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Original article

Serial changes in the concentrations of cortisol and thyroid hormones in Beagle dogs infected with *Babesia rossi*

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ABSTRACT

An experimental infection using *Babesia (B.) rossi* was performed in healthy male Beagle dogs to assess the changes in endocrine variables during disease. Two dogs were infected with a low dose (LD) of parasite inoculum (10^4 parasites) and three dogs were infected with a high dose (HD) (10^8 parasites). Basal serum cortisol, thyroxine (T4), and thyrotropin (TSH) concentrations were measured every second day. Samples were analyzed using a solid- phase, competitive chemiluminescent enzyme immunoassay (Immulyte® 2000, Siemens). Variables were compared between groups and timepoints using linear mixed models. In both groups, the median cortisol concentration increased, whilst the median T4 concentration decreased after infection, with a return towards baseline concentration post treatment. The highest cortisol and the lowest T4 concentrations were reached at 96 h and 108 h post infection, respectively, in the HD group and slightly later at 108 and 144 h post-infection, respectively, in the LD group. A higher cortisol concentration with a more rapid increase, and a lower T4 concentration with a more rapid decline, were associated with disease severity and a higher dose of parasite inoculum. The TSH concentration remained within the reference interval throughout the study period. This study illustrated the temporal changes in endocrine parameters during experimental *B. rossi* infection and demonstrated that cortisol and T4 tracked the severity of disease, albeit in opposite directions.

1. Introduction

Canine babesiosis is a common intraerythrocytic protozoal infection. The historical understanding of this infection posits that intravascular hemolysis and phagocytosis of erythrocytes results in anemia (Gray et al., 2010; Maegraith et al., 1957; Reyers et al., 1998). Yet, it is now commonly accepted that the body's disproportionate inflammatory response, rather than the infectious agent itself, results in the clinical signs most commonly seen (Clark, 2007; Clark and Jacobson, 1998). The complex pathophysiology, along with the severe clinical disease and unpredictable outcome in canine babesiosis, has catalyzed the investigation into a variety of biomarkers that may assist in the prediction of outcome. As such, multiple parameters have been associated with a poorer prognosis, including the degree of parasitemia (Böhm et al., 2006; Leisewitz et al., 2019a), hyperlactatemia (Eichenberger et al., 2016; Leisewitz et al., 2019b; Nel et al., 2004), hypoglycemia (Leisewitz et al., 2019b; Nel et al., 2004) and cytokine concentrations (Goddard et al., 2016; Leisewitz et al., 2019a). In addition, cortisol and thyroxine (T4) concentrations are considered two of the strongest predictors of outcome, despite the large body of research on new biomarkers in canine babesiosis (Leisewitz et al., 2019b; Schoeman et al., 2007b).

Our current understanding on the endocrine derangements in canine babesiosis has been limited to the clinical setting where heterogenous groups of dogs have been evaluated at varying stages of disease, with varying parasite loads. As a result, an experimental infection would provide insight into these endocrine changes from the onset of infection and would allow monitoring of these parameters as the disease progressed. Furthermore, a variety of disease states demonstrate a common

List of abbreviations: B., Babesia; HD, high dose; HPA, hypothalamic- pituitary- adrenal; HPT, hypothalamic- pituitary- thyroidal; IL, interleukin; LD, low dose; MODS, multiple organ dysfunction syndrome; PCR, polymerase chain reaction; RI, reference intervals; SIRS, systemic inflammatory response syndrome; T3, triiodothyronine; T4, thyroxine; TSH, thyrotropin.

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inflammatory pathway and share similar clinical signs, despite differences in the causative agents. These conditions commonly result in the systemic inflammatory response syndrome (SIRS) and may proceed to multiple organ dysfunction syndrome (MODS) (Bone et al., 1992; Jacobson, 2006). By considering experimental canine babesiosis as a model for inflammatory disease in both veterinary and human medicine, we may gain insight into the endocrine derangements associated with other diseases. The objective of this study was to describe the longitudinal changes in endocrine variables after experimental *Babesia (B). rossi* infection in Beagle dogs and to assess differences between dogs infected with a high (10^8 parasites) and a low dose (10^4 parasites) of parasite inoculum.

