Concentrations of cortisol, thyroid hormones and thyrotropin in Beagle dogs experimentally infected with *Babesia rossi*

by

ESTEE VAN ZYL

Submitted in partial fulfilment of the requirements for the degree of

MMedVet (Med)

in the Faculty of Veterinary Science

at the

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SUPERVISOR: Prof Johan Schoeman

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Declaration of originality

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Ethics statement

The author, whose name appears on the title page of this dissertation, has obtained, for the research described in this work, the applicable research ethics approval.

The author declares that she has observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the Policy guidelines for responsible research.

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Animal Ethics Committee

4 December 2019

Approval Certificate
New Application

AEC Reference No.:	REC050-19
Title:	The time course of endocrine parameters during experimental Babesia rossi infection in beagle dogs.
Researcher:	Dr E van Zyl
Student's Supervisor:	Prof JP Schoeman

Dear Dr E van Zyl,

The **New Application** as supported by documents received between 2019-03-25 and 2019-11-25 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-11-25.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	**Number**
Dogs (Canine)	6 (sampling completed) V003-18
Samples Blood smear	14 (Retrospective stored samples V003-18)
Whole blood in serum tube - Canine	7(Retrospective stored samples V003-18)

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-12-04.
3. Please remember to use your protocol number (REC050-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Faculty of Veterinary Science

Research Ethics Committee

Project Title	**The time course of endocrine parameters during experimental Babesia rossi infection in beagle dogs.**
Project Number	**REC050-19**
Researcher / Principal Investigator	**Dr E van Zyl**

Dlissertation / Thesis submitted for	**Masters**

Supervisor	**Prof JP Schoeman**

APPROVED	Date: **2019-08-26**
CHAIRMAN: UP Research Ethics Committee	Signature: 

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Animal Ethics Committee

PROJECT TITLE	**A dog model of severe haemolytic illness**
PROJECT NUMBER	**V003-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Prof. A Leisewitz**

STUDENT NUMBER (where applicable)	----------
DISSERTATION/THESIS SUBMITTED FOR	**Academic**

ANIMAL SPESIES/SAMPLES	**Beagles (Canine)**	
NUMBER OF ANIMALS	**6**	
Approval period to use animals for research/testing purposes		**July 2018 - July 2019**
SUPERVISOR	**Prof. A Leisewitz**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 29 June 2018
CHAIRMAN: UP Animal Ethics Committee	Signature 

S4285-15

agriculture,
forestry & fisheries

Department:
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Private Bag X138, Pretoria 0001

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Reference: 12/11/1/1/6

Professor Andrew Leisewitz
Department of Companion Animal Clinical Studies
Faculty of Veterinary Science
University of Pretoria
0110
Email: Andrew.Leisewitz@up.ac.za

Dear Prof Leisewitz,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Your application sent per email on 31 October 2018 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The study is approved as per the application form dated 19 Oct 2018 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
3. All potentially infectious material utilised, collected or generated during the study (other than the samples specified in this permit to be stored and distributed) are to be destroyed at the completion of the study. Records must be kept for five years for auditing purposes;

4. *Babesia rossi* parasites can be obtained from patients at the Onderstepoort Veterinary Academic Hospital, but can only be used for experimental infection once it is ascertained that there are no contaminating pathogens present in the stabilates;
5. Dogs for this study can only be sourced from the breeding colony at the Faculty of Veterinary Science, University of Pretoria;
6. This study can only be performed in the tick protected facility of the UPBRC, where windows may not be opened during the study and tick control is to be used to trap possible ticks entering and exiting the unit;
7. All dogs in the study must be treated orally with Bravecto every 3 months to ensure that they remain ectoparasite free for the duration of the study;
8. Following the experiment the dogs must be treated with diminazine aceturate to sterilize the babesia infection and the dogs can only leave the UPBRC after one month has elapsed since treatment;
9. Whole blood, serum, plasma and urine from the study may be stored in cryovials in the freezer of the Clinical Pathology Laboratory of the OVAH;
10. DNA, serum, plasma and urine from the study may be exported to the USA, in compliance with the import requirements of the importing country;
11. For any subsequent use or distribution of stored samples from this study, a separate section 20 or a section 20 amendment must be obtained from the Director: Animal Health;
12. Ethical approval for the study must be obtained from the relevant authority before the study may start;
13. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: A dog model of severe haemolytic illness
Researcher: Professor Andrew Leisewitz
Institution: Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria
Our ref Number: 12/11/1/1/6
Your ref: V003-18
Expiry date: December 2019

