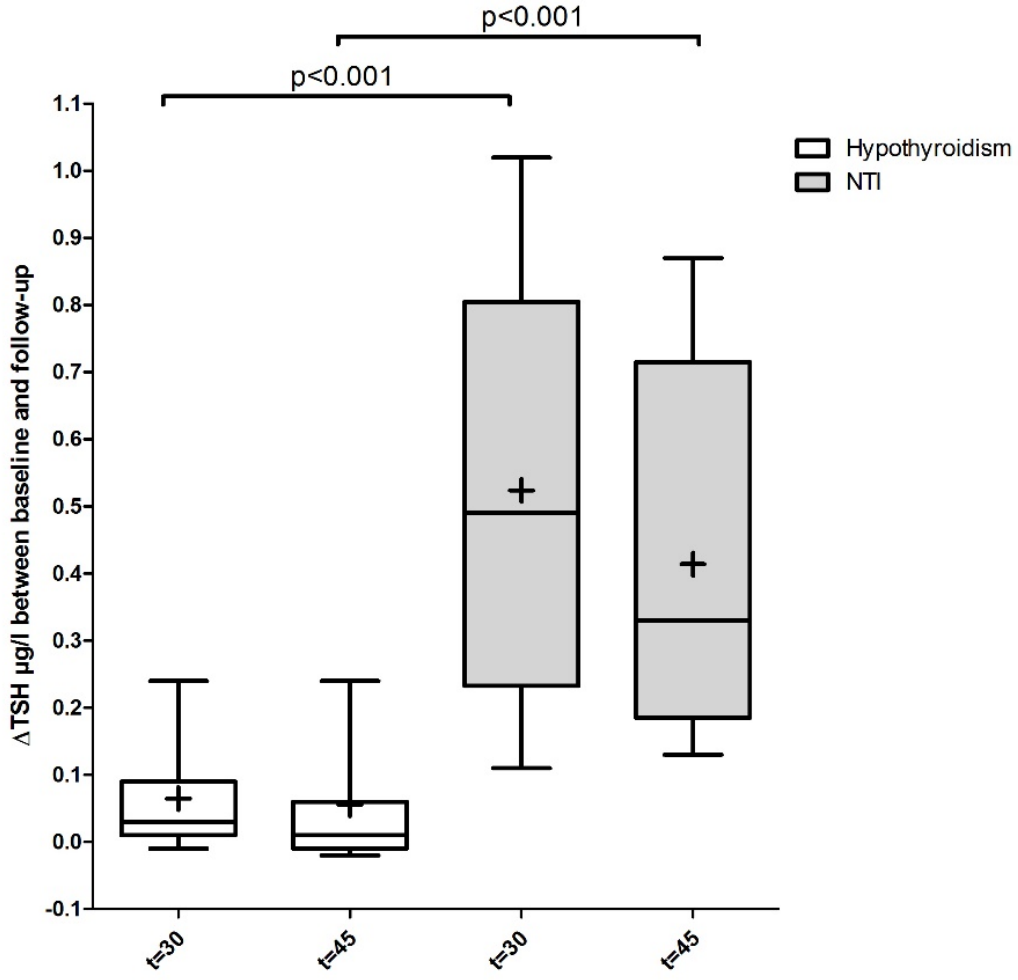

FIGURE 7. A BOXPLOT ILLUSTRATING THE DIFFERENCE (INCREMENT) BETWEEN THE BASELINE PLASMA TSH CONCENTRATION AND THE PLASMA TSH CONCENTRATIONS AT 30 AND 45 MINUTES FOR BOTH GROUPS AFTER TRH ADMINISTRATION. THE WHISKERS INDICATE THE MINIMUM AND MAXIMUM VALUES AND THE '+'SIGN INDICATES THE MEAN.

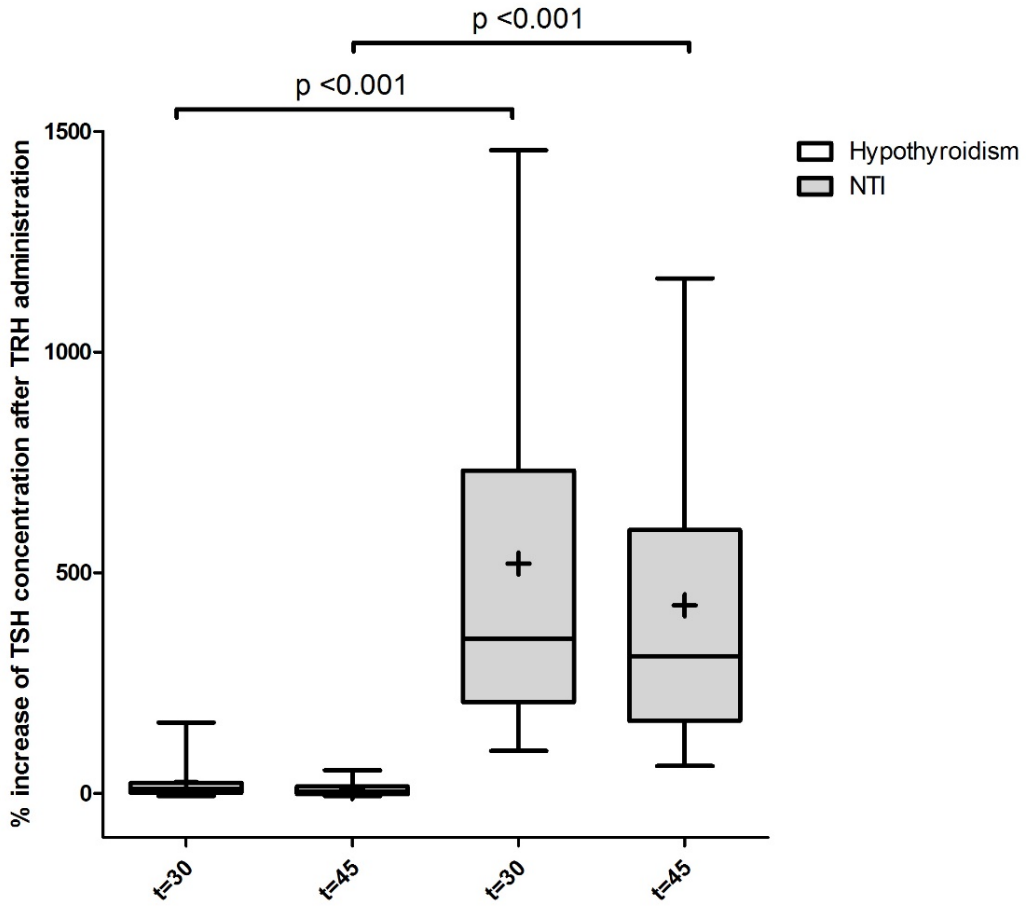

FIGURE 8. A BOXPLOT ILLUSTRATING THE PERCENTAGE INCREASE BETWEEN THE BASELINE PLASMA TSH CONCENTRATION AND THE PLASMA TSH CONCENTRATIONS AT T=30 AND AT=45 FOR BOTH GROUPS AFTER TRH ADMINISTRATION. THE WHISKERS INDICATE THE MINIMUM AND MAXIMUM VALUES AND THE '+'SIGN INDICATES THE MEAN.

