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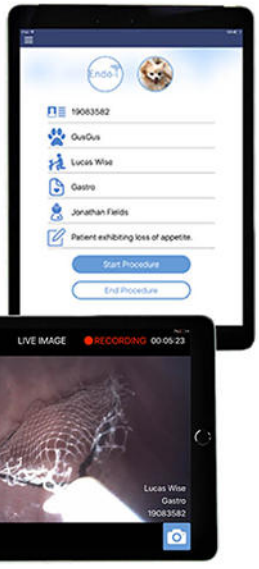
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Use of basal and TRH-stimulated plasma growth hormone concentrations to differentiate between primary hypothyroidism and nonthyroidal illness in dogs

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Background: A low plasma total thyroxine (TT_4) concentration in combination with a plasma TSH concentration within reference range does not distinguish between hypothyroidism and nonthyroidal illness (NTI) in dogs. Hypothyroidism is associated with TSH-releasing hormone (TRH)-induced increased release of growth hormone (GH).

Hypothesis: Basal and TRH-induced plasma GH concentrations can be used to distinguish hypothyroid dogs from NTI dogs.

Animals: Twenty-one dogs with signs consistent with hypothyroidism, a low plasma TT_4 concentration, and a plasma TSH concentration within reference interval.

Methods: Case control study. Thyroid scintigraphy was performed to classify dogs as having hypothyroidism or NTI. All dogs underwent a TRH stimulation test with measurement of plasma concentrations of GH and TSH before and 30 and 45 minutes after IV administration of TRH.

Results: Eleven of the dogs were classified as hypothyroid and 10 as having NTI. Basal plasma GH concentration in the hypothyroid dogs (3.2 μg/l; range, 2.0 to 12.5 μg/l) was significantly higher ($p<0.001$) than that in the NTI dogs (.73 μg/l; range, .45 to 2.3 μg/l), with minimal overlap, and increased ($p=.009$) after TRH administration in hypothyroid dogs, whereas it did not change in NTI dogs. At T=45, plasma GH concentrations in hypothyroid dogs and NTI dogs did not overlap. The plasma TSH concentration did not change significantly after TRH administration in hypothyroid dogs, whereas it increased ($p<.001$) in NTI dogs. At T=45, there was no overlap in percentage TSH increase from baseline between hypothyroid dogs.

Conclusions and Clinical Importance: Measurement of basal plasma GH concentration and concentrations of GH and TSH after TRH stimulation can distinguish between hypothyroidism and NTI in dogs.

KEYWORDS
canine, diagnosis, scintigraphy, thyroid

1 | INTRODUCTION

Hypothyroidism is one of the most common endocrinopathies in dogs.[1] Diagnosing hypothyroidism relies upon measurement of basal plasma

Abbreviations: $^{99m}TcO_4^-$, radioactive pertechnetate; GH, growth hormone; NTI, nonthyroidal illness; rhTSH, recombinant human TSH; ROC, receiver operating characteristics; TRE, thyroid hormone response element; TRH, TSH-releasing hormone; TSH, thyroid stimulating hormone; TT_4, total thyroxine.

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concentrations of total thyroxine (TT_4) or free T_4 and thyroid stimulating hormone (TSH). A low plasma TT_4 concentration is a sensitive variable for diagnosing hypothyroidism in dogs.[2] However, a number of factors other than the intrinsic thyroid function can affect the circulating TT_4 concentration, such as certain drugs and nonthyroidal illnesses (NTI).[1,3,4] A low plasma TT_4 combined with a high plasma TSH confirms primary hypothyroidism. However, 30%-38%[5,6] of hypothyroid dogs have a plasma TSH concentration within the reference range. Consequently, a low plasma TT_4 concentration in combination with a plasma TSH concentration within the reference range does not distinguish between hypothyroidism and NTI.