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -12- 1 3

SUBJECT: S20 PERMISSION FOR: A DOG MODEL OF SEVERE HAEMOLYTIC ILLNESS - LJvR

Acknowledgements

To my husband and family, thank you for your constant encouragement and endless love. Without your support, this would never have been possible. I am so grateful for all the sacrifices you have made to get me to this point.

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Summary

Concentrations of cortisol, thyroid hormones and thyrotropin in Beagle dogs experimentally infected with *Babesia rossi*

Experimental protozoal infections can provide novel insights into the host's response before presentation to the veterinarian. The aim of this study was to track endocrine variables longitudinally in an experimental *Babesia (B.) rossi* infection in beagle dogs and to assess changes in endocrine variables between dogs infected with a high (10^8 parasites) and a low dose (10^4 parasites) of parasite inoculum.

Six purpose bred castrated male beagle dogs were included in this prospective longitudinal observational study. The infectious inoculum was raised in a splenectomised dog. The remaining five dogs were randomly divided into two groups. Two dogs were infected with the low dose of parasite inoculum and three were infected with the high dose. Basal serum cortisol, thyroxine (T4), triiodothyronine (T3), and thyrotropin (TSH) concentrations were measured every second day, until the predetermined end points of infection. Samples were analysed using a solid- phase, competitive chemiluminescent enzyme immunoassay (Immulyte® 2000, Siemens). Once the end points were reached, the dogs were drug cured with diminazene aceturate (3.5 mg/kg subcutaneously).

In both groups, the median cortisol concentration increased and the median T4 and T3 concentrations decreased after infection with a return towards baseline concentration post treatment. The high dose group showed a rapid and more pronounced increase in cortisol concentration, whilst the low dose group demonstrated a slower and milder increase. One dog from the high dose group died during the study. This dog showed the highest recorded cortisol concentration of 610 nmol/L at 96 hours after infection, shortly before death. The high dose group showed a rapid and greater decline in T4 concentration whilst the low dose group showed a more gradual and milder decrease. The T3 concentration also decreased post infection and was significantly lower in the high dose group when compared to the low dose group, at certain timepoints. 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 rose, whilst T4 and T3 declined proportionate to the severity of disease which is associated with the dosage of the parasite inoculum.

Key terms:
Babesia rossi, babesiosis, dog, experimental, cortisol, T4, T3, TSH.