Plasma GH concentrations in the TRH stimulation test

The median baseline plasma GH concentrations before administration of TRH were significantly higher ($p<0.001$) in the hypothyroid dogs (3.2 µg/l, range 2.0 – 12.5 µg/l) than in the NTI dogs (0.73 µg/l, range 0.45 – 2.3 µg/l).

The plasma GH concentration increased significantly ($p=0.033$) in the hypothyroid dogs after TRH was administered as compared with the baseline concentration. At t=30, the median plasma GH concentration was 6.1 µg/l (range 2.0 – 54.4 µg/l) ($p=0.009$) and at t=45, the median GH concentration was 6.6 µg/l (range 2.4 – 100 µg/l) ($p=0.006$). No significant difference ($p=0.51$) was observed between t=30 and t=45.

There was no significant difference in plasma GH concentrations in the NTI group between baseline, t=30 and t=45 ($p=0.13$). The median GH concentration at t=30 was 1.1 µg/l (range 0.50 – 1.9 µg/l) and at t=45 0.75 µg/l (range 0.40 – 1.1 µg/l).

The median plasma GH concentrations were significantly higher in the hypothyroid dogs than in the NTI dogs at t=30 ($p<0.001$) and t=45 ($p<0.001$) (Figure 9).

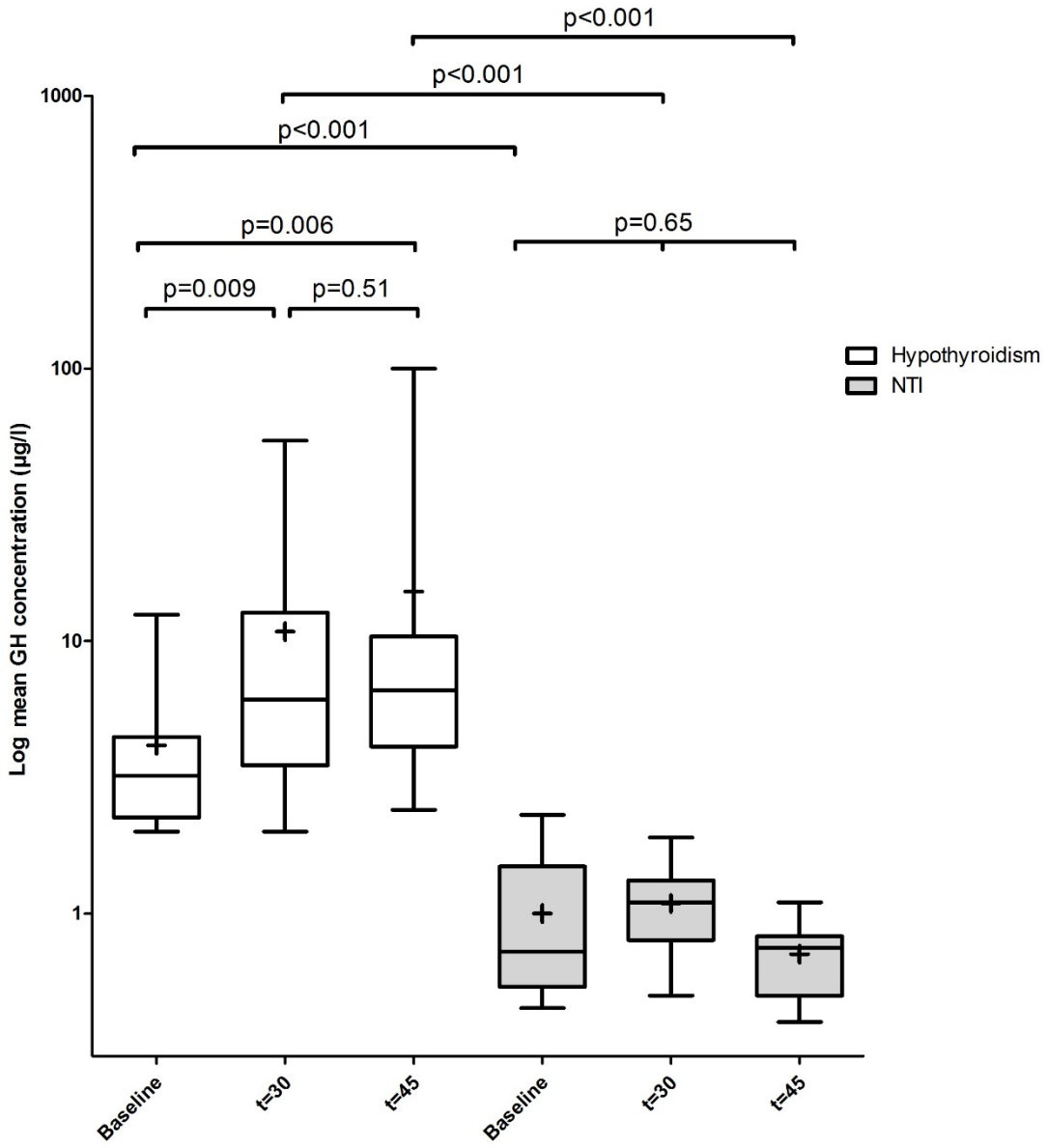

FIGURE 9. A BOXPLOT SHOWING THE PLASMA GH CONCENTRATIONS AT BASELINE, T=30 AND T=45 FOR BOTH GROUPS. THE WHISKERS INDICATE THE MINIMUM AND MAXIMUM VALUES AND THE '+'SIGN INDICATES THE MEAN FOR THE NTI GROUP ONLY ONE P-VALUE IS PROVIDED AS THE KRUSKAL WALLIS TEST DID NOT SHOW A DIFFERENCE BETWEEN THE THREE TIME POINTS (P=0.65) AND THEREFORE NO FURTHER ANALYSIS BETWEEN THE TIME POINTS WAS PERFORMED.

The increment in plasma GH concentrations at 30 and 45 minutes after TRH was administered was significantly different between the two groups. The GH concentration at 30 minutes increased with a median of 2.7 µg/l (range -0.95 – 41.9 µg/l) in the hypothyroid dogs as compared to 0.18 µg/l (range -1.3 – 0.65) in the NTI group (p=0.008). There was also a significant difference (p=0.001) of GH increment 45 minutes after TRH administration, with a GH concentration increase of 3.2 µg/l (range -0.35 – 87.5 µg/l) in the hypothyroid group versus 0.0 µg/l (range -1.6 – 0.40 µg/l) in the NTI group (Figure 10).

The median plasma GH concentration increase expressed as percentages at t=30 and t=45 was different between the hypothyroid dogs and NTI dogs, but only significantly at t=45 (p=0.002) and not at t=30

(p=0.07) (Figure 11). In hypothyroid dogs the median percentage increase in GH concentration was 88% (range -21 – 335%) at t=30 and 92% (range -8 – 700%) at t=45. In NTI dogs, the increase in GH concentration at t=30 was 15% (range -57 – 118%) and at t=45 -7% (range -72 – 78%).