To overcome this problem, other tests for diagnosing hypothyroidism are required. A TSH response test, using recombinant human TSH (rhTSH), is considered the gold standard.[7] But, even though rhTSH can be aliquoted and frozen for 4–8 weeks,[8] the high costs of the formulation prevent it from being a good diagnostic option for veterinary practitioners. The circulating free T_4 concentration is more specific than the plasma TT_4 concentration but less sensitive[9] and several free T_4 assays are not reliable.[10] Detection of antibodies against thyroglobulin does not prove that the dog has or will develop hypothyroidism[11] and negative results occur in dogs with hypothyroidism.[12] Histologic examination of thyroid tissue is regarded as the definitive test to identify thyroid disease, but taking thyroid biopsies is invasive and the presence of a lesion does not necessarily prove the existence of a functional abnormality.[13]

Imaging techniques have been investigated as an additional tool in diagnosing canine hypothyroidism. Scintigraphy, using radioactive pertechnetate ($^{99m}TcO_4^-$), is reported as a reliable method for diagnosing canine hypothyroidism, with in almost all cases a clear difference in thyroidal uptake of $^{99m}TcO_4^-$ between hypothyroid dogs and dogs with NTI.[14,15] Unfortunately, this diagnostic option is only available in a few referral centers, since specialist equipment and radiation-isolation facilities are needed.

Instead of a TSH stimulation test, a TSH-releasing hormone (TRH) stimulation test with measurement of the plasma TT_4 concentration has been proposed. Because many euthyroid dogs do not show a significant rise in the plasma TT_4 concentration, the TRH stimulation was discarded as diagnostic test for hypothyroidism in dogs.[16] Administration of TRH causes plasma TSH concentrations to rise significantly more in euthyroid dogs than in hypothyroid dogs.[17] This suggests that TRH stimulation tests with determination of the plasma TSH concentration can potentially differentiate hypothyroidism from NTI.

Another interesting observation is that hypothyroidism in dogs with a plasma TSH concentration above the reference range is associated with increased release of growth hormone (GH).[17,18] Moreover, plasma GH concentration increases significantly after TRH administration in these dogs with hypothyroidism, whereas this increase is absent in euthyroid dogs.[17]

The aim of our study was to evaluate whether a basal plasma GH concentration or a TRH stimulation test with measurements of GH and TSH plasma concentrations can differentiate between dogs with NTI and dogs with hypothyroidism that have a plasma TSH concentration within the reference range.

2 | MATERIALS AND METHODS

2.1 | Dogs

Inclusion criteria for dogs in our study were (1) presence of clinical signs compatible with hypothyroidism, (2) plasma TT_4 concentration below the reference interval (19–46 nmol/L) and a plasma TSH concentration within the reference interval (<0.60 μg/L), and (3) no use of medication that could affect test results during the last two months. A total of 21 dogs were included in our study. The dogs' median age was 7.4 years (range, 1–10 years). Median body weight was 28.8 kg (range, 14.2–75.0 kg). Fourteen dogs were male (4 neutered) and seven female (all neutered). 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 clinical signs. In addition, locomotion problems (lameness, stiff gait; n = 9), myxedema (tragic facial expression; n = 7), excessive snoring (n = 5), and a preference for warm places (n = 3) were noticed. At presentation, the time from onset of the signs ranged from 3 weeks to 2 years. All dogs were client-owned and owners signed an informed consent.

2.2 | $^{99m}TcO_4^-$ uptake

Scintigraphic imaging of the thyroid glands was performed with the Integrated ORBITER Gamma Camera System with Open Icon Workstation, equipped with a high resolution parallel-hole collimator. Dogs were injected IV with a dose of 111 to 166 MBq radioactive pertechnetate ($^{99m}TcO_4^-$) and after 45 minutes thyroid scintigraphy was performed. Based on the uptake pattern, dogs were subdivided into the hypothyroid group (no or limited uptake) or the NTI group (normal uptake).[15]

2.3 | TRH stimulation test

Blood samples for plasma TSH and GH measurements were collected 15 minutes before ($T = -15$), immediately before ($T = 0$), and 30 ($T = 30$) and 45 ($T = 45$) minutes after IV (cephalic vein) administration of 10 μg/kg body weight TRH (TRH, Protirelin 0.2 mg/mL; Ferring Arzneimittel GmbH, Fabrikstrasse 7, 24103 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 concentration analysis. Subsequently, the samples were centrifuged at 4°C and plasma samples were stored at −70°C until analysis.