2. Materials and methods

Ethical approval for the study was obtained from the University of Pretoria Animal Ethics Committee and Research Ethics Committee (V003-18) for a pilot investigation into the transcriptomic response to *B. rossi* infection in Beagle dogs (Smith et al., 2021). Our study collected secondary data from the original study and was approved by the Faculty Research Ethics committee (REC050-19). Six, six-month-old purpose--bred castrated male Beagle dogs were included in this prospective longitudinal experimental study. All dogs were habituated to the process of examination and sample collection through daily training and socialization with people. They were housed in a play-enriched environment as a group until the infection phase, at which time they were housed individually. The laboratory facility housing the dogs conformed to the requirements stipulated in the South African National Standards for the use and care of animals for scientific purposes (SANS 10386: 2008). Inclusion criteria required that the dogs be clinically healthy, current on vaccination and deworming schedules, and free from *Babesia, Ehrlichia, Theileria* and *Anaplasma* infections as determined by polymerase chain reaction (PCR) prior to experimental infection. One dog was randomly selected for splenectomy and infected with a cryopreserved parasite inoculum of wild type *B. rossi*. A viable parasite inoculum was raised in this splenectomized dog and used to infect the remaining five experimental dogs. The five experimental dogs had baseline samples taken on two separate occasions, four and two weeks prior to experimental infection, creating ten baseline control samples in total.

The above-mentioned cryopreserved parasite inoculum with which the splenectomized dog was infected, was created from a dog naturally infected with *B. rossi* that presented to the Outpatients clinic of the Onderstepoort Veterinary Academic Hospital. PCR was performed to confirm the presence of a *B. rossi* infection and to exclude co-infection with other blood-borne parasites. Fifty milliliters of blood were collected and divided into 5 × 10-ml heparinized tubes from the naturally infected dog. Forty-five milliliters of blood were decanted from the collection tubes into a 50-ml flask and kept on melting ice. Using a 1-ml pipette, 5 ml of dimethyl sulfoxide was added one drop at a time, with a waiting period of 10 s between drops, whilst simultaneously swirling the flask kept on melting ice. The blood was divided into 4-ml cryotubes kept on melting ice. The cryotubes were transferred to a Cool Cell container and stored in a -80°C freezer.

The cryopreserved ampules from the naturally infected dog were thawed in a water bath set at 37°C. The splenectomized dog was inoculated intravenously with 2 ml of the cryopreservate followed by a second inoculation, 24 h later. Parasitemia of the splenectomized dog was manually determined on venous blood collected twice daily, starting one day post infection, as previously described (Böhm et al., 2006). Upon detection of a parasitemia, citrated whole blood was collected from the splenectomized dog. A culture media (Culture Media RPMI 1640, Hepes, filtered water, sodium bicarbonate, sodium pyruvate and gentamycin) was used as the diluent to create serial dilutions of 10^8 and 10^4 parasitized erythrocytes per milliliter. The five experimental dogs were randomly divided into a high dose (HD) and a low dose (LD) group and inoculated intravenously with 1 ml of 10^8 and 10^4 parasitized erythrocytes, respectively. Basal serum cortisol, T4 and thyrotropin (TSH) concentrations were measured at 24, 72, 108, 144 and 192 h post infection in all experimental dogs. The HD group had additional samples taken at 96 and 120 h, since clinical deterioration necessitated more intensive monitoring. Predetermined end points upon which daily blood collection would cease were as follows:

- A hematocrit or PCV <15%
- A habitus score of 1+ (defined as lethargic and non-responsive)
- Neurological signs, whether due to neuroglycopenia or not
- Clinical evidence of lung pathology
- Evidence of oliguria (defined as a urine production of <1 ml/kg/h) with a serum creatinine >200 mmol/L (normal <140 mmol/L)
- Evidence of hemoconcentration (defined as a PCV >55%)
- A dog that lived to 20 days post infection

All dogs were to be drug cured with 3.5 mg/kg diminazene aceturate (Berenil® RTU 0.07 g/mL, Intervet, Kempton Park, South Africa) subcutaneously and re-homed as pets thereafter.