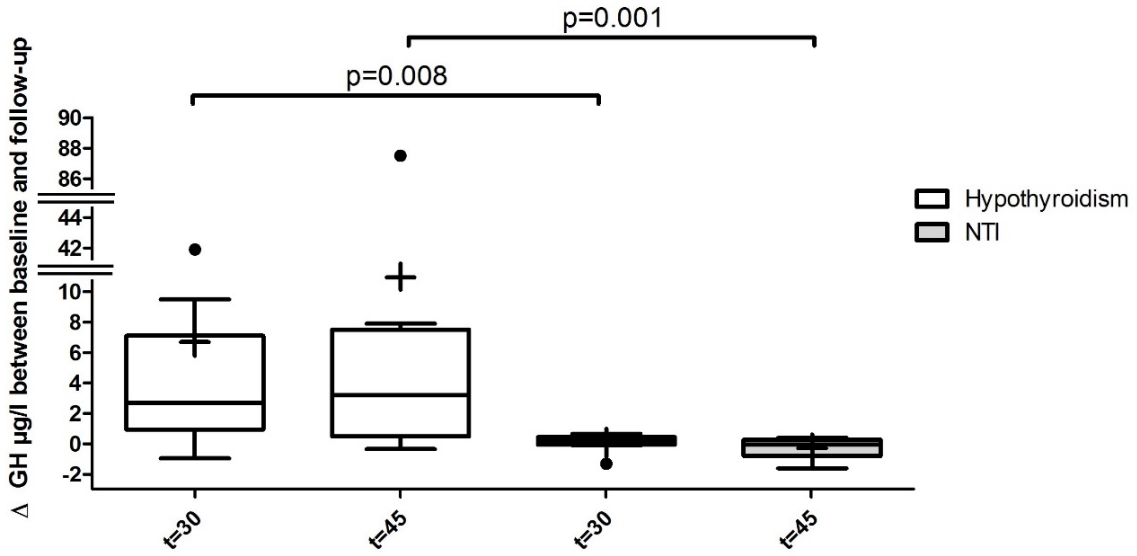

FIGURE 10. A BOXPLOT ILLUSTRATING THE DIFFERENCE BETWEEN THE BASELINE PLASMA GH CONCENTRATION AND THE PLASMA GH CONCENTRATIONS AT 30 AND 45 MINUTES AFTER TRH ADMINISTRATION. THE WHISKERS INDICATE THE LOWEST AND HIGHEST 1.5 IQR (TUKEY) AND THE DOTS REPRESENT THE MINIMUM AND MAXIMUM VALUES WHEN THESE ARE OUTSIDE THE 1.5 IQR. THE '+'SIGN INDICATES THE MEAN.

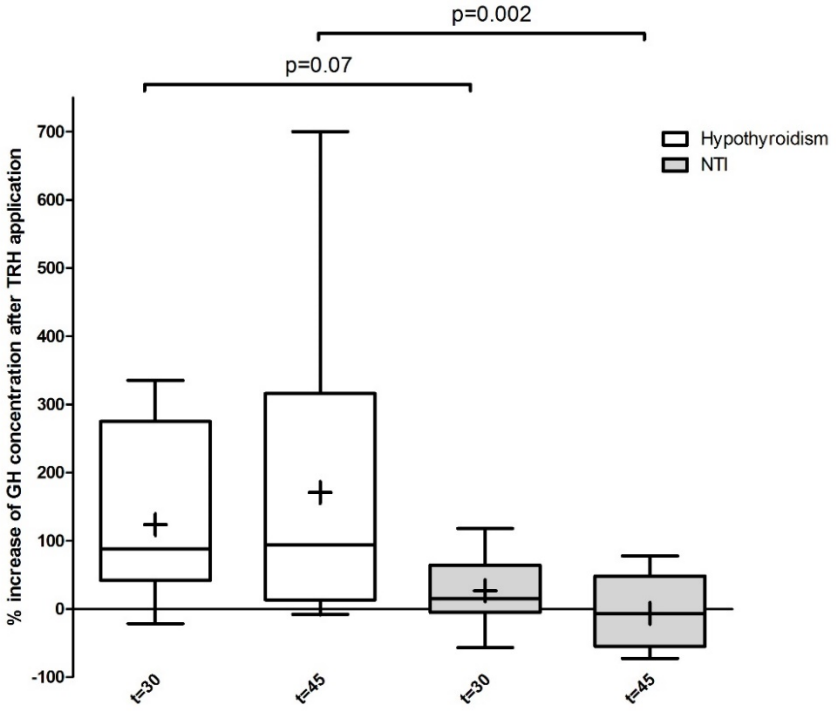

FIGURE 11. A BOXPLOT ILLUSTRATING THE PERCENTAGE INCREASE BETWEEN THE BASELINE PLASMA GH CONCENTRATION AND THE PLASMA GH CONCENTRATIONS AT 30 AND AT 45 MINUTES AFTER TRH ADMINISTRATION. THE WHISKERS INDICATE THE MINIMUM AND MAXIMUM VALUES AND THE '+'SIGN INDICATES THE MEAN.

GH – TSH ratio in the TRH stimulation test

The mean GH/TSH ratio at baseline was not significantly different (p=0.59) between hypothyroid dogs (17.08 ± 14.4, range 5.38 – 52.35) and NTI dogs (13.89 ± 12.2, range 1.37 – 32.0).

Within the hypothyroid group, there were no significant differences in the GH/TSH ratios between baseline and t=30 or t=45 (p=0.22). At t=30 the mean GH/TSH ratio was 26.98 ± 20.1 (range 4.26 – 77.71) and at t=45 37.21 ± 38.7 (range 5.45 – 142.86).

In the NTI dogs there was a significant difference (p=0.001) in the GH/TSH ratio between baseline, t=30 and t=45. At t=30 the GH/TSH ratio was 2.88 ± 3.0 (range 0.39 – 9.29) (p=0.007) and at t=45 1.88 ± 1.3 (range 0.36 – 3.75) (p=0.007).

The mean GH/TSH ratios were significantly higher in the hypothyroid dogs than in the NTI dogs at t=30 (p= 0.003) and at t=45 (p=0.013) (Figure 12).