2.4 | Assays

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 and as described previously.[19] The intra-assay coefficients of variation were 5.0 and 4.0% at TSH concentrations of 0.20 and 0.50 μg/L, respectively. The interassay coefficient of variation was

6.3% at a TSH concentration of 0.16 μg/L. The detection limit of the TSH assay was 0.03 μg/L.

Plasma GH concentrations were determined by a homologous radioimmunoassay, as described earlier.[20] The intra- and inter-assay coefficients of variation were 3.8 and 7.2%, respectively, and the sensitivity was 0.3 μg/L.

2.5 | Statistical analysis

IBM SPSS version 24 for Windows was used for statistical analysis. Sex and neutering status were tested with a chi-squared test. Body weight and age were compared with an independent samples *t*-test. Differences between basal plasma concentrations (as an average between $T = -15$ and $T = 0$) and concentrations at 30 and 45 minutes of TSH, GH, and GH/TSH ratio were tested with a Friedman test; when significant a Wilcoxon's signed rank test (with Bonferroni correction) was used as post-hoc test. Differences in hormone values between hypothyroid dogs and dogs with NTI were compared with a Mann-Whitney *U* test. Differences were considered significant at $P < .05$. Hormonal values are presented as median and range. Receiver operating characteristic (ROC) curve analysis was performed to determine optimal cut-off levels.

3 | RESULTS

Based on thyroid scintigraphy, 11 dogs were diagnosed with hypothyroidism and ten were classified as dogs with NTI. There were no significant differences in baseline characteristics between the hypothyroid and NTI dogs, except for sex. There were significantly ($P = .013$) more males in the hypothyroid group than in the NTI group.

The median basal plasma GH concentration in the hypothyroid dogs (3.2 μg/L; range, 2.0–12.5 μg/L) was significantly higher ($P < .001$) than that in the NTI dogs (0.73 μg/L; range, 0.45-2.3 μg/L). Receiver operating characteristic curve analysis revealed a cut-off level of 1.8 μg/L, which identified correctly all dogs with hypothyroidism and 9 of the 10 dogs with NTI.

No adverse effects were noted in the dogs after administration of TRH. The plasma GH concentration increased significantly ($P = .009$) after TRH administration in the hypothyroid dogs (Figure 1A) and post hoc analysis revealed significant differences in the time intervals $T = 0$ to $T = 30$ ($P = .009$) and $T = 0$ to $T = 45$ ($P = .006$). In the NTI group, the plasma GH concentration did not change significantly ($P = .15$) after TRH administration. At time points $T = 30$ and $T = 45$, the NTI group and hypothyroid group had no overlap in plasma GH concentrations. Receiver operating characteristic curve analysis revealed a cut-off level at $T = 30$ of 1.95 μg/L and at $T = 45$ of 1.75 μg/L, with complete distinction between hypothyroidism and NTI.

The median basal plasma TSH concentration in the hypothyroid dogs (0.31 μg/L; range, 0.09-0.47 μg/L) did not differ ($P = .11$) from that in the NTI dogs (0.11 μg/L; range, 0.03-0.51 μg/L). The plasma TSH concentration did not change significantly ($P = .06$) in the hypothyroid dogs after TRH administration, but it did increase significantly ($P < .001$) in the NTI group (Figure 1B). Post hoc analysis revealed