All clinical examinations and sample collections were performed between 8:00am and 10:00am each day, with the exception of a single day where clinical deterioration necessitated additional monitoring and sampling at 8:00pm. All blood samples were collected from the jugular vein with 21-gauge vacutainer needles (Precision GlideTM, UK). Serum samples were collected in serum vacutainer brand tubes (Beckton Dickinson Vacutainer Systems, UK), centrifuged, aliquoted and immediately stored at -80°C thereafter. Cortisol, T4 and TSH concentrations were analyzed as a single batch using a solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite® 2000, Siemens) as described in previous studies (Aicher et al., 2019; Proverbio et al., 2009; Wolff et al., 2020). The laboratory reference intervals (RI) for cortisol, T4 and TSH were 20-200 nmol/L, 13-41 nmol/L, and 0-0.45 ng/mL, respectively. Limit of quantification was 27.6 nmol/L for cortisol, 6.44 nmol/L for T4, and 0.03 ng/dL for TSH. Values below the limit of quantification were recorded as 27.5 nmol/L, 6.43 nmol/L, and 0.029 ng/dL respectively.

3. Statistical analysis

For analysis, variables expected to have a right-skewed distribution, i.e., cortisol and TSH, were log-transformed; T4 was not transformed. Linear mixed models with Bonferroni adjustment for multiple comparisons were used to compare concentrations within each experimental group to the baseline concentration, and between the LD and HD groups at each timepoint. Correlations between the variables, for all timepoints combined, were assessed using Spearman's rank correlation with Bonferroni adjustment. Statistical analysis was performed using Stata 15 (StataCorp, College Station, TX, USA). For all tests, significance was set at $P < 0.05$. Results were depicted as median and range.

4. Results

4.1. Study population and treatment

The dogs in the HD group were treated at 96 h post infection due to clinical collapse. Based on the severity of disease in the HD group, the LD group was treated at 108 h post infection, prior to the development of any predefined end points.

4.2. Percentage parasitemia

The HD group had higher venous parasitemias and required treatment at 96 h post infection based on habitus scores of 1+ (Table 1 and Fig. 1). In contrast, the LD group had lower parasitemias, reaching only a tenth of the parasite density seen in the HD group at similar time points. All dogs in the HD group developed habitus scores of 1+ and were

Table 1
Temporal changes in median peripheral parasitemia with ranges. Asterisks indicate the point of treatment in each group.

Time (hours)	Total	Parasitemia (%) LD	HD
Before infection	0 (0)		
After infection			
24		0 (0)	0.05 (0.05)
48		0 (0)	0.998 (0.69-1.06)
72		0.075 (0.05-0.1)	11.08 (6.74-14.81)
96		3.88 (3.15-4.61)	45.54* (34.95-59.8)
108		5.76* (4.71-6.81)	

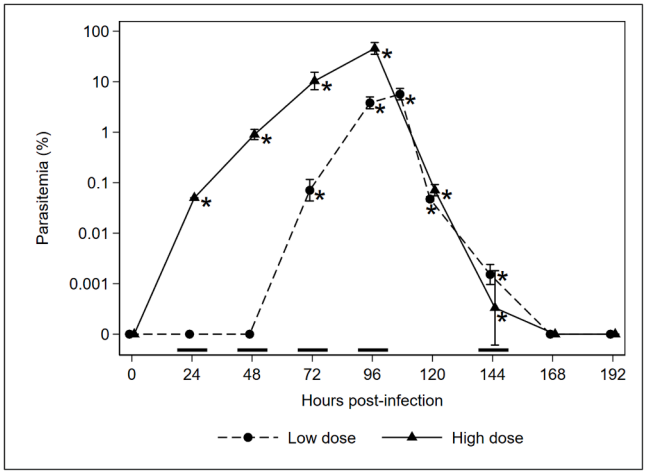

Fig. 1. *Line graph illustrating the temporal changes in parasitaemia percentage after experimental B. rossi infection.* Error bars indicate standard deviation; horizontal black bars indicate the timepoints at which the high and the low dose groups differ significantly from each other (P <0.05); asterisks indicate significant differences compared to the controls at time 0 (P <0.05).

collapsed (defined as the inability to stand unaided), whilst none of the dogs in the LD group were ever collapsed, nor reached any of the pre-determined end points.