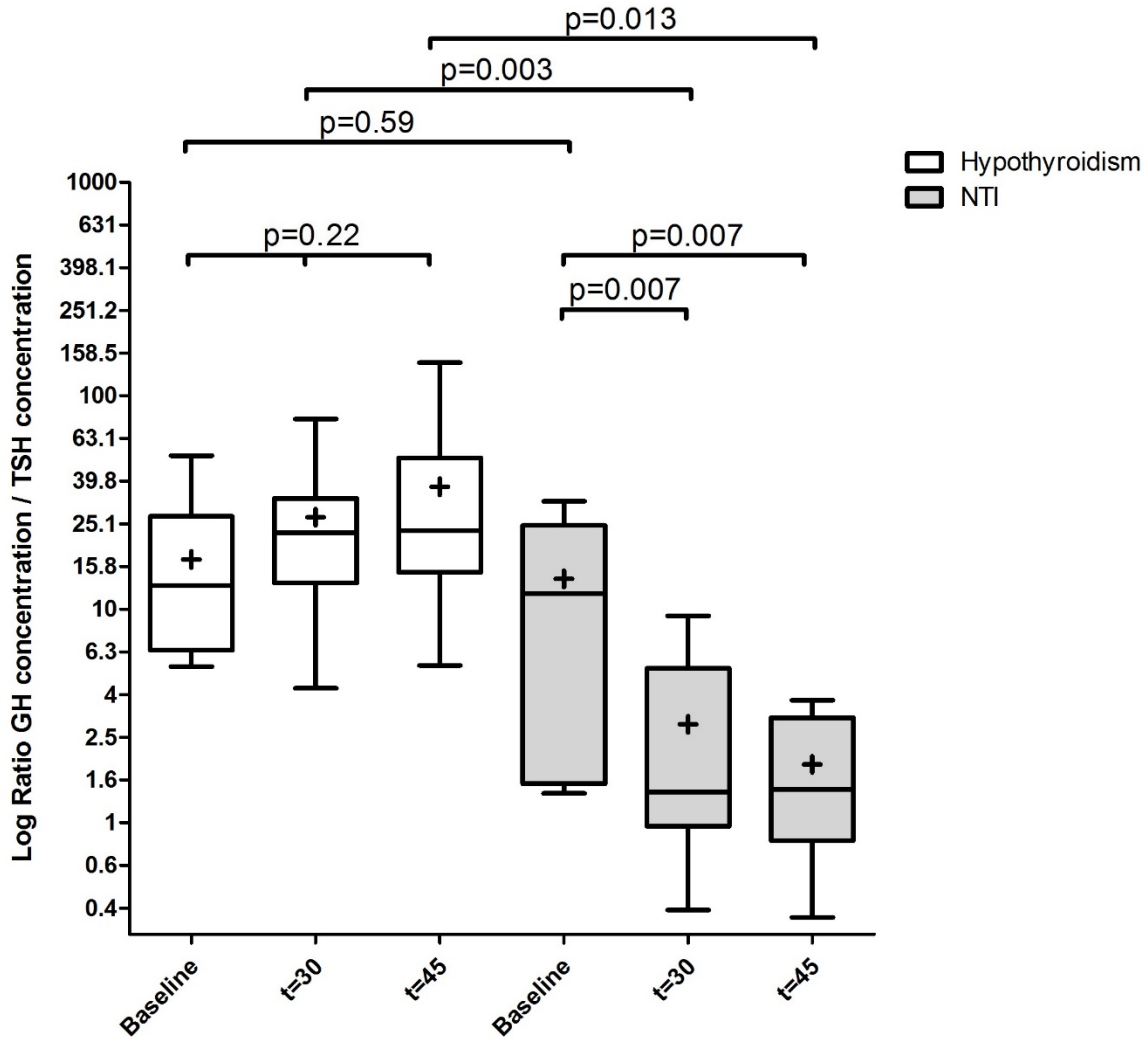

FIGURE 12. THE RATIO BETWEEN THE PLASMA GROWTH HORMONE CONCENTRATION DIVIDED BY THE PLASMA TSH CONCENTRATION AT ALL THREE TIME POINTS IN HYPOTHYROID DOGS AND DOGS WITH NTI. THE WHISKERS INDICATE THE MINIMUM AND MAXIMUM VALUES AND THE '+'SIGN INDICATES THE MEAN. FOR THE HYPOTHYROID GROUP ONLY ONE P-VALUE IS PROVIDED FOR THE WITHIN GROUP DIFFERENCE (BETWEEN BASELINE, T=30 AND T=45) AS THE KRUSKAL WALLIS TEST DID NOT SHOW A DIFFERENCE BETWEEN THE GROUPS (P=0.22) AND THEREFORE NO FURTHER ANALYSIS BETWEEN THE GROUPS WAS PERFORMED.

The median increment of the GH/TSH ratio at t=30 was 6.82 (range -9.23 – 50.54) in the hypothyroid dogs and -10.40 (range -25.87 – -0.37) in the NTI dogs (p=0.002). At t=45, the median increase was 9.48 (range -1.10 – 115.68) in the hypothyroid dogs and -10.50 (range -28.40 – -0.41) in the NTI dogs (p<0.001) (Figure 13).

When expressed as percentage increase in GH/TSH ratio, there were also significant differences between hypothyroid dogs and NTI dogs with respective median values at t=30 of 178% (range 69 - 349%) versus 24% (range 10 – 75%) (p<0.001) and at t=45 186% (range 88 – 526%) versus 12% (range 10 – 73%) (p<0.001) (Figure 14).

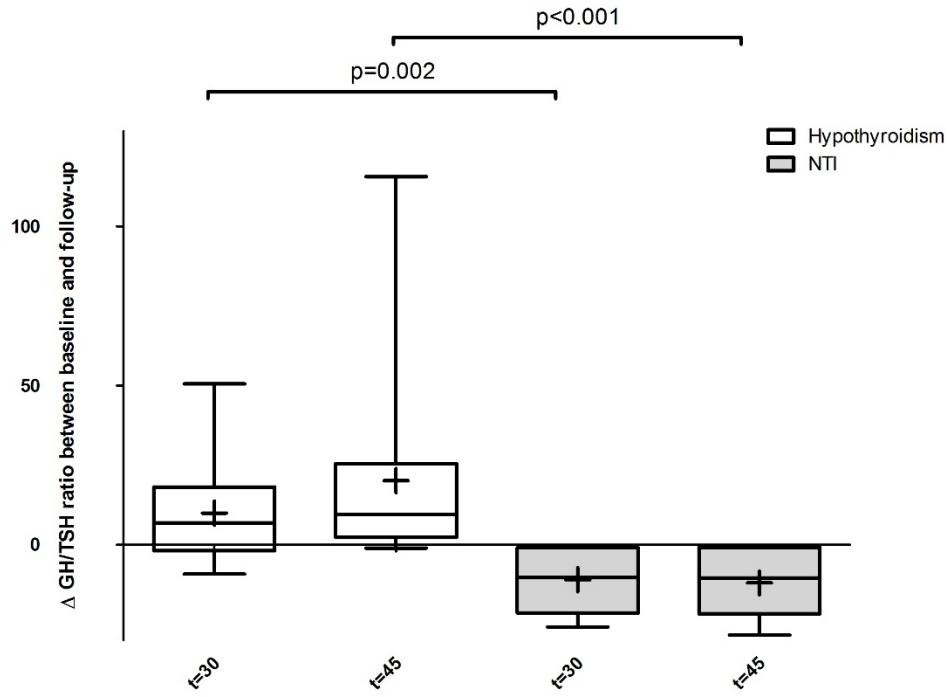

FIGURE 13. A BOXPLOT ILLUSTRATING THE DIFFERENCE (INCREMENT) BETWEEN THE BASELINE PLASMA GH/TSH RATIO AND THE PLASMA GH/TSH RATIOS AT 30 AND 45 MINUTES AFTER TRH ADMINISTRATION. THE WHISKERS INDICATE THE MINIMUM AND MAXIMUM VALUES AND THE '+' SIGN INDICATES THE MEAN.

