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# Relationship between thyroid function and sex hormones in female German shepherd dogs

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## Abstract

**Background:** Several variables influence the serum concentration of thyroid hormones in dogs, including breed, age, drugs, and concurrent diseases. However, data regarding the interaction between thyroid function and the estrous stage of female dogs are limited.

**Hypothesis:** Estrous stage may influence thyroid function in German Shepherd dogs.

**Methods:** Longitudinal, observational, non-randomized cohort study. The dogs were monitored during the complete estrous cycle, and different stages were determined by vaginal cytology. Two blood samples were collected at the beginning and end of each stage to analyze the following: total thyroxine (TT4), free thyroxine (fT4), total triiodothyronine (TT3), free triiodothyronine (fT3), canine thyrotropin (cTSH), progesterone, 17-β-estradiol, triglycerides, and cholesterol concentrations. Hematological and biochemical evaluations were performed at the beginning and end of the study period.

**Animals:** Seventeen German Shepherds were included, of which 7 were bred during the study period. One dog was excluded for estrus interruption and another for suspected hypothyroidism.

**Results:** Serum concentrations of T4, fT4, and fT3 were negatively correlated with age. Total thyroxine demonstrated significant changes in serum concentrations between estrous stages, with higher concentrations in estrus and diestrus. Total thyroxine concentrations were positively correlated with progesterone concentrations and negatively correlated with 17-β-estradiol concentrations. Free thyroxine did not show significant variations but was positively correlated with progesterone concentrations. Canine TSH concentrations were positively correlated with 17-β-estradiol concentrations. No significant differences in thyroid hormones and cTSH concentrations were observed between diestrus during pregnancy and pseudopregnancy.

**Conclusions:** Different stages of estrus can influence the measurement of TT4 in female dogs.

**Abbreviations:** 95% CI, 95% confidence interval; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANA test, antinuclear antibodies test; AST, aspartate transferase; cTSH, canine thyroid-stimulating hormone; ENCI, Italian Kennel Club (Ente Nazionale Cinofilia Italiana); fT3, free triiodothyronine; fT4, free thyroxine; GGT, gamma-glutamyl-transferase; TRH, thyrotropin releasing hormone; TT3, total triiodothyronine; TT4, total thyroxine.

KEYWORDS
canine, dog, estradiol, hormones, progesterone, thyroxine, triiodothyronine

## 1 | INTRODUCTION

Assessment of thyroid function in dogs is challenging. In veterinary medicine, it has been reported that serum concentrations of thyroid hormones are affected by a series of non-thyroidal factors, which can compromise the specificity of the available tests.[1,2] Among these, age has been reported as an influencing factor, with older dogs having total thyroxine (TT4) concentrations below the reference intervals (RIs) and dogs aged <3 months having TT4 concentrations 5 times higher than those in adults.[1] Breed also should be considered when assessing thyroid function.[3-6] Although intense physical activity can decrease thyroid hormone concentrations, brief exercise has little effect on these concentrations.[7-12] Suppression of total triiodothyronine (TT3) and TT4 in non-thyroidal diseases has been widely reported, with a larger decrease in patients with severe disease and worse prognosis.[13-16] Finally, drugs such as glucocorticoids, phenobarbital, aspirin, ketoprofen, carprofen, clomipramine, and sulfonamide-containing antibiotics can influence the serum concentrations of thyroid hormones and pituitary canine thyrotropin (thyroid-stimulating hormone; cTSH).[17-20]

When the specific stage of the female reproductive cycle is not considered, and dogs are simply classified as female or male, sex does not have an apparent effect on thyroid hormone concentration.[21] However, studies conducted in humans, with reference to menopausal women, and in an ovariectomized rat model, inducing iatrogenic hyperestrogenism and hyperprogesteronism, highlight the possible influence of sex hormones on thyroid secretory activity.[22-29]

Data on the interaction between thyroid function and reproductive status in female dogs are limited. A previous study identified significant variation in TT4 and fT4 concentrations in female Beagles, suggesting the need for further investigation.[21] As reported in human medicine and rat models, sex hormones can influence thyroid secretory activity in dogs. In the context of the estrous cycle, such an effect, mediated by the predominance of progesterone or 17-β-estradiol depending on the phase considered, could result in significant changes in the hormones normally investigated in thyroid function assessment. We aimed to monitor total and free thyroxine (fT4), total and free triiodothyronine (fT3), canine thyrotropin, progesterone, and 17-β-estradiol concentrations over the course of the estrous cycle in adult and clinically healthy female German shepherds. We hypothesized that the different phases of estrus may influence the assessment of thyroid function in female dogs.

## 2 | MATERIALS AND METHODS

### 2.1 | Study design

This longitudinal, observational, non-randomized cohort study included 17 dogs over a period of 1.5 years (2012-2013).

### 2.2 | Ethical approval

Each dog owner signed a written informed consent form before enrollment, as required by regulations. The study was conducted between the 2nd half of 2012 and the end of 2013, before enactment of Legislative Decree No. 26 on March 4, 2014, and the establishment of the Animal Welfare Board. This opinion was postponed to allow publication of the data obtained.

### 2.3 | Dogs

The enrolled dogs had to be intact female German Shepherds, and consequently registration with the Italian Kennel Club (ENCI: Ente Nazionale Cinofilia Italiana) was required: adult, sexually mature with the first estrus cycle already occurring, aged ≤6 years, and without any history of endocrine, reproductive, metabolic, or immune-mediated disease. All dogs had to be clinically healthy, with hematology and serum biochemistry results in normal ranges, without any disease or use of any drugs or iodine supplementation in the previous 60 days. Furthermore, no drugs or supplements were provided without medical consent during the trial. The uncontrolled variables were diet and living environment; all dogs were fed a commercial maintenance diet and were living in the same district in northeastern Italy.

### 2.4 | Study protocol

Seventeen clinically healthy female German Shepherd dogs aged 9 months to 6 years were monitored during a complete estrous cycle (anestrus, proestrus, estrus, and diestrus), and the different phases were determined by vaginal cytology using the Harris-Schorr staining method and progesterone concentration.[30] Cytological sampling and progesterone measurements were performed every 4-5 days during proestrus and estrus, with an average of 2 samplings for each phase, and every 30-40 days during the diestrus and anestrus phases, with an average of 2 samplings for each phase. Cytological assessment of the different estrous stages followed guidelines applied in previous studies.[31] Slide readings were performed in duplicate by a diplomate of the European College of Animal Reproduction and a second operator employed daily in the clinical setting. Cytological specimen collection and smear preparation were performed by a third operator.