List of abbreviations

ACTH- Adrenocorticotropic hormone

APACHE- Acute physiology and chronic health evaluation

APPLE- Acute patient physiologic and laboratory evaluation

B.- Babesia

CBC- Complete blood count

CBG- Cortisol binding globulin

CIRCI- Critical illness related corticosteroid insufficiency

CRH- Corticotropin-releasing hormone

DMSO- Dimethyl sulfoxide

ESS- Euthyroid sick syndrome

fT3- Free triiodothyronine

fT4- Free thyroxine

HPA- Hypothalamic- pituitary- adrenal

HPT- Hypothalamic- pituitary- thyroidal

ICU- Intensive care unit

IL- Interleukin

IQR- Interquartile range

MODS- Multiple organ dysfunction syndrome

mRNA- Messenger ribonucleic acid

OVAH- Onderstepoort Veterinary Academic Hospital

PaO2- Partial pressure of oxygen

PCR-RLB- Polymerase chain reaction- reverse line blot

PCV- Packed cell volume

RBC- Red blood cell

rT3- Reverse triiodothyronine

SIRS- Systemic inflammatory response syndrome

T3- Triiodothyronine

T4- Thyroxine

TBG- Thyroid hormone binding globulins

TNF- α- Tumour necrosis factor alpha

TSH- Thyrotropin

OVARU – Onderstepoort Veterinary Animal Research Unit

List of Figures

Chapter 1: Literature review

1.1 Canine babesiosis

Canine babesiosis is a common, and often fatal, intraerythrocytic protozoal infection in Southern Africa. The historical understanding of this infection posits that intravascular haemolysis and phagocytosis of both parasitised and non-parasitised red blood cells results in anaemia. [1-3] Building onto this initial understanding of the disease process, it is well recognised that babesiosis, malaria and many viral diseases all share a common inflammatory cytokine process, which explains the similarity in clinical signs seen across a variety of infectious diseases. [4-8]

It is now commonly accepted that it is the body's disproportionate inflammatory response, rather than the infectious agent itself, that results in the clinical signs we most commonly see. [5,9] A variety of diseases that all have a marked inflammatory component will typically share similar clinical signs despite the differences in the causative agent. These conditions commonly result in the systemic inflammatory response syndrome (SIRS) and may proceed to multiple organ dysfunction syndrome (MODS). [10,11] An increase in pro- inflammatory cytokines is seen in canine babesiosis and higher concentrations have been associated with disease severity and mortality. [12,13] Indeed, an exaggerated immune response is a hallmark in severe babesiosis and results in a shock- like state characterised by SIRS and MODS. [1,3,14-16] This amplified inflammatory response has been termed the 'cytokine storm' and has been associated with a variety of conditions including heatstroke, pancreatitis, and an assorted array of infectious agents. This common host response across a variety of disease states would suggest that investigation into *Babesia (B.) rossi* may provide insight into the pathobiology of other disease conditions that share a similar inflammatory response. [5-7,9]

The complex pathophysiology, along with the severe clinical disease and unpredictable outcome in canine babesiosis, has catalysed the investigation into a variety of biomarkers that may assist in prediction of outcome. Multiple parameters have been associated with a poorer prognosis including the degree of parasitaemia; [12,17] hyperlactataemia; [16,18,19] hypoglycaemia; [16,18] pro- inflammatory cytokine concentrations; [12,13] hyperbilirubinaemia; and the presence of

haemoconcentration. A large body of research has been dedicated to investigating new biomarkers in canine babesiosis, yet cortisol and thyroxine (T4) concentrations are still considered to be two of the strongest predictors of outcome. [16,20,21]

1.2 Cortisol in human literature

Cortisol plays a fundamental role in critical illness by regulating metabolism, vascular tone, and immune functions. It increases blood glucose concentration through induction of insulin resistance, gluconeogenesis and glycogenolysis; whilst also stimulating lipolysis and proteolysis. It contributes to the maintenance of vascular tone by regulating sodium clearance and enhancing the vasoconstrictor action of catecholamines; and regulates the immune system by preventing expression of pro- inflammatory mediators, thereby counteracting uncontrolled inflammation associated with sepsis. Cortisol is well known for its immunosuppressive effect. Yet, it also improves opsonisation and enhances the production of immunoglobulins. [22-26]

During critical illness, a variety of factors including cytokine release, hypoglycaemia, and hypoxaemia all stimulate the hypothalamus to release corticotropin-releasing hormone (CRH). Interleukin (IL)- 1 and IL- 6 further stimulate the hypothalamic- pituitary- adrenal (HPA) axis and amplify the action of CRH. This increased CRH activity ultimately results in increased cortisol release. [25,27,28] Multiple other factors such as vasopressin, angiotensin and the noradrenergic system further stimulate the HPA axis. [29] In addition, the biological half- life of exogenous cortisol in moribund patients with fatal shock has been shown to increase. This would suggest that high cortisol concentrations in critical illness may be due to a combination of increased production along with impaired removal. [30] When Boonen et al. investigated the theory of pituitary- independent mechanisms that could provide an explanation for elevated cortisol despite low adrenocorticotropic hormone (ACTH) concentrations in critically ill patients, they found that cortisol synthesis was 83% higher in intensive care unit (ICU) patients compared to healthy controls, yet cortisol clearance was simultaneously reduced by 50%. These findings confirmed that decreased cortisol metabolism developed and contributed to elevated cortisol concentrations and ACTH suppression during severe illness. [31] Similar alterations in glucocorticoid metabolism have been shown in dogs with naturally occurring sepsis. [32]