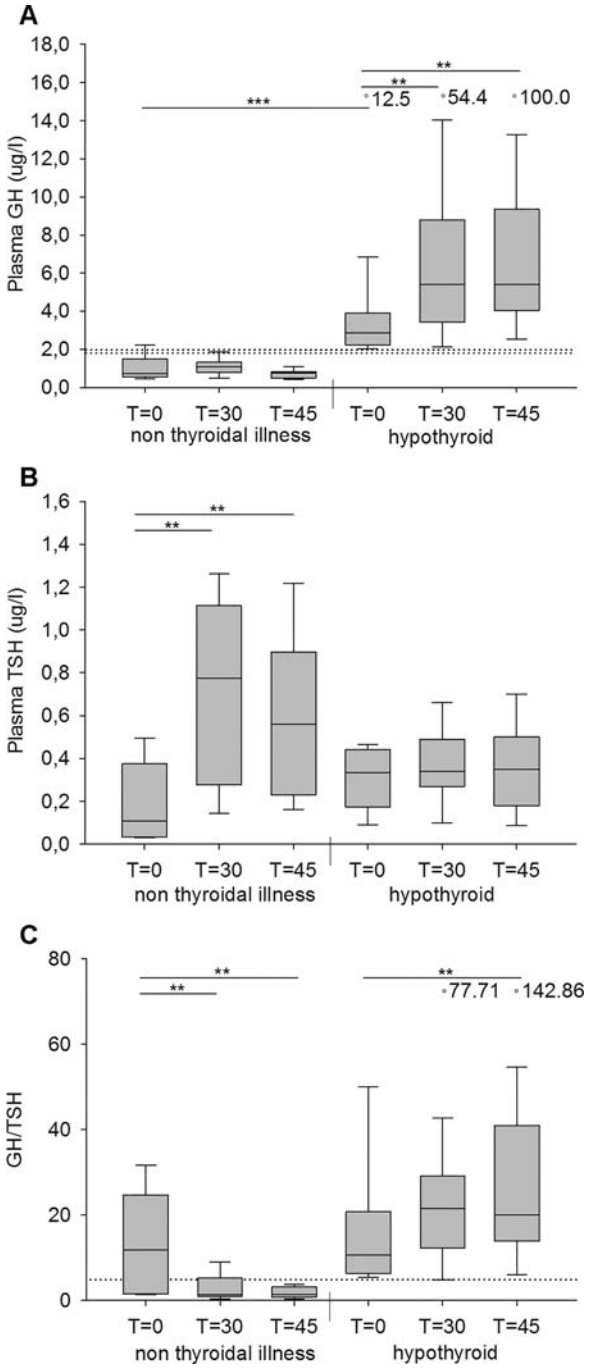


FIGURE 1 Boxplots showing plasma GH concentrations (μg/L; panel A), plasma TSH concentrations (μg/L; panel B) and the ratio between the plasma concentrations of GH and TSH (GH/TSH; panel C) at baseline, and at 30 and 45 minutes after TRH administration in 10 dogs with NTI and 11 dogs with hypothyroidism. One outlier is plotted individually. Double asterisks (**) indicate significant ($P < .01$) difference. Triple asterisks (***) indicate significant ($P < .001$) difference in baseline plasma GH concentrations between groups. Dotted lines in panel A represent cut-off levels (1.95 μg/L for $T = 30$ and 1.75 μg/L for $T = 45$). At $T = 30$ and $T = 45$, there was no overlap between hypothyroid dogs and dogs with NTI. The dotted line in panel C represents the cut-off level (4.6) at $T = 45$. At $T = 45$, there was no overlap between hypothyroid dogs and dogs with NTI

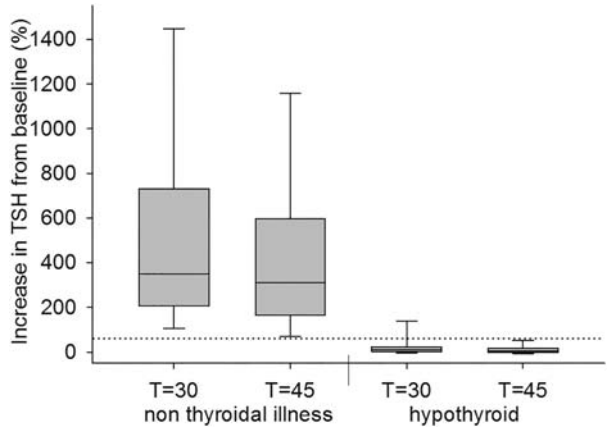


FIGURE 2 Boxplot showing the percentage increase in plasma TSH concentrations (%) from baseline, at 30 and 45 minutes after TRH administration in 10 dogs with NTI and 11 dogs with hypothyroidism. The dotted line represents the cut-off level (57%) at T=45. At T=45, there was no overlap between hypothyroid dogs and dogs with NTI