The following criteria were applied in the interpretation of the vaginal smears: first, anestrus referred to a predominance of basal and parabasal cells, round in shape and nucleated, characterized by a high nucleus-to-cytoplasm ratio. Rare leukocytes and bacteria and abundant amorphous extracellular material could be observed. Second, proestrus referred to the predominance of intermediate, polygonal,

**TABLE 1** Median and interquartile range of serum hormones and lipids concentrations during each stage of the estrus cycle.

| | Proestrus | | Estrus | | Diestrus | | Anestrus | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Median | IQR | Median | IQR | Median | IQR | Median | IQR | (n) | RIs |
| cTSH (ng/mL) | 0.11 | 0.06-0.14 | 0.15 | 0.07-0.27 | 0.11 | 0.06-0.18 | 0.11 | 0.07-0.19 | 107 | <0.5 ng/mL |
| TT4 (µg/dL) | 2.1 | 1.7-2.2 | 2.5 | 2.15-3.05 | 2.6 | 2.4-2.92 | 2.2 | 2-2.5 | 107 | 1.0-4.7 µg/dL |
| fT4 (ng/dL) | 0.88 | 0.69-0.99 | 1.04 | 0.86-1.29 | 0.89 | 0.75-1.16 | 0.94 | 0.78-1.09 | 107 | 0.6-3.7 ng/dL |
| TT3 (ng/mL) | 0.47 | 0.37-0.6 | 0.57 | 0.44-0.62 | 0.54 | 0.43-0.63 | 0.57 | 0.43-0.72 | 107 | 0.45-1.5 ng/mL |
| fT3 (pg/mL) | 2.41 | 2.16-3.19 | 2.9 | 2.65-3.75 | 2.45 | 2.05-3.67 | 2.95 | 2.29-3.79 | 107 | [a] |
| P (ng/mL) | 0.84 | 0.46-1.53 | 16.3 | 3.43-21.79 | 18.65 | 2.42-31.7 | 0.54 | 0.21-0.86 | 107 | [b] |
| $E_2$ (pg/mL) | 17.7 | 4-34.8 | 8.4 | 4.5-22 | 5 | 4.5-13.2 | 6.2 | 4.5-14.2 | 107 | [b] |
| TRIG (mg/mL) | 74 | 51-85.2 | 68.5 | 52-76.5 | 69 | 54.7-80.2 | 64 | 57-69 | 105 | 10-114 mg/mL |
| CHOL (mg/mL) | 190.5 | 157.7-214.5 | 207 | 168.5-244.5 | 206 | 166-245.7 | 180 | 159.5-206.5 | 105 | 101-275 mg/mL |

*Note*: On the right are reported the reference intervals applied for each variable and the total number of determinations on which the data are calculated (total, n).

Abbreviations: CHOL, total cholesterol; cTSH, canine thyroid stimulating hormone; E2, 17-β-estradiol; fT3, free triiodothyronine; fT4, free thyroxine; IQR, interquartile range; P, progesterone; RIs, reference intervals; TRIG, triglycerides; TT3, total triiodothyronine; TT4, total thyroxine.

[a]Reference interval not available at the time.

[b]Unique reference range for all the phases of the estrous cycle not available.

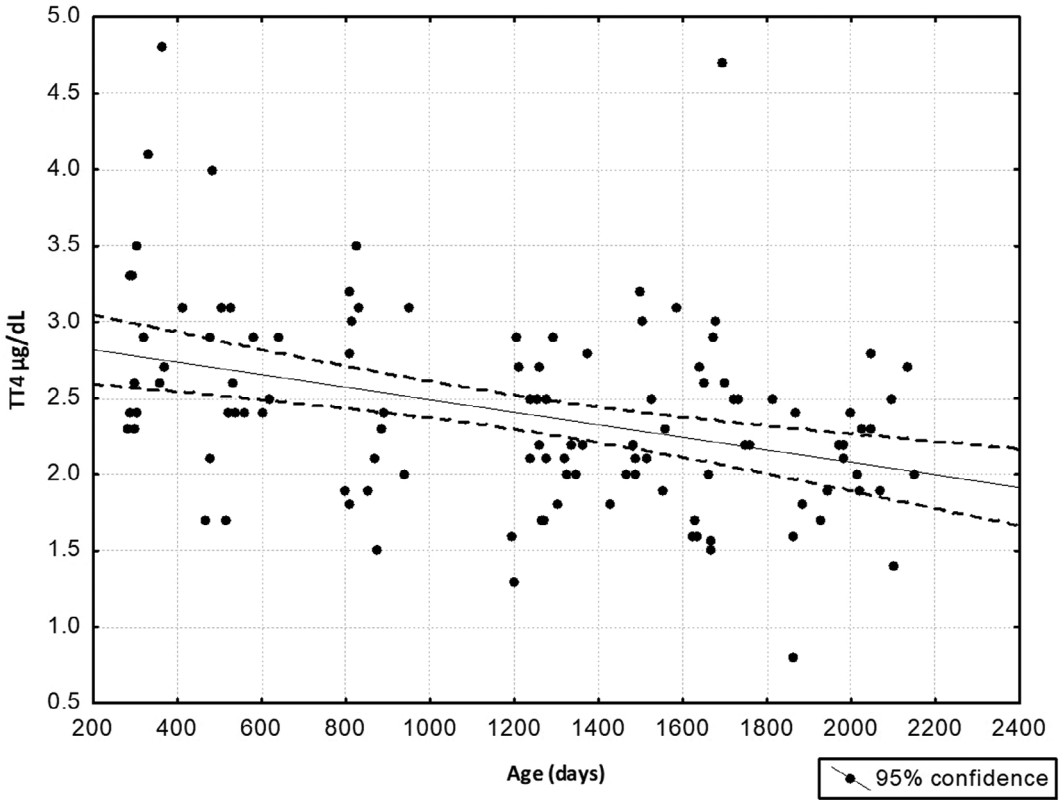


**FIGURE 1** Association between total thyroxine (TT4) and age (expressed in days), results from correlation test. A significant negative correlation was found between TT4 and age. Regression coefficient (*r*) and *P* value (*P*): $r = -0.367$; $P < .05$. Reference interval for TT4: 1.0-4.7 µg/dL. 95% confidence, 95% confidence interval; TT4, total thyroxine.

and nucleated superficial cells. Abundant erythrocytes and moderate numbers of leukocytes and bacteria as well as moderately amorphous extracellular materials could be present. Third, estrus referred to the predominance of anucleated superficial cells or cells with a low nucleus-to-cytoplasm ratio. Erythrocytes, leukocytes, and bacteria as well as amorphous extracellular materials were scarce. Finally, diestrus