4.3. Hypothalamic-pituitary-adrenal (HPA) axis

The response of the HPA axis to experimental *B. rossi* infection at different infectious doses is illustrated in Table 2 and Fig. 2. Both groups demonstrated a rise in cortisol concentration with peaks reached at the point of treatment in each group, followed by a decline in cortisol concentration post treatment. In the HD group, a higher cortisol concentration with a more rapid increase was noted, followed by a more rapid decline towards baseline values post treatment. When compared to baseline control samples, a significantly higher cortisol concentration was seen at 72 h ($P < 0.001$), 96 h ($P < 0.001$), 108 h ($P < 0.001$), 120 h ($P < 0.001$) and 144 h ($P < 0.001$) in the HD group; and at 108 h ($P = 0.042$) in the LD group. No samples were collected from the LD group at the 96- and 120 h timepoints due to ethical concerns related to excessive sampling. As a consequence, it is impossible to determine whether the peak in cortisol concentration may have occurred at one of these time points in the LD group. A more rapid increase with a significantly higher cortisol concentration was seen in the HD group when compared to the LD group at 72 h ($P = 0.001$), 108 h ($P < 0.001$) and 144 h ($P = 0.012$) post infection. One dog from the HD group died during the study. This

Table 2
Temporal changes in median cortisol concentrations with ranges. Asterisks indicate the point of treatment in each group.

Time (hours)	Total	Cortisol (nmol/L) LD	HD
Before infection	27.5 (27.5-43.6)		
After infection			
24		27.5 (27.5)	27.5 (27.5-36.4)
72		27.5 (27.5)	83 (41.9-83.9)
96			315* (223-610)
108		52.15* (34.5-69.8)	172 (94.6-251)
120			155 (116-194)
144		41.4 (40.3-42.5)	84 (61-107)
192		27.5 (27.5)	31 (27.5-34.5)

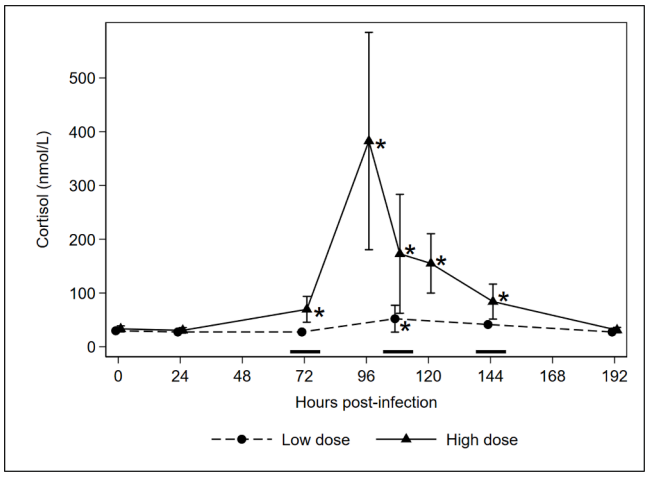

Fig. 2. *Line graph illustrating the temporal changes in median cortisol concentration after experimental B. rossi infection.* Error bars indicate standard deviation; horizontal black bars indicate the timepoints at which the high and the low dose groups differ significantly from each other (P <0.05); asterisks indicate significant differences compared to the controls at time 0 (P <0.05).

dog showed the highest cortisol concentration of 610.0 nmol/L at 96 h post infection, shortly before death.