The HPA axis displays temporal changes during the progression of critical illness, with distinct differences seen in acute and chronic disease. During the initial phase, the HPA axis is predominantly under the control of CRH stimulation, which is characterised by elevated ACTH and cortisol concentrations. In contrast, chronic disease is typically characterised by low ACTH and high cortisol concentrations. The elevated cortisol despite suppressed ACTH concentrations would suggest that cortisol production in chronic disease is primarily stimulated by non- ACTH driven factors such as cytokines and vasoactive substances, along with impaired metabolism of cortisol already in circulation. [31,33-35]

The prognostic value of cortisol concentration during critical illness has been extensively researched in human literature. Several studies have found that higher basal cortisol concentrations are associated with a poorer outcome [36-46] whilst others have found contrary results [47,48] and a few studies identified no significant difference between cortisol concentrations and outcome. [49-51] There is still confusion regarding the role of cortisol in critical illness and its association with prognosis. However, most studies suggest that higher cortisol concentrations are associated with diseased states and poorer outcome.

1.3 Cortisol in veterinary literature

Although fewer studies have been published on cortisol changes in canine critical care, the findings appear to mirror those of human literature. When endocrine function was assessed in puppies confirmed with parvoviral diarrhoea, median cortisol concentration on admission was significantly higher in diseased puppies when compared to healthy controls. Furthermore, the cortisol concentration decreased over the subsequent days in the affected dogs that survived, as opposed to the non-survivors where cortisol concentrations remained elevated. [52] These findings were strongly supported by a later study that showed significantly higher cortisol concentrations on admission, in puppies with parvoviral diarrhoea when compared to healthy controls. The cortisol concentration at 72 hours post admission was strongly associated with mortality. The survivors showed a decline in cortisol concentration after treatment, whilst in the non- survivors, cortisol concentration remained persistently elevated. [53]

Prittie et al. assessed a group of critically ill dogs and found no significant difference between median basal cortisol, cortisol post ACTH stimulation, and endogenous ACTH concentration, in survivors versus non- survivors. These findings conflicted with the previous study; however, the sample size was small, and the dogs possessed a large variety of illnesses with varying duration of chronicity which may have confounded the results. [54] An additional study in dogs with lymphoma and non- haematopoietic neoplasia produced an unexpected conclusion. Basal cortisol and cortisol concentration post ACTH stimulation showed no difference between healthy dogs and dogs with neoplasia, or between dogs with mild, moderate, or severe disease. Furthermore, basal plasma endogenous ACTH concentration showed no difference between dogs with lymphoma and non- haematopoietic neoplasia. This study most likely possessed the same limitations seen with Prittie et al. as the investigation utilised a heterogenous group of patients with varying chronicity of disease. [55]

A large experimental study created a canine model for pneumonia- induced sepsis by introducing *Staphylococcus aureus* isolates into the right caudal lung lobes of beagle dogs by means of a bronchoscope. The authors found that total cortisol, free cortisol, and ACTH levels all increased markedly after bacterial challenge before decreasing back to baseline levels. At 24 hours, these parameters correlated significantly with survival time and each parameter moderately differentiated between survivors and non- survivors. The authors concluded that the recovery period provided valuable information for prognostication, as survivors showed lower cortisol and ACTH levels at 24 hours post bacterial challenge. [56]