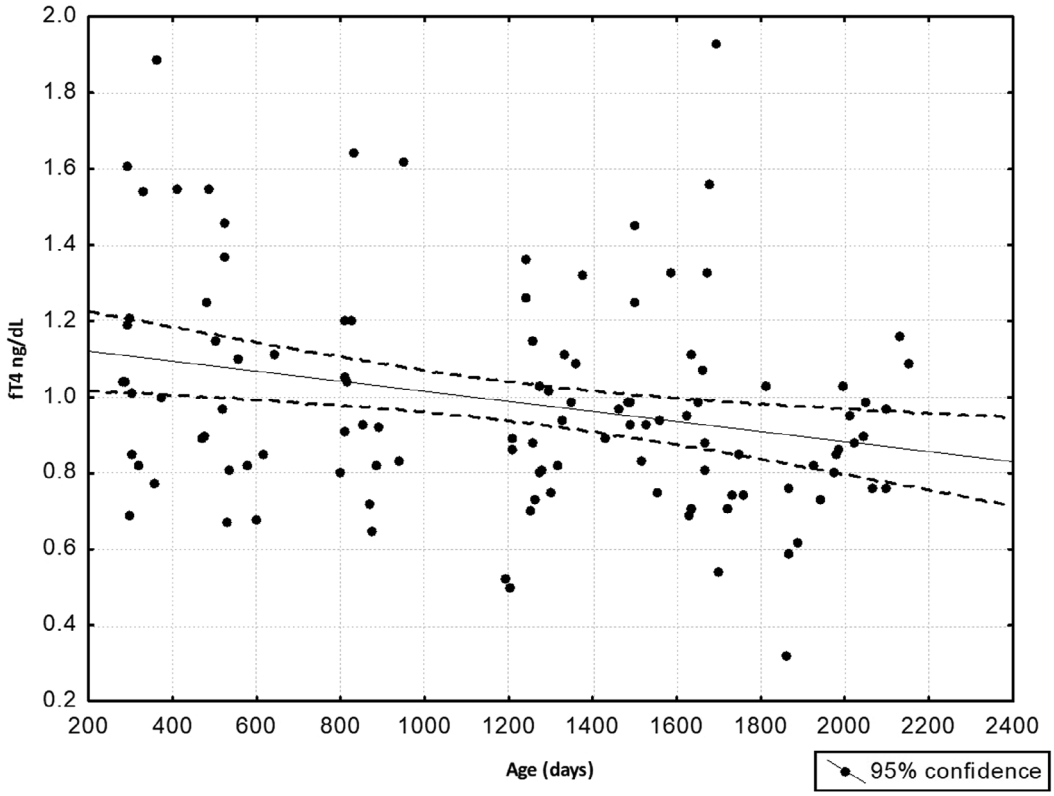


**FIGURE 2** Association between free thyroxine (fT4) and age (expressed in days), results from correlation test. A significant negative correlation was found between fT4 and age. Regression coefficient (*r*) and *P* value (*P*): $r = -0.266$; $P < .05$. Reference Interval for fT4: 0.6-3.7 ng/dL. 95% confidence, 95% confidence interval; fT4, free thyroxine.

referred to the predominance of intermediate, basal, and parabasal cells. Occasional foam cells, moderate leukocytes and bacteria, and abundant amorphous extracellular materials were observed.[31]

Two blood samples were collected from the cephalic vein at the beginning and end of each phase of the estrous cycle between 11 am and 2 pm and after 12 to 24 hours of fasting.[32] Thyroid hormones (TT4, fT4, TT3, fT3), cTSH, progesterone, and 17-β-estradiol concentrations were assessed in each blood sample. Serum triglyceride and cholesterol concentrations were monitored on each occasion to better interpret altered thyroid function.

At the beginning and end of the study, a CBC and measurements of serum urea, creatinine, glucose, total protein, albumin, total bilirubin, total cholesterol and triglyceride concentrations and alanine transaminase, aspartate aminotransferase, alkaline phosphatase and gamma-glutamyl transferase activities were performed. Seven dogs were bred during the study, and hormone concentrations were evaluated during pregnancy.

## 2.5 | Analytical procedures

A total of 5 mL blood was collected at each sampling. Specifically, 1 mL of the sample, collected in $K_3$-EDTA (Miniplast; LP Italiana S. p.A., Milan, Italy), was used for CBC and blood smear examination. Blood smears were prepared and examined immediately after collection by an experienced veterinarian at the clinic's in-house laboratory. The remaining blood was transferred to a plain tube (Vacuette; Greiner Bio-One GmbH, Kremsmünster, Austria) at a maximum capacity of 5 mL. After 10 minutes, the plain tubes were centrifuged at 1000 × *g* for 20 minutes to obtain serum, which then was transferred to plain conical tubes (Eppendorf AG, Hamburg, Germany). The aliquoted serum was stored at 4°C, and then sent to the veterinary laboratory of the University of Milan or to IDEXX laboratories for the remaining analytical procedures. The samples were transported inside refrigerated containers and processed within 24 hours of collection.

An automated enzyme immunoassay analyzer (AIA-360; Tosoh Bioscience, Tokyo, Japan)[33] was used to measure TT4, fT4, TT3, fT3, progesterone, and 17-β-estradiol concentrations, whereas cTSH was measured by chemiluminescence (Immulite 1000; Siemens Medical Solutions-Diagnostics, USA). The reference ranges for the applied methods are listed in Table 1. All biochemical variables were assessed using an automated spectrophotometer (Cobas Mira; Roche Diagnostic Systems, Basel, Switzerland). The CBC was performed using an Abacus Junior Vet (Diatron Messtechnik GmbH, Austria).

## 2.6 | Data analysis

Sample size calculations were performed based on the mean concentrations of cTSH and fT4, for which historical in-clinic data for German Shepherd females of different ages are available. A 2-way Student's

*t* test ($\alpha = .05$) for the mean cTSH concentration of 0.15 ng/mL had a power of 80% to detect a 30% difference with a group size of 15 subjects. Similarly, for fT4, the group size sufficient to detect a 30% difference, at significance level $\alpha = .05$, was 11 subjects. Pearson's *r* correlation test was performed among thyroid hormones and cTSH vs age, thyroid hormones, cTSH vs sex hormones, 17-β-estradiol, and cholesterol concentrations. A 1-way analysis of variance (ANOVA) was performed to compare the average concentrations of thyroid and pituitary hormones among different stages of the estrous cycle and between diestrus during pregnancy and pseudopregnancy. Owing to the large variability, a logarithmic transformation of the progesterone data was performed for analysis. Concerning 17-β-estradiol measurement, 5 pg/mL was the limit of detection (LOD) of the method in use. Values below the LOD were recorded as 4.5 in the statistics. Data were tested for normality before applying ANOVA. A 2-tailed *t* test, with Welch correction in the case of unequal variances, was performed to compare thyroid hormone and cTSH serum concentrations between diestrus and pregnancy. The effect size (Cohen's d) or root mean square standardized effect was calculated to measure the magnitude and strength of significant findings. Statistical analyses were performed using Statistica 7.1 (Statsoft, Inc., USA) and Med Calc 16.1.2 (MedCalc Software bvba); the former also was used for power analysis.