4.4. Hypothalamic-pituitary-thyroidal (HPT) axis

The changes in T4 concentration after experimental *B. rossi* infection at different infectious doses is illustrated in Table 3 and Fig. 3. The HD group's T4 concentration declined rapidly whilst the LD group's T4 concentration declined slowly, prior to each group's point of treatment. The T4 concentration in both groups continued to show a mild decline post treatment, before increasing. When compared to baseline samples, a significantly lower T4 concentration was seen at 72 h ($P < 0.001$), 96 h ($P < 0.001$), 108 h ($P < 0.001$), 120 h ($P < 0.001$), 144 h ($P < 0.001$) and 192 h ($P < 0.001$) in the HD group. The T4 concentration in the LD group was significantly higher than baseline samples at 24 h ($P < 0.001$), yet significantly lower at 108 h ($P < 0.001$) and 144 h ($P < 0.001$). There was a significantly lower T4 concentration in the HD group than in the LD group at 108 h ($P < 0.001$) post infection. The single dog that died during this study had a T4 concentration below the limit of quantification at 96 h post infection, shortly before death.

The changes in TSH concentration are illustrated in Table 4 and

Table 3
Temporal changes in median T4 concentrations with ranges. Asterisks indicate the point of treatment in each group.

Time (hours)	Total	T4 (nmol/L) LD	HD
Before infection	38.97 (25.4-61.6)		
After infection			
24		47.45 (44.8-50.1)	51.6 (45.7-54.8)
72		37.55 (32.6-42.5)	27.5 (24.5-29.3)
96			7.14* (6.43-8.74)
108		26.1* (21.4-30.8)	6.43 (6.43)
120			7.45 (6.43-9.06)
144		16.655 (8.71-24.6)	18 (14.9-21.1)
192		36.4 (35-37.8)	34.4 (34.4)

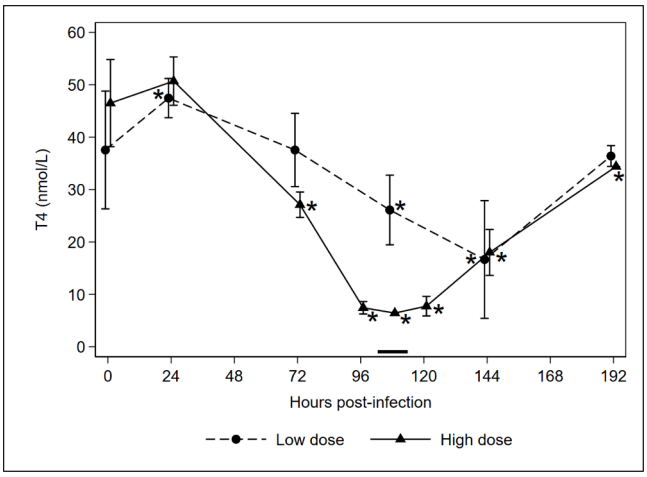

Fig. 3. *Line graph illustrating the temporal changes in median T4 concentration after experimental B. rossi infection.* Error bars indicate standard deviation; horizontal black bars indicate the timepoints at which the high and the low dose groups differ significantly from each other (P <0.05); asterisks indicate significant differences compared to the controls at time 0 (P <0.05).

Table 4
Temporal changes in median TSH concentration with range. Asterisks indicate the point of treatment in each group.

Time (hours)	Total	TSH (ng/dL) LD	HD
Before infection	0.073 (0.032-0.178)		
After infection			
24		0.114 (0.058-0.169)	0.097 (0.066-0.169)
72		0.09 (0.063-0.117)	0.066 (0.035-0.07)
96			0.033* (0.032-0.31)
108		0.228* (0.191-0.264)	0.029 (0.029)
120			0.036 (0.029-0.043)
144		0.06 (0.035- 0.084)	0.043 (0.038-0.047)
192		0.082 (0.071-0.092)	0.107 (0.094-0.119)