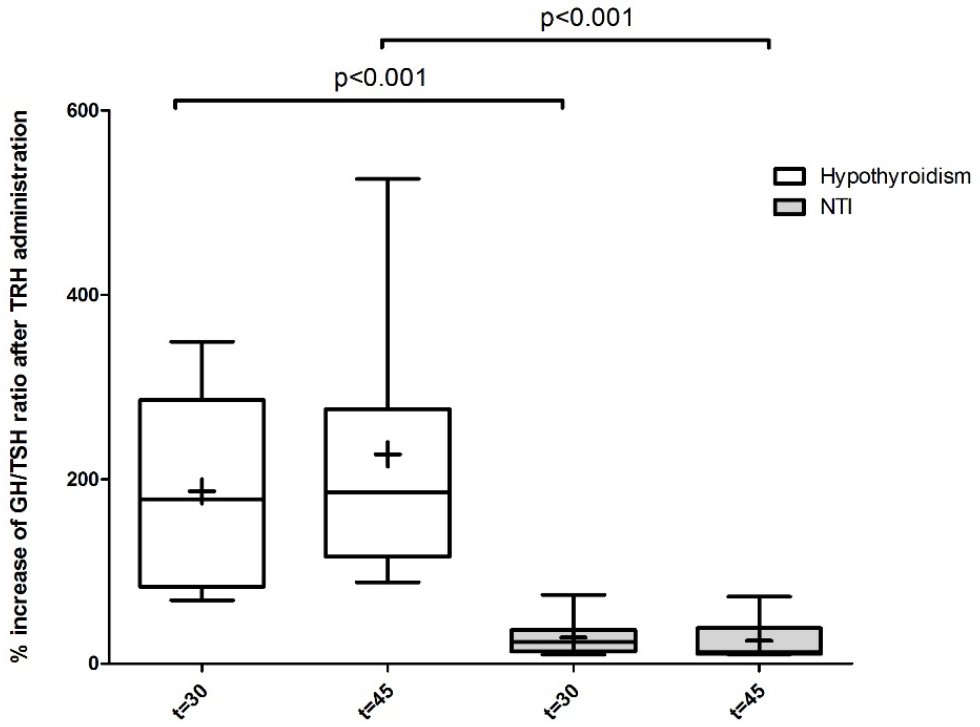

FIGURE 14. A BOXPLOT ILLUSTRATING THE PERCENTAGE INCREASE BETWEEN THE BASELINE PLASMA GH/TSH RATIO AND THE PLASMA GH/TSH RATIOS AT 30 AND AT 45 MINUTES AFTER TRH ADMINISTRATION. THE WHISKERS INDICATE THE MINIMUM AND MAXIMUM VALUES AND THE '+'SIGN INDICATES THE MEAN.

Threshold values to differentiate between hypothyroidism and NTI

For five outcomes, there was no overlap in values between the hypothyroid dogs and dogs with NTI. The percentage increase of TSH at t=45, the absolute concentration of GH at t=30 and t=45, the GH/TSH ratio at t=45 and the percentage increase of GH/TSH at t=45 could fully differentiate hypothyroid dogs from NTI dogs. For the percentage increase of TSH at t=45, there was no overlap between 52% and 62% increase. The cut-off value to distinguish between hypothyroid and NTI dogs was 57%. Values below this cut-off value are diagnostic for hypothyroidism. For the absolute GH concentration at t=30 there was no overlap between 1.9 µg/l and 2.0 µg/l. A cut-off value > 1.95 µg/l was diagnostic for hypothyroidism. At t=45 there was no overlap between 1.1 µg/l and 2.4 µg/l. A cut-off value > 1.75 µg/l was diagnostic for hypothyroidism. For the GH/TSH ratio, there was no overlap between 3.75 and 5.45. A cut-off value > 4.60 was diagnostic for hypothyroidism. Finally, for the percentage increase of GH/TSH ratio at t=45 there was no overlap between 73% and 88%, A cut-off value > 80.5% was diagnostic for hypothyroidism. Logically, all values within the above mentioned ranges resulted in an area under the ROC curve of 1.00, corresponding with 100% sensitivity and 100% specificity for diagnosing hypothyroidism.

Discussion

Dogs clinically suspected of hypothyroidism with both a low plasma TT4 concentration and a plasma TSH concentration within the reference range are difficult to distinguish from dogs with NTI. No definitive diagnosis can be made without an invasive, not widely available, or expensive diagnostic work-up. This study shows that a relatively simple TRH stimulation test with measurement of plasma concentrations of TSH and GH can differentiate between dogs with primary hypothyroidism and dogs with NTI. In dogs with hypothyroidism, the plasma TSH concentration did not change after TRH was administered, while the plasma GH concentration significantly increased. Interestingly, the opposite was observed in dogs with NTI which showed a significant increase in plasma TSH concentration after administration of TRH, but no differences in plasma GH concentrations.

Baseline characteristics

A possible confounding factor between the hypothyroid and NTI group was gender, with almost all dogs in the hypothyroid group being male, whereas in the NTI group the larger part was female. However, gender has not been identified as a risk factor for hypothyroidism, and is therefore unlikely to influence the results [3,5]. Dogs did not differ in other baseline characteristics. Especially the neutering status was similar, which could be important as neutered dogs have been reported to be at increased risk for developing hypothyroidism [8].

Basal TSH

To the best of our knowledge, no studies are available which directly compare basal TSH plasma concentrations between hypothyroid dogs and NTI dogs that both have basal TSH plasma concentrations within the reference range. Data from previous studies showed that basal TSH plasma concentrations are lower in NTI dogs than in hypothyroid dogs [13,22,27]. However, in all these studies, also dogs with plasma TSH concentrations above the reference range were included. These results can therefore not be compared with the results of the current study. In our study, where only dogs with a basal TSH within the reference range were included, the basal TSH plasma concentration in the hypothyroid dogs tended to be higher than in the NTI dogs. This difference was statistically significant when basal plasma TSH concentrations used for inclusion were compared (0.40 vs 0.21 µg/l, p=0.002). However, the difference was not significant when the basal plasma TSH concentrations just before TRH administration were compared (0.30 vs 0.19 µg/l, p=0.13). The mean basal plasma TSH concentration in hypothyroid dogs was 0.1 µg/l lower than the plasma TSH concentration used for inclusion in the same dogs. A possible explanation might be the time that had passed between the measurements, as Diaz-Espiñeira et al. have shown that basal TSH concentrations decrease over time in hypothyroid dogs [11]. However, this is not very likely as the time between these measurements was only a few weeks in most dogs. Both the plasma TSH concentration used for inclusion and plasma TSH concentration obtained during the study just before TRH administration had a great overlap between hypothyroid dogs and NTI dogs and are therefore clearly insufficient to discriminate between hypothyroid and NTI dogs.