When records were retrospectively reviewed from dogs admitted to ICU where serum cortisol concentration was measured within the first 3 days of admission, higher cortisol concentration was found to be a good predictor of mortality. [57] Another prospective study in dogs hospitalised for a variety of diseases assessed cortisol concentration on admission and again 24 hours later. The results of this study showed that serum cortisol concentration measured 24 hours after hospitalisation was a significant and independent predictor of mortality, with a cut-off of 182 nmol/L predicting mortality with a sensitivity of 89.5% and a specificity of 61.9%. [58]

1.4 Critical illness related corticosteroid insufficiency

Activation of the HPA axis is believed to be an essential adaptation during critical illness. Yet, multiple studies have documented proof of impaired adrenal function in critically ill people defined by low plasma cortisol concentrations and an inadequate response to an ACTH stimulation test, represented by delta cortisol. [59] Critical illness related corticosteroid insufficiency (CIRCI) has been associated with hypotension that is unresponsive to fluid and vasopressor therapy. Consequently, clinicians have investigated the use of cortisol supplementation to improve survival in these individuals. Tumour necrosis factor alpha (TNF-α) and corticostatin concentrations increase with infection and they have both been implicated in contributing towards adrenal insufficiency. TNF- α has been shown to impair ACTH secretion whilst corticostatin competes with ACTH for receptor binding. [25,27] Septic shock has also been associated with cerebral pathology including haemorrhage and ischaemic necrosis, which may result in decreased synthesis of CRH and ACTH. [60,61]

Several studies have identified the presence of adrenal insufficiency in patients with sepsis. [39,44,49,62,63] Furthermore, some articles have identified a poorer prognosis for patients displaying low basal cortisol concentrations or low delta cortisol, [39,43,44] whereas others did not. [40,47,49,51] But there is controversy surrounding the definition of CIRCI, as no consensus exists regarding the interpretation of an ACTH stimulation test in critically ill patients. It is important to remember that the reference values we use were derived from a healthy population and it is unclear what should be considered normal adrenal function in the critically ill. Studies have shown a large disparity in the prevalence of adrenal insufficiency, largely based on whether the diagnosis was determined using basal cortisol concentrations, delta cortisol, or a combination of these two. [64]

Not only has the definition of CIRCI come into question, but the existence of this condition is also met with differing opinions. Venkatesh and colleagues (2015) argued that the theory of CIRCI predicts that critically ill patients with lower basal cortisol should have higher mortality rates, yet this has not been consistently proven in the literature. In contrast, most studies found higher cortisol concentrations to be associated with mortality. [25,65] Another setback arose when variations in cortisol measurements between different methods and laboratories were identified. Many immunoassays showed poor agreement in septic serum and exhibited varying

levels of cross- reactivity to steroids other than cortisol. A very large portion of the literature on CIRCI has been based on patients with sepsis therefore, cortisol measurements by means of immunoassays in these patients may have resulted in an over- or underestimation of true cortisol concentrations. [66]

It must also be emphasised that the cortisol response to ACTH is inversely related to basal cortisol concentrations. The concept that the adrenal glands can only be stimulated to a maximum threshold suggests that the small response seen with a high basal cortisol concentration is due to maximal stimulation and is not an indicator of adrenal insufficiency. Therefore, studies that have interpreted delta cortisol without considering the basal cortisol concentration should be carefully scrutinised. [24,49] Schoeman and Herrtage (2008) performed cortisol to ACTH ratios in dogs with babesiosis to investigate the presence of relative adrenal insufficiency in this population. Patients with adrenal insufficiency should, by definition, possess low cortisol despite a high ACTH concentration, resulting in a low cortisol to ACTH ratio. Yet dogs in this study that were diagnosed with relative adrenal insufficiency based solely on a low delta cortisol value possessed significantly higher cortisol to ACTH ratios. This clearly illustrates the danger of diagnosing relative adrenal insufficiency based purely on delta cortisol levels as the patients in this study with the lowest delta cortisol levels clearly possessed a strong adrenal response to ACTH. [67]