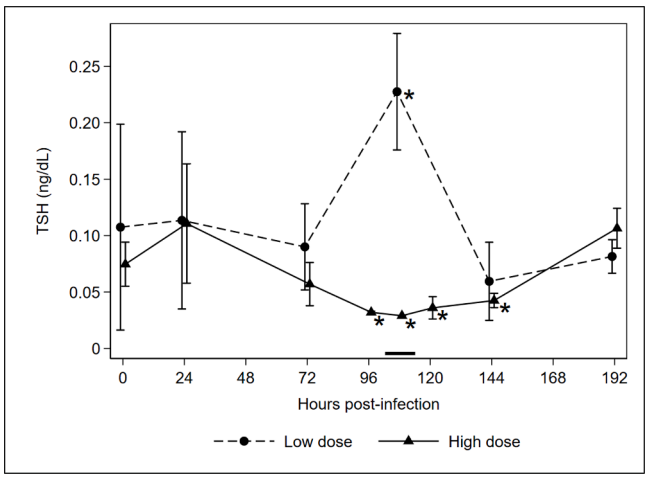

Fig. 4. *Line graph illustrating the temporal changes in median TSH concentration after experimental B. rossi infection.* Error bars indicate standard deviation; horizontal black bars indicate the timepoints at which the high and the low dose groups differ significantly from each other (P <0.05); asterisks indicate significant differences compared to the controls at time 0 (P <0.05).

Fig. 4. Both the HD and the LD group showed an initial decline in TSH concentration 72 h post infection, but a divergence between the two groups was seen thereafter. The TSH concentration in the HD group continued to decline, 108 h post infection, whilst the LD group's TSH concentration increased dramatically until the point of treatment in this group. The TSH concentration in the LD group showed a rapid decline after treatment, 144 h post infection, before increasing 192 h post infection. There was a significant difference between the baseline and the HD group at 96 h ($P < 0.001$), 108 h ($P < 0.001$), and 120 h ($P = 0.003$) post infection. A significant difference was only seen between the baseline and the LD group at 108 h ($P < 0.001$) post infection. Although the TSH concentration in the HD group was often lower than that of the LD group, a significant difference between the two groups was only seen at 108 h ($P < 0.001$). Despite these variations, the TSH concentrations remained within RI throughout the study period for both groups. Cortisol was negatively correlated with T4 ($r_s = -0.856$, $P < 0.001$) and TSH ($r_s = -0.698$, $P < 0.001$), whilst T4 showed a positive correlation with TSH ($r_s = 0.732$, $P < 0.001$).

4.5. Discussion

This study is the first to illustrate the temporal changes in endocrine parameters during an experimental *B. rossi* infection in dogs. The cortisol concentration increased more rapidly in the HD group than in the LD group, which was consistent with clinically more severe disease. Similar changes and patterns in cortisol concentration have been demonstrated in experimental canine staphylococcal pneumonia (Cortes-Puch et al., 2014) and in puppies that survived natural parvovirus infection (Schoeman et al., 2007a). Yet, different timelines in these cortisol changes were seen between the studies, most likely due to inherent organism variation in incubation period and infectious pathophysiology. The dog in the HD group that died showed the highest cortisol concentration (610 nmol/L at 96 h post infection) shortly before death. In puppies with parvoviral diarrhea, the highest cortisol concentration of 529 nmol/L was recorded in a puppy that died (Schoeman et al., 2007a). Moreover, in a clinical study of canine babesiosis, the highest cortisol concentration of 562 nmol/L was also seen in a dog that died (Schoeman et al., 2007b). Our data agrees with most human and animal studies that have shown an increase in cortisol concentration in critically ill dogs compared to healthy controls (Boonen et al., 2013; Cortes-Puch et al., 2014), with higher cortisol concentration predicting increased severity of disease (Annane et al., 2000; Christ-Crain et al.,

2007; Sam et al., 2004; Schoeman et al., 2007a; Schoeman and Herrtage, 2008a, Schoeman et al., 2007b, Yuki et al., 2019; Swales et al., 2020). The mechanism underlying increased cortisol concentration during critical illness is thought to be multifactorial and results from a combination of increased glucocorticoid production (Bethin et al., 2000; Franchimont et al., 2002) along with impaired metabolism, resulting in a prolonged cortisol half-life (Boag et al., 2020; Boonen et al., 2013).