TSH after TRH stimulation

After administration of TRH, the plasma TSH concentration increased significantly in the NTI dogs, whereas no change was observed in the hypothyroid dogs. The plasma TSH concentration seemed to reach its peak 30 minutes after TRH administration in NTI dogs. The TSH plasma concentration decreased significantly between 30 minutes and 45 minutes after TRH administration, although the concentration at t=45 was still significantly higher than the baseline concentration. The absolute plasma TSH concentration was higher in the NTI dogs than in the hypothyroid dogs 30 minutes after TRH

administration; however there was quite some overlap between groups. This is in line with the findings of Diaz-Espiñeira et al., where a 4RH test (including TRH) did not cause a significant change in plasma TSH concentration in hypothyroid dogs whereas in the NTI dogs a significant increase occurred [27]. In another study, it was also found that administration of TRH had no statistically significant effect on the plasma TSH concentration of hypothyroid dogs, whereas it rose significantly in healthy dogs [26]. A study from the nineties showed a significantly lower percentage increase of TSH concentration in response to TRH administration in hypothyroid dogs (median 24%) as compared to healthy dogs (median 207%) and euthyroid dogs with concurrent disease (median 167%) [22]. It should be noted that all above mentioned studies also included hypothyroid dogs with a basal plasma TSH concentration above the reference range and are therefore not identical to our study population. Because the baseline plasma TSH concentration tended to be higher in the hypothyroid dogs than in the NTI dogs of our study, the differences became more apparent when the absolute increment and percentage increase in plasma TSH concentration after TRH administration were analysed. The most pronounced difference between hypothyroid dogs and NTI dogs was found for the percentage increase in plasma TSH concentration 45 minutes after TRH administration, with no overlap between the two groups (maximum of 52% increase in the hypothyroid dogs and minimum increase of 62% in the NTI dogs). The percentage increase of plasma TSH concentration after TRH administration was therefore the best measure of TSH to distinguish between hypothyroid dogs and NTI dogs.

Underlying mechanisms: TSH

Various explanations have been proposed for the fact that the basal TSH plasma concentration is within the reference range in some dogs with hypothyroidism and that dogs with hypothyroidism have a blunted or absent TSH response to TRH administration.

Kooistra et al. demonstrated that the secretion of TSH in dogs with hypothyroidism is pulsatile (pulses with a relatively low amplitude). They suggested that this pulsatile secretion pattern could explain the non-elevated TSH levels found in some dogs with hypothyroidism [15]. However, these ultradian fluctuations are an unlikely explanation for the TSH concentrations that are not in the most upper limit of the reference range, which are also encountered in some dogs with hypothyroidism.

Furthermore it has been postulated that a failure of the TSH assay to detect all isomers of circulating TSH or concurrent secondary hypothyroidism could be reasons for a low TSH measurement in some dogs with hypothyroidism and might contribute to the absent TSH response to TRH [7,21,29].

Finally, in a study of dogs with induced hypothyroidism, the plasma TSH concentration rapidly increased as expected after thyroidectomy. Hereafter, the plasma TSH concentration gradually lowered in time until after 3 years, values similar to the initial values from before induced hypothyroidism were reached. In these dogs, the response of TSH after administration of TRH was even faster diminished; the increase in TSH remained unchanged in the first 2 months, but decreased thereafter. The authors proposed that in dogs with hypothyroidism the TSH producing cells in the pituitary gland might become desensitized for TRH after a while due to persistent stimulation of thyrotrophs via the negative feedback loop [11].

Basal GH

In our study the mean basal plasma GH concentration was significantly higher in dogs with hypothyroidism than in dogs with NTI, which is in agreement with the findings of Diaz-Espiñeira et al. [27]. However, in this study also dogs with a plasma TSH level above the reference range were included. In other studies basal plasma GH concentrations in hypothyroid dogs have been compared to healthy dogs. In these studies it was also demonstrated that dogs with hypothyroidism had elevated basal plasma GH concentrations [11,16,26,30,31]. Also in man, rats and pigs, a correlation has been found between GH secretion

and primary hypothyroidism. However, primary hypothyroidism in these species has been associated with decreased levels of GH [32-34].

GH after TRH administration

In our study, levels of GH significantly increased in dogs with hypothyroidism after administration of TRH. This observation extends earlier findings of Diaz-Espiñeira et al., where hyper-responsiveness of GH after TRH stimulation was reported in dogs with induced primary hypothyroidism. The TRH stimulation test resulted in a significant increase in plasma GH concentrations after 30 and 45 minutes [11]. An earlier study of these authors also reported a significant increase of GH after TRH stimulation (at 10 and 20 minutes) in dogs with primary hypothyroidism [26]. These dogs all had a basal TSH concentration above the reference value. A similar GH response to TRH has been reported in humans [31,35].

In contrast to dogs with hypothyroidism, the plasma GH concentration of NTI dogs did not increase significantly after administration of TRH in our study. The only study to the knowledge of the author which investigated the response of TRH administration on GH in dogs with NTI, is a study of Diaz-Espiñeira et al. Contrary to our findings, they report a significant increase in 2 of the 5 time points (both within 15 minutes after administration of 4RH, no differences were observed after 20, 30 and 45 minutes). However, in this study a 4RH test was used as a stimulation test, instead of solely administrating TRH, so the increment in GH might very well have been a response to GHRH [27]. Other studies in healthy dogs showed that plasma GH concentrations did not increase after administration of TRH [26,36].

For the GH values, the best distinction between hypothyroid dogs and NTI dogs could be made based on the absolute GH concentrations at 45 minutes after TRH administration. No overlap between hypothyroid dogs (minimum concentration of 2.4 µg/l) and NTI dogs (maximum concentration of 1.1 µg/l) was observed. At 30 minutes after TRH administration, there was also no overlap between the two groups. However, the difference between the plasma GH concentration in hypothyroid dogs (2.0 µg/l) and NTI dogs (1.9 µg/l) was much smaller than at 45 minutes after TRH administration.

Other promising parameters could be the GH/TSH ratio and the percentage increase of GH/TSH ratio, for which both at t=45 no overlap was observed between hypothyroid dogs (minimum ratio of 5.45 and 88% respectively) and NTI dogs (maximum ratio of 3.75 and 73%, respectively).

Underlying mechanisms: GH

Several mechanisms have been proposed in order to explain the elevated basal and TRH-stimulated plasma GH concentrations in dogs with hypothyroidism. One of the most recent findings is the existence of bihormonal, thyrosomatotrophic cells in dogs [11], which previously already had been described in humans and rats [37-39]. These cells may be somatotrophs transformed into stimulated thyrotrophs and are immunoreactive for both GH and TSH. These thyrosomatotrophic cells may therefore explain the elevated GH levels in dogs with primary hypothyroidism as they could release TRH induced GH. However, we previously described that thyrotrophs might become desensitised for TRH as a possible explanation for the absent increase of TSH after TRH stimulation and the low basal TSH concentration in hypothyroid dogs. An explanation for this discrepancy could be that the TRH receptors on the thyrotrophs are more likely to become desensitised than the TRH receptors on the thyrosomatrophs. The amount of TSH secreted by these thyrosomatrophs is probably insufficient to influence the total TSH concentrations [11].