## 3 | RESULTS

### 3.1 | Study population

In total, 17 clinically healthy German Shepherds were included in the study. All of the recruited subjects had previously participated in dog shows. Nonetheless, in the medical history, no intense physical activity in the previous 6 months was reported for any of the dogs. Moreover, for the 2 subjects still included in dog shows, exhibition activity was suspended for the entire study period according to the ENCI regulations.

At the beginning of the trial, the age of the population ranged from 280 to 2014 days (median, 1238 days; interquartile range [IQR], 516-1630 days). Serum concentrations of the investigated hormones and blood lipids during different phases of the estrous cycle are presented in Table 1 (median and IQR).

Of the tested females, 7 were mated and diagnosed as pregnant during the study period. For these subjects, with the exception of 1 that was excluded from further analysis because of suspected hypothyroidism, the hormone trends between diestrus during pregnancy or pseudopregnancy also were evaluated.

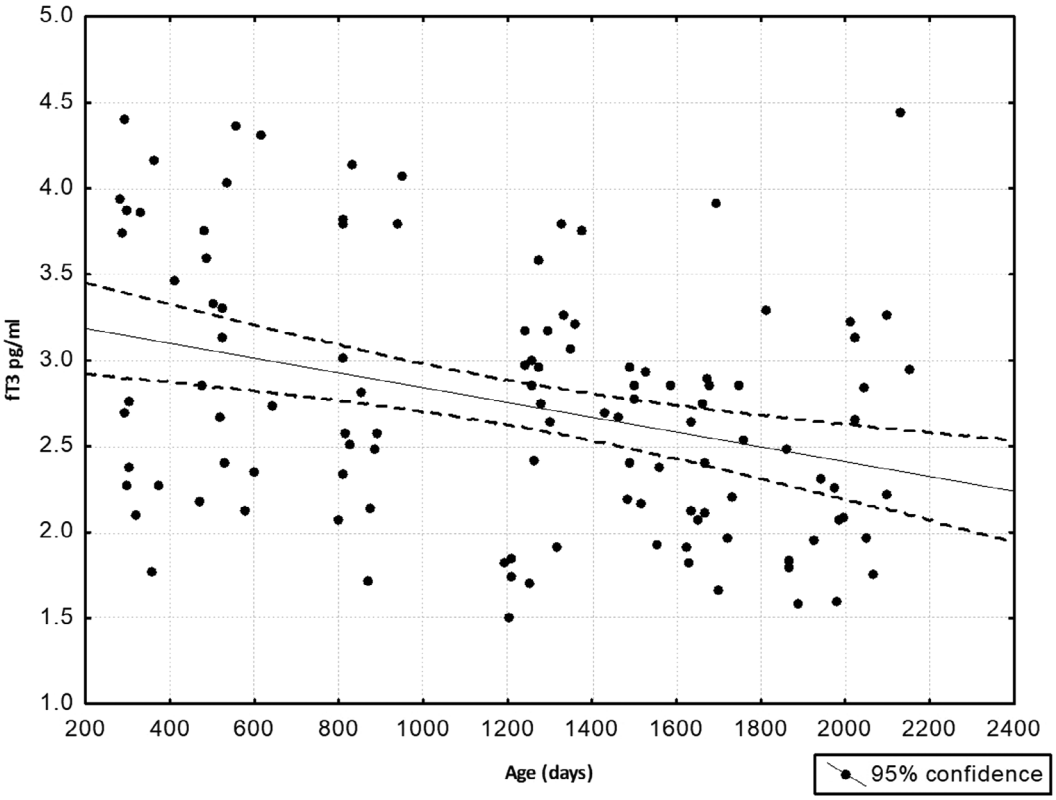


**FIGURE 3** Association between free triiodothyronine (fT3) and age (expressed in days), results from correlation test. A significant negative correlation was found between fT3 and age. Regression coefficient (*r*) and *P* value (*P*): $r = -0.335$; $P < .05$. 95% confidence, 95% confidence interval; fT3, free triiodothyronine.

### 3.2 | Exclusion of dogs

Two dogs were excluded from the study. The first had abnormally increased cTSH concentrations and tested positive for thyroglobulin autoantibodies. The second patient had interrupted estrus twice. The presence of a large ovarian cyst subsequently was diagnosed by ultrasound examination.

### 3.3 | Associations among thyroid hormones, cTSH, and age

A significant negative correlation between age and TT4 (n = 107; $r = -0.367$; $P < .05$), fT4 (n = 107; $r = -0.266$; $P < .05$), and fT3 (n = 107; $r = -0.335$; $P < .05$) was identified (Figures 1-3). No correlation was observed between age and serum TT3 or cTSH concentrations.

### 3.4 | Thyroid hormones and cTSH concentrations in the different stages of estrus

The 1-way ANOVA indicated that only TT4 serum concentrations were significantly different during the different phases of the cycle. In particular, concentrations measured during the estrus and diestrus stages were higher than those in the proestrus and anestrus stages ($F_{3,55} = 5.14$; $P = .03$) with an effect size of 0.641 (large). During proestrus, the TT4 concentration was significantly lower than that in all the other phases ($P = .003$; Figure 4).

Of the 107 determinations, only 1 serum TT4 concentration was below the reference range of the method (Figure 1). Of the 107 serum fT4 determinations, 5 were below the reference range of the method (Figure 2). None of the subjects included in the statistical analysis had serum cTSH concentrations above the reference range at any time during the follow-up period.

No significant differences were detected in thyroid hormone concentrations among diestrus, pregnancy, and pseudopregnancy. In addition, cTSH concentration during diestrus and pregnancy was not significantly different according to the 2-tailed $t$ test ($P = .05$), although a higher cTSH concentration during pregnancy was evident.