The concept of decreased cortisol metabolism during critical illness can also have clinical implications on the interpretation of CIRCI. Hypercortisolaemia can be maintained through mechanisms other than increased production by the adrenal glands therefore, the adrenal response to ACTH should not be evaluated in isolation. Impaired glucocorticoid metabolism has been demonstrated in critically ill humans and dogs, and clinicians should be cognizant of this alteration when diagnosing CIRCI.[31,32]

Lastly, the use of etomidate in previous studies has enhanced controversy regarding the true incidence of adrenal insufficiency. Etomidate is an anaesthetic agent that was commonly used to facilitate endotracheal intubation in ventilator patients. We now know that this drug directly interferes with glucocorticoid synthesis by causing reversible inhibition of the 11- hydroxylase enzyme, resulting in decreased cortisol secretion. It has been shown that a single dose of etomidate may inhibit cortisol synthesis for at least 24 hours in critically ill patients and increases the risk of adrenal insufficiency by 12-fold. [68,69] The results from earlier studies that

used etomidate need to be carefully interpreted. It has been proposed that these studies were ultimately flawed in identifying the incidence of adrenal insufficiency and, at best, purely illustrated that a decreased mortality rate could be seen in patients with iatrogenic adrenal insufficiency who had been supplemented with exogenous cortisol. [48,70,71]

There is limited information regarding the presence and incidence of CIRCI in canine critical illness. No evidence of CIRCI was identified in a group of critically ill dogs that presented with varying diseases [54] or in dogs naturally infected with acute and subclinical ehrlichiosis. [72] Schoeman and Herrtage (2008) were unable to find any indication of adrenal insufficiency in dogs infected with *B. rossi* after measuring the cortisol to ACTH ratios, which they suggested had more benefits over the conventional ACTH stimulation test due to its assessment of the entire HPA axis. [67] However, Burkitt and colleagues (2007) suggested the presence of CIRCI in 48% of septic dogs and found that low delta cortisol was associated with increased incidence of systemic hypotension and mortality. Unfortunately, this study assessed delta cortisol without reporting the basal cortisol concentration, which makes interpretation difficult.[73] CIRCI was later diagnosed in 37% of critically ill dogs based on delta cortisol after an ACTH stimulation test. Again, the basal cortisol concentration in the individual dogs was not clear, which hampers interpretation. [74] The existence of CIRCI in veterinary science is undoubtedly still up for debate.

1.5 Thyroid hormones in human literature

Thyroid hormones play an integral role in the control of homeostasis and affect a variety of systems in the body. They assist growth, metabolism, and maintenance of the cardiovascular, reproductive, and central nervous systems. They have positive inotropic and chronotropic effects on the heart; they enhance gastrointestinal motility; and stimulate glucose absorption. [35,75,76]

Systemic illness in both human and veterinary literature has been associated with decreased serum thyroid hormone concentrations. This decrease despite normal thyroid function has been termed the “euthyroid sick syndrome” (ESS) and is believed to result from a variety of different mechanisms. These include inhibition of the 1 and 2 deiodinase enzymes, resulting in decreased

conversion from T4 to triiodothyronine (T3), decreased concentration of thyroid hormone binding globulins (TBG), decreased affinity and binding of thyroid hormones to TBGs, or the presence of circulating substances that inhibit binding. Decreased production of thyrotropin (TSH) in severe illness secondary to cytokines and reactive oxygen species has also been reported. [35,76-82] Human literature is filled with reports that link low thyroid hormone concentrations with disease states, as well as disease severity and outcome. [43,75,83,84]