Another mechanism that could be responsible for the high values of GH in hypothyroid dogs might be the presence of a negative thyroid hormone response element (TRE). The human and rat GH gene contains a positive TRE, meaning that thyroid hormones stimulate the expression of GH genes and thus GH production in the pituitary gland [40-42]. In line with this observation, hypothyroidism in humans and rats

results in low basal GH plasma concentrations [31-33]. Interestingly, the opposite is observed in hypothyroid dogs as shown in previous studies and the current study. Therefore, a possible explanation for the absence of a decrease in GH in dogs with hypothyroidism could be the lack of a TRE, as Lantinga-van Leeuwen et al. could not identify a TRE in dogs [43]. However, this does not explain why hypothyroid dogs have significant higher basal GH concentrations than NTI dogs. An explanation for the difference in basal GH concentrations between hypothyroid and NTI dogs would be the existence of a negative TRE within the GH gene in dogs, leading to a reduced suppression of GH expression [16]. Although the previously mentioned study of Lantinga-van Leeuwen did not demonstrate the existence of a TRE in dogs, analysis might have been insufficient to truly rule out its existence. Negative TREs have already been found in other canine genes and might therefore also be present in the canine GH gene [16].
Finally, a third possible explanation which might in part be responsible for the elevated GH plasma concentration and the response to TRH in hypothyroid dogs could be the influence of SS. In rats, induced hypothyroidism is associated with decreased SS content and release from the hypothalamus [33,44]. Although no data is available for dogs, a theoretical reduced SS secretion from the hypothalamus might result in elevated basal GH secretion. However, whether this explanation holds could be discussed as the administration of SS did not result in a decrease of GH in dogs, which would be expected based on the same theoretical grounds of SS being an inhibitor of GH release [11]. This is in contrast to humans, where administration of SS inhibits the GH response to TRH in hypothyroid patients [45].

Differentiating between dogs with hypothyroidism and dogs with NTI

As discussed previously, for five outcomes there was no overlap between hypothyroid dogs and NTI dogs, resulting in 100% sensitivity and 100% specificity. The percentage increase of TSH at t=45, the absolute concentration of GH at t=30 and t=45, the GH/TSH ratio at t=45 and the percentage increase of the GH/TSH ratio 45 minutes after TRH administration could fully differentiate between hypothyroid dogs and NTI dogs. Hence, these measures are promising candidates to simply and inexpensively distinguish hypothyroid dogs from NTI dogs in the setting of a primary veterinary practice. The threshold values in this population to differentiate hypothyroid dogs from NTI dogs were: for the percentage increase in TSH concentration <57%; for the absolute GH concentration at t=30 >1.95 µg/l and at t=45 >1.75 µg/l; for the GH/TSH ratio >4.60 and for the percentage increase in GH/TSH ratio >80.5%. However, this study was neither designed to analyse diagnostic accuracy nor to calculate threshold values of these tests. Still, our values do provide valuable reference values which might guide future studies on diagnostic accuracy and threshold values. It should be noted that two dogs were excluded from the analysis which otherwise could have influenced the sensitivity and specificity of the outcomes.

Strengths and weaknesses

A power calculation was performed to determine the population size. Our results showed that this power calculation was adequate as significant differences between the two groups were found and we therefore did not introduce a type 2 error. To overcome variations due to pulsatile excretion, baseline values were measured twice. Scintigraphy was used as a golden standard to determine if dogs had hypothyroidism or NTI. For two dogs however, no final diagnosis could be made based on $^{99m}TcO_4^-$ uptake. One dog was excluded based on an inconclusive scintigraphy result because it showed a decreased uptake of $^{99m}TcO_4^-$, but the thyroid glands were still clearly visible. No distinction could therefore be made between hypothyroidism and NTI based on the scintigraphy. Another dog was classified as NTI based on the scintigraphy, however, after an intense diagnostic workup no cause for the clinical signs could be identified. Also, the GH and TSH values of this dog in the current study corresponded with the hypothyroid group. Based on these findings, the scintigraphy result might have been inaccurate and

therefore a diagnostic treatment with L-thyroxine was started. The effect of this treatment will be evaluated after six weeks, which unfortunately is outside the timeframe of the current thesis. Concerns for the reliability of thyroid scintigraphy on some results of individual animals have been expressed in earlier studies. However, in most cases dogs could definitely be classified as euthyroid or hypothyroid [46]. Another study showed scintigraphy to have the highest discriminatory power to differentiate between hypothyroidism and NTI, as some results of the TSH stimulation test overlapped between the two groups in contrast to results from $^{99m}TcO_4^-$ uptake [13].

Future research

As discussed previously, the current study was not designed to establish threshold values or to test the diagnostic accuracy. A larger cohort study is necessary to answer these questions. In a larger group of dogs, also other variables such as the influence of breed, gender, neutering status and duration of symptoms could be tested. The current results did not seem to be influenced by these factors, but this study was underpowered to reliably perform these sub-analyses. In the future it might also be interesting to assess how reliable the test is when it is used in dogs with less overt clinical signs of hypothyroidism as clinicians in the primary veterinary practice might also want to rule out hypothyroidism in these dogs if they have a low T4 and TSH within the reference range.

Conclusion

A differential response to TRH administration was observed in dogs with hypothyroidism and dogs with NTI. Plasma TSH concentrations after TRH administration increased significantly in dogs with NTI, whereas they remained unchanged in dogs with hypothyroidism. The contrary was found for GH. Plasma GH concentrations after TRH administration significantly increased in dogs with hypothyroidism, whilst they remained unchanged in dogs with NTI. The outcome parameters without any overlap between the two groups were mostly observed 45 minutes after TRH administration. Therefore, plasma concentrations of TSH and GH 45 minutes after administration of TRH can differentiate between hypothyroid dogs and NTI dogs that have clinical signs of hypothyroidism, a low basal plasma TT4 concentration and a basal plasma TSH concentration within the reference interval. This is a promising test which might be used in primary veterinary practice as it could be a relatively non-invasive, inexpensive and widely available test. The exact threshold values and accuracy need to be determined in a larger cohort of dogs.

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Appendix

		All dogs	Hypothyroid dogs	NTI dogs
Baseline Characteristics	Age	p=0.200	p=0.196	p=0.200
	Weight	p=0.200	p=0.077	p=0.200
	TSH used for inclusion	p=0.200	p=0.136	p=0.200
Plasma TSH concentrations	Baseline	p=0.200	p=0.200	p=0.076
	t=30	**p=0.024**	p=0.200	p=0.200
	t=45	p=0.145	p=0.200	p=0.200
	Percentage increase between baseline and t=30	**p=0.001**	**p=0.001**	**p=0.011**
	Percentage increase between baseline and t=45	**p=0.001**	**p=0.015**	**p=0.015**
	Increment between baseline and t=30	**p=0.040**	**p=0.025**	p=0.200
	Increment between baseline and t=45	p=0.081	**p=0.007**	p=0.200
Plasma GH concentrations	Baseline	**p=0.004**	**p=0.014**	p=0.051
	t=30	**p<0.001**	**p=0.002**	p=0.200
	t=45	**p<0.001**	**p<0.001**	p=0.200
	Percentage increase between baseline and t=30	**p=0.018**	**p=0.020**	p=0.200
	Percentage increase between baseline and t=45	**p<0.001**	**p=0.019**	p=0.200
	Increment between baseline and t=30	**p<0.001**	**p=0.003**	**p=0.043**
	Increment between baseline and t=45	**p<0.001**	**p<0.001**	p=0.200
GH/TSH ratio	Baseline	p=0.072	p=0.195	p=0.200
	t=30	**p=0.015**	p=0.183	**p=0.038**
	t=45	**p<0.001**	p=0.101	p=0.182

TABLE 1. THE DISTRIBUTION OF THE DATA WAS ASSESSED WITH THE KOLMOGOROV-SMIRNOV TEST. P-VALUES <0.05 ARE HIGHLIGHTED IN BOLD AND REPRESENT A NON-NORMAL DISTRIBUTION.