### 3.5 | Associations among thyroid hormones, cTSH, and progesterone

Considering thyroid and pituitary hormones vs sex hormones, the Pearson test indicated a significant and positive correlation between TT4 (n = 107; $r = 0.40289$; $P < .001$) and fT4 (n = 107; $r = 0.260$;

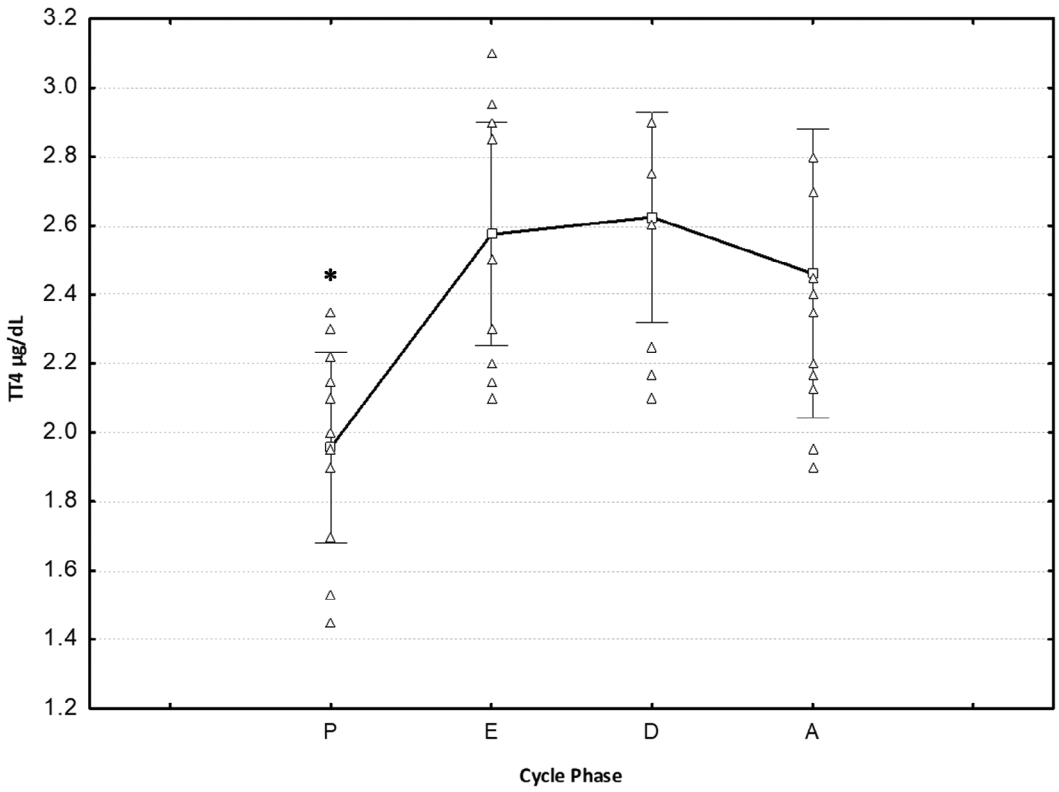


**FIGURE 4** Distribution of total thyroxine (TT4) concentrations (µg/dL) around the mean in the different phases of the cycle. The vertical bars represent the ±95% confidence interval (CI). Mean value is represented by the squares. During proestrus TT4 concentration was significantly lower compared to all other phases ($P = .003$), as indicated by the asterisk. Reference interval for TT4: 1.0-4.7 µg/dL. A, anestrus; D, diestrus; E, estrus; P, proestrus; TT4, total thyroxine.

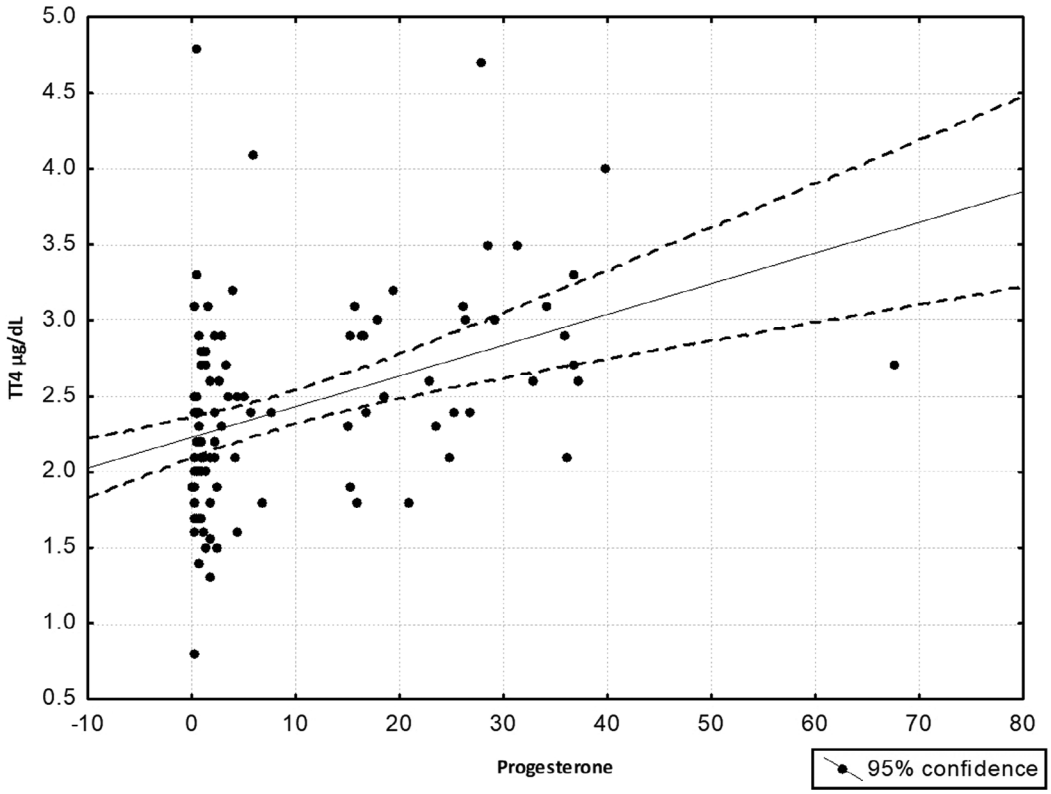


**FIGURE 5** Association between total thyroxine (TT4) and progesterone, results from correlation test. A significant positive correlation was found between TT4 and progesterone. Regression coefficient (*r*) and *P* value (*P*): $r = 0.40289$; $P < .001$. Reference interval for TT4: 1.0-4.7 µg/dL. 95% confidence, 95% confidence interval; TT4, total thyroxine.

$P = .007$) with progesterone. No correlation between progesterone and cTSH, TT3, or fT3 was identified (Figures 5 and 6).

### 3.6 | Associations among thyroid hormones, cTSH, and 17-β-estradiol

Canine TSH was positively and significantly correlated with 17-β-estradiol (n = 107; $r = 0.2$; $P = .04$; Figure 7), whereas TT4 was negatively and significantly correlated with 17-β-estradiol (n = 107; $r = -0.3179$; $P = .001$; Figure 8). Concentrations of 17-β-estradiol below the LOD were recorded only in the diestrus and anestrus phases. No other correlations between sex hormones and fT4, TT3, or fT3 concentrations were identified.