significant differences in the time intervals $T = 0$ to $T = 30$ ($P = .005$) and $T = 0$ to $T = 45$ ($P = .005$), but with some overlap of the absolute values. At $T = 30$, the median percentage increase in plasma TSH concentration from baseline in hypothyroid dogs (10%, range, −6%-161%) was significantly lower ($P < .001$) than in dogs with NTI (351%, range, 96%-1457%). Also at $T = 45$ the median percentage increase in plasma TSH concentration from baseline in hypothyroid dogs (4%, range, −6%-52%) was significantly lower ($P < .001$) than in dogs with NTI (311%, range, 62%-1167%; Figure 2), and no overlap was present between hypothyroid dogs and NTI dogs at this time point. Receiver operating characteristic curve analysis revealed a cut-off level at $T = 45$ of 57%.

When the ratio between GH and TSH (the GH/TSH ratio) was calculated (Figure 1C), the median basal GH/TSH ratio in the hypothyroid dogs (12.85; range, 5.38–52.35) did not differ ($P = .53$) from that in the NTI dogs (11.85; range, 1.37-32.00). The GH/TSH ratio decreased significantly ($P = .001$) after TRH administration in the NTI group; post hoc analysis revealed significant differences in the time intervals $T = 0$ to $T = 30$ ($P = .005$) and $T = 0$ to $T = 45$ ($P = .005$). In contrast, the GH/TSH ratio increased significantly ($P = .038$) after TRH administration in the hypothyroid group and post hoc analysis revealed a significant difference in the time intervals $T = 0$ to $T = 45$ ($P = .008$) but not in $T = 0$ to $T = 30$ ($P = .091$). At $T = 45$, no overlap was present in the GH/TSH ratio between the dogs with NTI and the hypothyroid dogs. Receiver operating characteristic curve analysis revealed that at $T = 45$ a GH/TSH ratio > 4.60 corresponded with hypothyroidism and a value < 4.60 with NTI.

4 | DISCUSSION

Diagnosing primary hypothyroidism can be challenging, as NTI can also lead to a decreased plasma TT_4 concentration. Our study shows that measurement of basal plasma GH concentration and plasma concentrations of GH and TSH after TRH stimulation can distinguish between hypothyroidism and NTI in dogs. The study was unique in that only dogs were enrolled with a low plasma TT_4 concentration and a plasma TSH concentration within the reference range, that is, characteristics that may be found both in hypothyroid dogs and dogs with NTI. These animals pose the greatest diagnostic challenge in veterinary practice, and this results in overtreatment with l-thyroxine of many dogs with NTI and underdiagnosing true primary hypothyroid dogs.

In our study, thyroidal $^{99m}TcO_4^-$ uptake was used as diagnostic test to differentiate between NTI and hypothyroid dogs. This test has proved to have the highest discriminating power between the two groups, with hypothyroid dogs having little to no uptake of $^{99m}TcO_4^-$.[14,15,21] For practitioners this diagnostic test is hardly helpful, because it is only available in a few referral centers, since specialist equipment and radiation-isolation facilities are required.

In our study, the basal plasma GH concentration was increased in the hypothyroid dogs, which is in accordance with previous studies in hypothyroid dogs with a plasma TSH concentration above the reference range.[18,22] In contrast, in some mammals, such as sheep, primary hypothyroidism does not seem to affect GH secretion,[23] whereas in humans, rats and pigs primary hypothyroidism is associated with low circulating GH concentrations.[24–28] The human and rat pituitary GH gene contains a positive thyroid hormone response element (TRE),[29] and as a consequence the low thyroid hormone concentrations of hypothyroidism will result in low expression of pituitary GH in these species. Indeed, in hypothyroid rats decreased pituitary content of both GH mRNA and GH protein have been reported.[25,27] The lack of a decrease in GH release in dogs with primary hypothyroidism might be explained by the absence of a positive TRE within the canine GH gene, as sequence analysis of the promoter region and intron 1 of the canine GH gene has not demonstrated the existence of a TRE in this species.[30]

Apart from direct effects on transcription of the GH gene, hypothyroidism might affect pituitary GH release via an influence on the hypothalamic hormones somatostatin and TRH. Somatostatin is not only the main inhibitory factor for pituitary GH release, but also the main inhibitory factor for pituitary TSH release. In case of primary hypothyroidism, the decreased feedback signal from thyroid hormones on the hypothalamic-pituitary axis should result in increased pituitary TSH production and release, which might be partly mediated by less somatostatin secretion, which will also result in less suppression of GH release.