A more rapid and significant decline in T4 concentration was seen in the HD group with more severe disease. Our findings mirrored an experimental study where healthy dogs were injected intravenously with endotoxin (Panciera et al., 2003). The single dog that died in our study had the lowest T4 concentration (below the limit of quantification) at the 96 h timepoint, shortly before death. Similar to our study, the T4 concentrations recorded in puppies with parvoviral diarrhea that died were all below the limit of quantification at 24 h after admission (Schoeman et al., 2007a), whilst 6 out of 7 dogs that died from natural *B. rossi* infection had T4 concentrations below the limit of quantification on admission (Schoeman et al., 2007b). Although different assays were used between the studies, the trend can be appreciated and emphasizes the need for a more sensitive assay with a lower limit of quantification for future studies. Systemic illness in both human and veterinary literature has been associated with decreased serum thyroid hormone concentrations (Elliott et al., 1995; Giunti et al., 2017; Nishii et al., 2019; Panciera et al., 2003; Pashmakova et al., 2014; Zygner et al., 2015) and a lower T4 concentration often reflected severity of disease (Kantrowitz et al., 2001; Mooney et al., 2008; Schoeman and Herrtage, 2008b).

Although the TSH concentrations in the HD group were often lower than that of the LD group, a significant difference was only seen at 108 h and the TSH concentrations remained within laboratory RI throughout the study period, which would suggest limited clinical utility in using TSH as a prognostic tool in this patient population. Lower TSH concentrations have been associated with disease severity in people admitted to intensive care units (Nylén et al., 2006; Sumita et al., 1994) but similar to our study, the TSH concentrations often remained within RI. When dogs with non-thyroidal disease were compared to clinically normal controls, the serum TSH concentration remained within the RI regardless of disease severity (Kantrowitz et al., 2001). Similarly, TSH concentrations remained within the RI in dogs with natural *B. canis* infection (Zygner et al., 2015) and TSH concentration was not associated with outcome in dogs with natural *B. rossi* infection (Schoeman et al., 2007b). Furthermore, TSH was considered a less sensitive predictor of outcome than T4 concentration in puppies with parvoviral diarrhea (Schoeman and Herrtage, 2008b). In the HD group, TSH concentrations mimicked the changes in T4 concentrations with a decline in TSH post infection that gradually increased again post treatment. The TSH concentration for this group often measured below the limit of quantification therefore, the true TSH nadir could not be fully appreciated. A similar problem occurred when endotoxin was experimentally administered to healthy dogs (Panciera et al., 2003). They postulated that the poor sensitivity of the TSH assay may have contributed to the lack of significant findings (Panciera et al., 2003) and this may very well have been the same scenario in our study. The TSH concentrations in the LD group showed an unexpected increase at the 108 h timepoint and declined again thereafter. These assays were repeated to investigate the possibility of laboratory error, but the results remained consistent. This short-lived increase in TSH in the LD group may have been due to a transient loss in negative feedback inhibition associated with lower thyroid hormone concentrations. It is possible that this surge in TSH is a normal physiological phenomenon in response to lowering T4 levels in the presence of permissively low cortisol concentrations. In contrast, the cortisol concentration in the HD group was too high to allow for a surge in TSH, resulting in a form of euthyroid sick phenomenon with suppression of TSH by high cortisol. Consequently, we may have identified a differential TSH suppression pattern between the HD and LD groups. Further experimental studies with a larger population size would be required to illuminate whether this transient surge in TSH concentration is a consistent finding in mildly sick dogs. Systemic illness in both human and veterinary literature has been associated with decreased serum thyroid hormone concentrations, as seen in the current study. It is believed to result from a variety of different mechanisms, including inhibition of the 1 and 2 deiodinase enzymes resulting in decreased conversion from T4 to triiodothyronine (T3); cortisol and cytokine-induced inhibition of TSH synthesis; or the presence of circulating inhibitors to receptor binding (Elliott et al., 1995; Maxime et al., 2007; Pashmakova et al., 2014; Peeters et al., 2005; Zygner et al., 2015). Following on from the above, a negative correlation was indeed noted between cortisol and the thyroid hormones tested. Previous investigations have shown that pharmacological doses of dexamethasone and physiological concentrations of cortisol were both associated with suppressed TSH secretion (Re et al., 1976) and when patients were injected with interleukin (IL)-6, a rise in cortisol preceded a decline in TSH concentration (Torpy et al., 1998). This is to be expected, since TSH stimulates the release of T4 under physiological conditions (Elliott et al., 1995; Pappa et al., 2011; Pashmakova et al., 2014). During the recovery phase of disease, TSH has been found to increase and precede the rise in T4 and T3 concentration (Nylén et al., 2006). This preceding rise was not clearly demonstrated in our study (barring the transient surge noticed in the LD group) and may have been masked by the lower sampling frequency.