### 3.7 | Association among cholesterol and 17-β-estradiol

Cholesterol was negatively and significantly correlated with 17-β-estradiol (n = 105; $r = -0.3355$; $P = .0001$) and was significantly higher during diestrus ($F_{4,100} = 3.698$; $P = .001$) compared to the other phases of the cycle. No significant correlations between total triglycerides and 17-β-estradiol and between total triglycerides and total cholesterol and progesterone were observed.

### 3.8 | Vaginal smears

Total agreement between the 2 operators involved was found in the interpretation of vaginal smears. Vaginal cytology results also were consistent with the data on serum progesterone concentration.

## 4 | DISCUSSION

We aimed to analyze variations in serum concentrations of thyroid hormones and cTSH during different phases of the estrous cycle. To recruit a completely homogeneous population, each variable previously identified as a confounding factor was considered. In dogs, age, breed, previous drug treatments, concurrent diseases, and intense exercise have been established to influence thyroid function assessment.[1,2]

Specifically, in terms of breed-related variability, studies on large dog populations have found significant differences in serum concentrations of thyroid hormones and cTSH among common breeds, warranting breed-specific RIs. Collies, Samoyeds, Keeshonds, and Whippets have been reported to have higher cTSH concentrations than Golden Retriever, Alaskan Malamute, English Setter, and Siberian Husky breeds.[10,34] Breed-specific RIs have been reported for Greyhounds, Salukis, Sloughis, Whippets, Scottish Deerhounds, and

Basenjis, with lower fT4 and TT4 concentrations than the general canine population, casting doubt on the reliability of a general RI for the species.[3,4,6,10,34]

Regarding the German Shepherd breed, a recent study[35] measured TT4, fT4, and cTSH serum concentrations using the chemiluminescence method in 57 dogs, dividing the population into 2 groups according to age, and a separate third group of police dogs. The mean and median concentrations of TT4 and cTSH in this population were lower and higher, respectively, than the general RIs determined in a previous study, as described for other breeds such as Collie, Keeshond, and Samoyed.[34,35] However, this finding requires further investigation and appropriate statistical analyses. In contrast to that study,[35] the median concentrations of cTSH and TT4 measured in our sample population were consistent with the general RIs mentioned above.[34] For TT4, the use of different analytical methods in the 2 studies should be considered. In general, no study has aimed to determine specific RIs for the German Shepherd breed. An insufficient sample size, in our study, also could be a factor explaining these discrepancies.

The variance in TT4 concentrations among the different phases of the estrous cycle emerged as a significant finding. Specifically, we identified higher concentrations of TT4 in estrus and diestrus, when compared to concentrations measured in proestrus and anestrus.

A previous study identified higher serum TT4 concentrations in the progesterone-dominant phases of the cycle for the first time in female dogs.[21] These were defined as the phases in which progesterone secretion was predominant. This condition is characteristic of estrus after the peak concentration of luteinizing hormone and diestrus. Therefore, our finding of higher concentrations of TT4 during estrus and diestrus compared to proestrus and anestrus may be in agreement with the results of the aforementioned study.[21]

Moreover, in accordance with the previous study,[21] no significant differences in serum TT4 concentrations were observed between diestrus and pregnancy.[21] However, in that study, serum TT4 concentrations were significantly lower during estrus than during diestrus and pregnancy. Significantly higher TT3 concentrations were detected in diestrus than in any other reproductive state. However, these findings were not confirmed in our study. Thus, the different breeds of the recruited dogs as well as the different inclusion criteria, sample sizes and analytical methods should be considered as potential confounding factors.

Supporting the finding of higher TT4 concentrations in progesterone-dominant phases, a positive correlation was identified between progesterone and TT4. This finding was confirmed in a previous study that compared pregnant and non-pregnant bitches in diestrus with healthy euthyroid control subjects.[36] The TT4 serum concentrations were higher in pregnant and pseudo-pregnant bitches than in

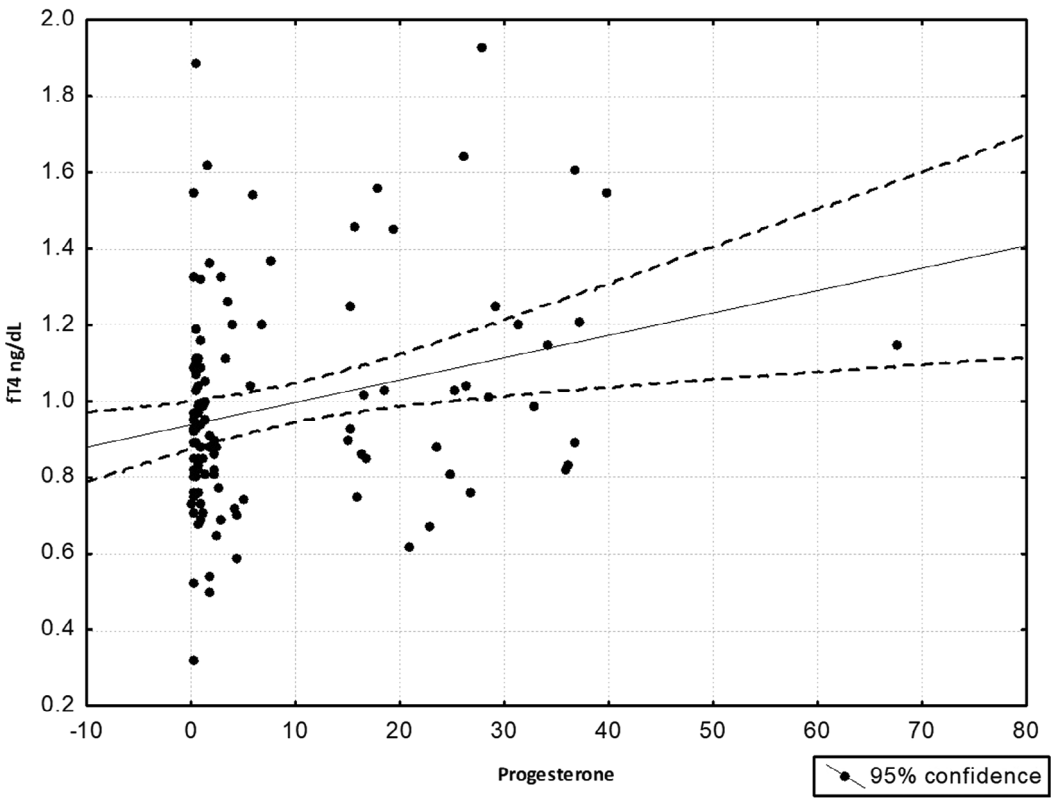


**FIGURE 6** Association between free thyroxine (fT4) and progesterone, results from correlation test. A significant positive correlation was found between fT4 and progesterone. Regression coefficient (*r*) and *P* value (*P*): $r = 0.260$; $P = .007$. Reference interval for fT4: 0.6-3.7 ng/dL. 95% confidence, 95% confidence interval; fT4, free thyroxine.