The decreased feedback signal from thyroid hormones on the hypothalamic-pituitary axis will not only result in less somatostatin secretion, but also in more TRH secretion. This increased TRH secretion might be one of the reasons for the increased basal plasma GH concentration in our hypothyroid dogs. In healthy humans and euthyroid rats administration of TRH elicits no or only a small transient rise in plasma GH concentrations, whereas in humans and rats with primary hypothyroidism TRH administration results also in significantly increased plasma GH concentrations.[31,32] Similarly, TRH administration caused a paradoxical rise in plasma GH concentrations in our hypothyroid dogs, as described previously.[17]

The aim of our study was to investigate whether basal and TRH-induced plasma GH concentrations can be used to differentiate

between NTI dogs and dogs with primary hypothyroidism with a low plasma T_4 concentration and a plasma TSH concentration within the reference range, as they pose the main diagnostic challenge. Using a cut-off level for the basal plasma GH concentration of 1.8 μg/L identified correctly 100% of the dogs with hypothyroidism and 9 out of 10 dogs with NTI. Because GH is secreted in a pulsatile fashion,[33] it might be expected that dogs with NTI might have a plasma GH concentration above 1.8 μg/L at some time points of the day. A basal plasma GH concentration below 1.8 μg/L, however, strongly indicates that hypothyroidism is unlikely. More certainty can be obtained when a TRH stimulation test is performed. At 45 minutes after TRH administration no overlap was seen between the NTI and hypothyroid dogs in plasma GH concentrations. All dogs with primary hypothyroidism had a plasma GH concentration above 1.75 μg/L, and all NTI dogs had a value below 1.75 μg/L. Although the results of our study show that measurement of circulating GH concentration might be very helpful to distinguish between hypothyroidism and NTI in dogs, the limited availability of a reliable canine GH assay is currently a limiting factor in the application of this method in veterinary practice.

As may be expected, TRH administration resulted in a significant increase in the plasma TSH concentration in the NTI dogs. However, a significant TRH-induced increase in plasma TSH concentration was absent in the hypothyroid dogs. This lack of TSH response to TRH stimulation in our hypothyroid dogs has been reported before.[5,17,34] An explanation for this lack of TSH increase upon TRH stimulation has been suggested to be exhaustion of thyrotropes[35] or desensitization for TRH stimulation.[22] Another possibility is that with time isoforms of TSH are produced that are not measured by the available TSH assays.[5] By using the percentage increase in plasma TSH concentration after TRH administration, a complete distinction between hypothyroid dogs and NTI dogs was found.

Taking into account the opposite effects of TRH administration on circulating concentrations of GH and TSH in the NTI dogs and hypothyroid dogs, we calculated the ratio between GH and TSH. Using a cut-off value for the GH/TSH ratio of 4.60, the distinction between the NTI dogs and hypothyroid dogs was even more clear than with only the plasma GH concentration.

The data from our study are promising but research in a larger cohort of dogs is needed to further verify the usability and reliability of the TRH stimulation test as a method of differentiating primary hypothyroidism and NTI in dogs. For example, in some dogs with NTI, such as during sulfonamide treatment[4] and in dogs with untreated hypoadrenocorticism[36] an increased circulating TSH concentration may be present, and it would be interesting to investigate the effects of TRH administration in these dogs. Furthermore, it might be that dogs with a low plasma T_4 concentration because of hypercortisolism, that is, dogs with NTI, will have a limited response in plasma TSH concentration after TRH administration.[37]

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

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