Dog	Breed	Age (Years)	Weight (kg)	Gender	Symptoms	Duration symptoms	Diagnosis based on scintigraphy
1	Alaskan Malamute	1.8	34	F*	S,L,W,Lo, C	7 Months	NTI
2	Stabyhoun	9.1	25.4	F*	L,Sn,C	Few months	NTI
3	German Wirehaired Pointer	4.7	34.5	M	S,C	Few months	NTI
4	Cross-bred	10,7	28.8	F*	L,W	1 Year	NTI
5	Cross-bred	7.4	28.8	M	S,L,W,Lo,Wa	1 Year	NTI
6	Cross-bred	8.11	36.9	F*	L,W	3 weeks	NTI
7	Rhodesian Ridgeback	9.2	48.0	F*	S,L,W,C	Unknown	NTI
8	Siberian Husky	7.2	32.9	F*	L,W,Lo	6 Months	Unclear, started diagnostic treatment
9	Scottish Shepherd	7.1	27.5	M	L,Lo	5 Months	NTI
10	Cross-bred	6.0	36	F*	L,W,Sn	6 Months	NTI
11	Beagle	6.7	17.1	M	S,C	> 1 Year	NTI
12	American Cocker Spaniel	8.6	14.2	F*	S,L,Sn	> 1 Year	Hypothyroidism
13	Leonberger	5	75.0	M*	S,LW,Lo	Few months	Hypothyroidism
14	Golden Retriever	7.8	37.9	M	S	Unknown	Hypothyroidism
15	English Cocker Spaniel	7.6	17.6	M	S,W	2,5 Months	Hypothyroidism
16	Beagle	7.8	18.9	M*	S,L,W,Lo,M	Unknown	Hypothyroidism
17	Golden Retriever	4.1	49.5	M	S,L,W,Lo,M,Wa	6 Months	Hypothyroidism
18	Rhodesian Ridgeback	3.6	50.1	M	S,L,M,Wa	1 Year	Hypothyroidism
19	German Shepherd	6.5	50.8	M*	S,L,W,M,Sn	1.5 Years	Hypothyroidism
20	Small Münsterländer	8.9	23.6	M*	S,L,Lo,M,Sn	2 Months	Hypothyroidism
21	Nova Scotia Duck Tolling Retriever	4.2	25.0	M	S,L,W,Lo,M	2 Years	Hypothyroidism
22	Beagle	9.8	20.4	M	S,L,Lo,M	1.5 Years	Hypothyroidism
23	Cross-bred	4.8	25.4	M*	L, W, C	1 Year	Unclear

TABLE 2. CHARACTERISTICS OF STUDY POPULATION. THE AGE IS PRESENTED IN YEARS (NOT MONTHS). F= FEMALE, M=MALE, *= NEUTERED. SYMPTOMS: S= SKIN PROBLEMS, L= LETHARGY, W= WEIGHT GAIN, LO=LOCOMOTION, M=MYXOEDEMA, SN=SNORING, WA= WARM PLACES, C=CONCURRENT PROBLEMS. THE DURATION OF THE SYMPTOMS IS AN ESTIMATE MADE BY THE OWNER.

Dog	Plasma TSH concentration (µg/l) (before and after TRH stimulation)				Diagnosis
	t=-15	**t=0**	**t= 30**	**t=45**	
1	0,03	0,03	0,14	0.16	NTI
2	0,07	0,07	1,09	0,83	NTI
3	0,14	0,15	0,63	0,40	NTI
4	0,05	0,05	0,31	0,25	NTI
5	0,27	0,32	0,92	0,72	NTI
6	<0,03	<0,03	0,44	0,38	NTI
7	0,52	0,49	0,99	0,82	NTI
8	0,27	0,29	0,30	0,29	unclear, started diagnostic treatment
9	0,37	0,36	1,27	1,23	NTI
10	0,04	0,03	0,18	0,17	NTI
11	0,45	0,36	1,18	1,10	NTI
12	0,22	0,25	0,27	0,24	hypothyroidism
13	0,26	0,25	0,28	0,25	hypothyroidism
14	0,35	0,32	0,34	0,35	hypothyroidism
15	0,10	0,07	0,08	0,08	hypothyroidism
16	0,12	0,11	0,30	0,12	hypothyroidism
17	0,49	0,44	0,49	0,70	hypothyroidism
18	0,38	0,38	0,47	0,44	hypothyroidism
19	0,17	0,18	0,17	0,18	hypothyroidism
20	0,45	0,43	0,50	0,50	hypothyroidism
21	0,40	0,40	0,43	0,38	hypothyroidism
22	0,50	0,42	0,70	0,70	hypothyroidism
23	0,35	0,32	0,63	0,61	Unclear

TABLE 3. PLASMA TSH CONCENTRATIONS BEFORE (T=-15 AND T=0) AND AFTER (T=30 AND T=45) TRH ADMINISTRATION.

Dog	Plasma GH concentration (µg/l) (before and after TRH stimulation)				Diagnosis
	t=-15	**t=0**	**t= 30**	**t=45**	
1	0,7	0,7	1,3	0,6	NTI
2	0,2	0,9	1,2	0,8	NTI
3	1,4	3,2	1,0	0,7	NTI
4	1,9	1,3	1,9	0,9	NTI
5	0,3	0,6	0,5	0,8	NTI
6	0,8	0,6	1,1	1,1	NTI
7	0,6	0,9	1,1	0,8	NTI
8	1,5	6,5	7,9	6,2	unclear, started diagnostic treatment
9	0,5	<0,5	< 0,5	<0,5	NTI
10	1,5	0,5	0,9	0,5	NTI
11	1,6	1,3	1,4	0,4	NTI
12	1,5	2,5	7,5	9,0	hypothyroidism
13	4,0	3,4	6,4	4,2	hypothyroidism
14	1,7	2,6	4,5	3,8	hypothyroidism
15	5,6	3,3	3,5	4,1	hypothyroidism
16	3,4	3,4	6,1	6,6	hypothyroidism
17	3,2	1,8	4,7	10,4	hypothyroidism
18	2,9	1,8	2,0	2,4	hypothyroidism
19	2,2	2,3	3,2	4,2	hypothyroidism
20	4,3	2,1	12,7	7,8	hypothyroidism
21	8,5	5,7	14,2	13,6	hypothyroidism
22	17,5	7,5	54,4	>100	hypothyroidism
23	0,3	0,3	1,2	1,0	unclear

TABLE 4. PLASMA GH CONCENTRATIONS BEFORE (T=-15 AND T=0) AND AFTER (T=30 AND T=45) TRH ADMINISTRATION.