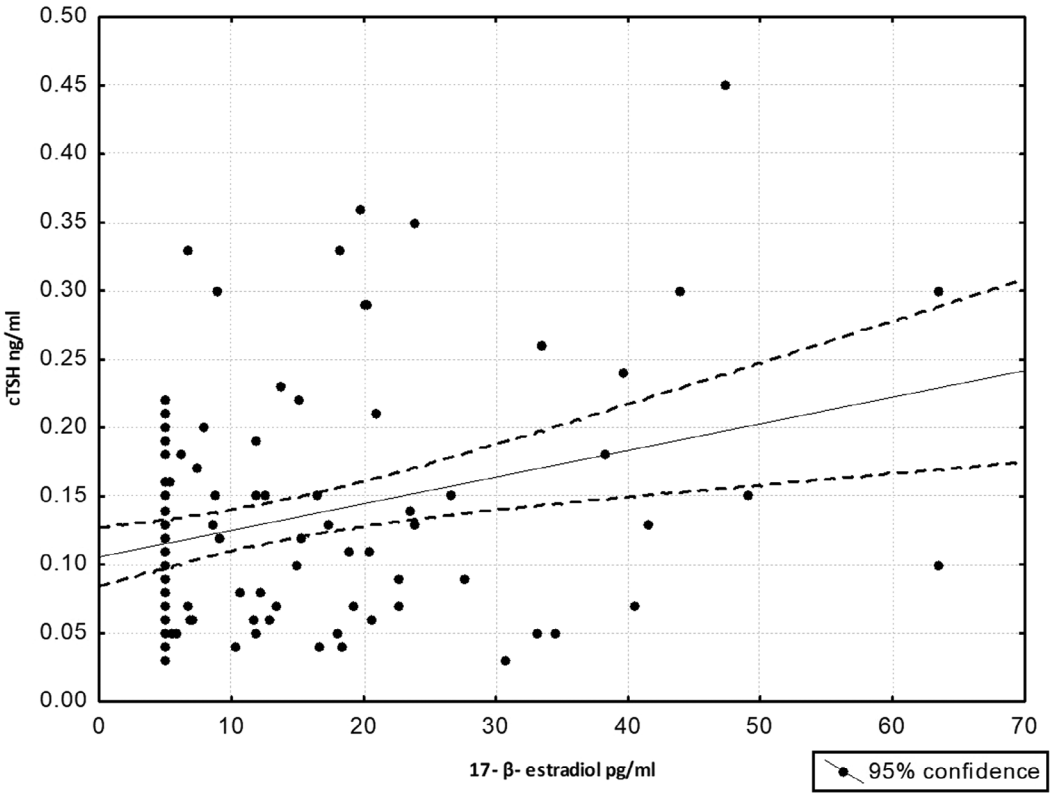


**FIGURE 7** Association between canine thyrotropin (cTSH) and 17-β-estradiol, results from correlation test. A significant positive correlation was found between cTSH and 17-β-estradiol. Regression coefficient (*r*) and *P* value (*P*): $r = 0.2$; $P = .04$. Reference interval for cTSH: <0.5 ng/mL. 95% confidence, 95% confidence interval; TT4, total thyroxine.

euthyroid dogs or healthy controls. Furthermore, consistent with our study, no significant differences were reported between diestrus and pregnant bitches.[36]

A study performed in postmenopausal women receiving progestin treatment for 12 weeks reported a significant increase in serum fT4 concentrations compared with placebo controls.[29] However, the mechanism by which progesterone increases thyroid secretory activity remains unclear. However, unlike estrogen, progesterone stimulates an increase in TT4 secretion without leading to significant differences in serum concentrations of thyroxine-binding globulin. This difference may account for the concurrent increase in the free fraction of hormones.[29] In our study, a significant positive correlation between fT4 and progesterone was identified, although it did not result in significant variance in fT4 concentrations among the different estrous phases.

In addition, in the abovementioned studies in humans, TSH concentrations did not decrease significantly during progestin treatment, although a downward trend was observed.[29] Similarly, a study conducted on ovariectomized rats reported that progesterone treatment had no effect on serum or pituitary TSH concentrations or on the response to thyrotropin-releasing hormone (TRH).[28] In our study, cTSH did not correlate with progesterone, indicating that the stimulus for thyroid secretory activity during progesterone-dominant phases is likely outside the context of the pituitary-thyroid axis.

In our study, lower serum TT4 concentrations were measured during proestrus than in all other phases of estrus. Interestingly, except for a single diestral fT4 measurement, the remaining fT4 ($n = 4$) and TT4 ($n = 1$) results that were below the RI were recorded during proestrus. Specifically, the first subject had a single proestral fT4 concentration below the RI with a normal TT4 concentration. The second subject had both fT4 determinations in the proestral phase below the RI, with normal TT4 concentrations. Finally, a third participant had fT4 and TT4 concentrations below the RI in a single proestrus sample. In each subject, the concurrent serum cTSH concentrations were within the normal range.

The normality of the concurrent concentrations of cTSH in all 4 subjects and normal TT4 in 3 of 4 subjects led to exclusion of a diagnosis of hypothyroidism. Even in subjects in which fT4 and TT4 in the proestrus were both below the RI, the normalization of these results at the next determination (4 days later) and the total absence of concurrent chronic diseases led to the interpretation of these results as fluctuations, which may be related to the phase of the estrous cycle, in the context of euthyroidism. Although the number of TT4 and fT4 concentrations below the reference range was extremely low (1 in 107 and 5 in 107 patients, respectively), this finding should be considered relevant. The use of a non-dialytic method for the measurement of fT4 should be considered.