This experimental study was able to use a homogenous group of purpose-bred Beagle dogs who were infected at identical timepoints, with a known inoculum size. Both peripheral parasitemia (Böhm et al., 2006; Leisewitz et al., 2019a) and a collapsed clinical state have been identified as indicators of disease severity (Leisewitz et al., 2019a; Leisewitz et al., 2019b) and support the notion that the dogs in the HD group were more severely ill than those in the LD group. Endocrine parameters have previously been investigated as measures of disease severity in dogs diagnosed with natural *B. rossi* infection. Median serum cortisol and plasma ACTH concentrations were significantly higher, and serum T4 concentrations were significantly lower, in dogs with natural *B. rossi* infection that died in comparison to dogs that survived after treatment in hospital, or dogs that were stable enough to be managed as outpatients (Schoeman et al., 2007b). These findings were later mirrored in the largest cohort of molecularly confirmed natural *B. rossi* infections published to date, where low T4 and high cortisol concentration, along with high bilirubin and high urea, were identified as the parameters that were most predictive of outcome (Leisewitz et al., 2019b). In dogs with natural *B. rossi* infection, basal and ACTH-stimulated cortisol concentrations were both significantly higher, whilst T4 and free T4 concentrations were significantly lower, in hypoglycemic dogs in comparison to those with normoglycemia and hyperglycemia (Schoeman and Herrtage, 2007). Infected dogs also had significantly higher basal cortisol concentrations than control dogs, and basal and ACTH-stimulated cortisol concentrations were both significantly higher in dogs that died compared to those that were hospitalized and survived and compared to those that were healthy enough to be treated as outpatients. Delta cortisol and cortisol to ACTH ratios did not provide any significant predictive value for the different outcome groups. However, the median delta cortisol was markedly lower in dogs that died, but only because of the severely elevated basal cortisol concentrations (Schoeman and Herrtage, 2008a). These studies illustrate the significant and repeatable endocrine derangements that occur during *B. rossi* infection and the value they may have in prognostication. Furthermore, by considering experimental canine babesiosis as a model for inflammatory disease in both veterinary and human medicine, we can gain insight into these endocrine changes during the initial and progressive stages of disease.

5. Limitations

Due to the experimental nature of the study, ethical approval could not be acquired for a larger number of dogs. The limitations associated

with a smaller sample size were partially offset by the experimental nature of the study and rigorous attempts to ensure a homogenous experimental group. All dogs were of the same age and breed and sampling was performed at the same time each day for all group members, with the exception of one day where clinical deterioration necessitated additional monitoring and sampling in the evening. Dogs in the LD and HD groups each received an identical dose of parasite inoculum at the same time as the other dogs in their respective groups. Furthermore, the baseline samples were taken from the experimental dogs prior to infection to ensure that the baseline samples matched the study population as closely as possible. Admittedly, by creating a homogenous group of young experimental dogs, the changes recorded may not be representative of clinically infected dogs in older age categories. In addition, the HD group achieved a parasitemia that is very rarely seen in naturally acquired infections and therefore only models severe forms of natural disease.

Serum samples were collected at 24, 72, 108, 144 and 192 h post infection in all experimental dogs. Due to severe clinical deterioration, the HD group had additional serum samples taken at 96 and 120 h post infection to provide more intensive monitoring. This additional sampling could not be justified in the LD group who was clinically stable, resulting in critical timepoints where comparisons could not be made between the two groups. Ideally, all patients should have had samples taken at identical timepoints throughout the study, but ethical guidelines were prioritized in this instance.

Finally, many of the T4 and TSH concentrations were below the limit of quantification, hampering true interpretation of the results. An alternative next generation assay with a lower limit of quantification may have provided more meaningful information, especially with reference to positive predictive values for non-survival.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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