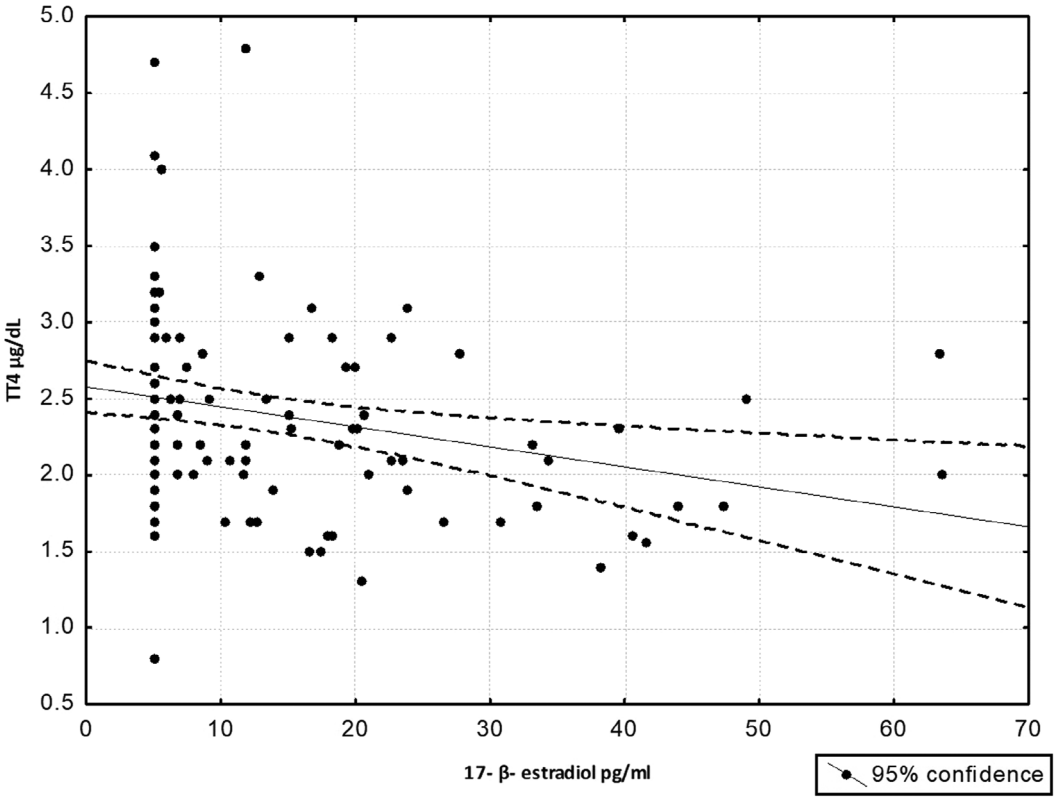


**FIGURE 8** Association between total thyroxine (TT4) and 17-β-estradiol, results from correlation test. A significant negative correlation was found between TT4 and 17-β-estradiol. Regression coefficient (*r*) and *P* value (*P*): $r = -0.3179$; $P = .001$. Reference interval for TT4: 1.0-4.7 µg/dL. 95% confidence, 95% confidence interval; TT4, total thyroxine.

Defining proestrus as the estrogen-dominant phase, the negative correlation identified between 17-β-estradiol and TT4 further supported previous findings. Although not documented in dogs, it has been extensively reported in rat models.[23,24,26] Specifically, chronic treatment with relatively high-dose 17-β-estradiol for 4 weeks induces hyperplasia of thyroid cells in ovariectomized rats, in the presence of decreased secretory activity of the gland.[24,27] The mechanism by which estrogen modulates thyroid activity has not yet been elucidated. Direct estrogen-inhibitory action on thyroid function has been proposed using specific thyroidal estrogen receptors.[37-39] A role for hyperestrogenism in suppressing the ability of the pituitary gland to respond to TRH stimulation in ovariectomized rats also has been hypothesized.[22]

Our study found a positive correlation between 17-β-estradiol and cTSH concentrations in female dogs. However, no significant differences in TSH concentration in the estrogen-dominant stage of the cycle were observed in our study. It is conceivable that because thyrotropin secretion from the pituitary gland is primarily dependent on the stimulus of TRH and negative feedback from thyroid hormones, the hypothetical decreased concentration of TT4 associated with estrogenic action may induce an increase in serum cTSH. A negative correlation was identified between 17-β-estradiol and total cholesterol concentrations. This finding is consistent with previous reports that identified significantly higher serum cholesterol concentrations in spayed bitches than in unspayed female dogs.[40]

As for 17-β-estradiol concentrations below the LOD, as mentioned above, they were recorded entirely in the diestrus and anestrus phases. This finding reflects the normal pattern that generally occurs during the estrous cycle of the dog. Our result is consistent with previous reports in the literature.[41]

Perhaps because of the homogeneity of the samples examined, our study not only confirmed the previously reported negative correlation between TT4 and fT4 concentrations and age but also identified a similar trend for fT3 concentrations.[1] To the best of our knowledge, ours is the first study to identify a negative correlation between serum fT3 concentration and age in dogs. However, this result should be interpreted with caution, given the use of a method for measuring fT3 with no published validation data for dogs. For TT3, no such association was demonstrated. Thus, explaining why this correlation did not occur in TT3 is difficult. However, it has been widely reported that the measurement of this hormone in the diagnosis of hypothyroidism has poor specificity and sensitivity.[1] Moreover, mechanisms such as increased secretion of TT3 or increased conversion of TT4 to TT3 have been hypothesized to occur in response to decreased thyroid secretory activity. These mechanisms could explain the absence of a correlation with age for this variable.[1]

Regarding the interpretation of vaginal smears, total agreement between the 2 operators involved can be explained by the absence of complex cases and adherence to international criteria for the

categorization of the estrous phase. For this reason, the finding was not subjected to further statistical analysis.

One of the limitations of our study is the measurement of fT4 using a non-dialytic method. For the measurement of fT3, TT3, 17-β-estradiol and TSH, methods commonly used in clinical practice have been applied. For those tests, no published validation work is available for the dog. This situation represents another limitation. However, in the case of TSH, fT4, and TT3, previous studies have reported the use of these methods in dogs.[42-48] Additionally, for all of the methods applied, internal validation demonstrated good accuracy (coefficient of variation < 7%). The decision to make the sample population as homogeneous as possible in terms of age, physical activity, and breed certainly lent more robustness to the correlations identified but decreased the possibility of identifying subjects that, because of borderline thyroid function, had cycle phase-related variability that may have affected the proper diagnosis of euthyroidism.[41]

Considering our findings, the stage of the estrous cycle is a factor that influences serum concentrations of TT4 in female dogs. Although significant, changes in TT4 concentrations detected at different estrous stages or during pregnancy may be clinically unremarkable when investigating altered thyroid function. In our study, based on TT4 fluctuations during the estrous cycle, no subject was improperly classified as having hypothyroidism. However, some TT4 and fT4 concentrations were below the RI in proestrus with normal cTSH concentrations. Additional studies with larger and more heterogeneous sample populations are needed to verify that this variance does not have clinical and diagnostic impact. In conclusion, the time of the estrous cycle at which the sample was obtained may not be decisive in shifting the diagnosis from euthyroidism to hypothyroidism, although investigations during anestrus may involve fewer variables for this difficult diagnosis.

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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 Ethical Committee of the University of Milan approved this protocol (41/2017).

## HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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