1.6 Thyroid hormones in veterinary literature

There is very limited data concerning the hypothalamic- pituitary- thyroidal (HPT) axis in canine critical illness. Elliot et al. (1995) studied the thyroid hormone alterations in critically ill dogs to determine the incidence of ESS and to assess its prognostic value. They found that ESS was commonly seen in critical illness and was mostly associated with decreased serum T4 concentrations. No significant difference was found between the baseline and post stimulation T4 concentrations for survivors versus non- survivors. Yet, mean baseline T3 concentrations were significantly lower in non-survivors. There was also a significant difference between the post stimulation mean serum T3 concentration of survivors compared to non- survivors. [78] Intravenous administration of endotoxin to healthy experimental dogs resulted in the classic thyroid hormone modifications associated with ESS, namely decreased serum T3 and T4 concentrations, in combination with increased reverse triiodothyronine (rT3) concentration. [85] Another study found that median serum T4, T3 and free thyroxine (fT4) concentrations were significantly lower in dogs with non- thyroidal disease compared to clinically normal controls. Furthermore, dogs with severe disease were more likely to have low serum concentrations of T4, T3 and fT4 than dogs with mild disease however, serum TSH was more likely to remain within the reference range regardless of the severity of disease. [86] In a study assessing dogs involved in motor vehicle accidents, low T4 and fT4 concentrations were again identified as potential markers of mortality, whilst TSH was not predictive of outcome. [87]

Thyroid function in tumour- bearing dogs, with and without chronic weight loss, was compared with non- tumour- bearing dogs, with and without chronic weight loss. T4, T3 and free triiodothyronine (fT3) concentrations were significantly lower in dogs with chronic weight loss and these changes corresponded to the degree of weight loss, regardless of the presence or

absence of neoplasia. This would suggest that decreased thyroid hormone levels were most likely related to an aberrant nutritional state or the severity of illness, and not to tumour- burden. [88]

When serum thyroid hormones on admission were retrospectively evaluated in dogs diagnosed with acute pancreatitis, parvoviral enteritis, or septic peritonitis, dogs with septic SIRS had significantly lower T4 and T3 concentrations when compared to healthy controls and dogs with non- septic SIRS. A low T4 concentration was the only thyroid hormone that was predictive of mortality. [89] In puppies with parvoviral diarrhoea, median T4 concentration on admission was significantly lower when compared to healthy controls. Furthermore, a significantly lower T4 concentration was seen in non- survivors compared to survivors throughout the first three days of treatment. [52] Schoeman and Herrtage (2008) further investigated serial serum concentrations of T4, fT4 and TSH and its association with mortality in puppies diagnosed with severe parvoviral diarrhoea. They concluded that the median T4 and fT4 concentrations were significantly lower in non- survivors on days 1 to 4, whilst the median serum TSH concentration on day 1 was significantly higher in non-survivors. This study showed an initial elevation in TSH combined with low T4 and fT4 concentrations in the non- survivor group. This illustrated that suppression of the thyroidal axis is dependent on factors other than a decreased production of TSH from the pituitary gland. [90] In contrast, a later study did not find a significant difference in fT3 and fT4 concentrations between puppies with parvoviral diarrhoea and healthy controls. Furthermore, these thyroid hormones did not allow differentiation between survivors and non- survivors in the patient population. The authors suggested that the opposing results between these two studies may have been related to differences in virulence factors and viral strains. [53]

Pashmakova et al. (2014) assessed dogs hospitalised with sepsis or SIRS to determine whether thyroid hormone derangements occurred with these syndromes and whether these alterations related to illness severity and prognosis with comparison to the acute patient physiologic and laboratory evaluation (APPLE) score. Their results showed that median T4 concentrations were significantly below reference intervals for dogs with sepsis and SIRS, whilst median fT4 concentrations were decreased compared to healthy control dogs but were still within the normal range. TSH concentrations were significantly lower in dogs with SIRS compared to control dogs but no significant difference was found between dogs with SIRS and those with sepsis. This study found no association between serum thyroid hormone concentrations and

outcome. [76] These findings were later mirrored in another study where T4 concentration on admission to ICU showed no association with mortality or with the length of hospital stay. [91]

1.7 Endocrine parameters in *B. rossi* infections

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 that died in comparison to dogs that survived after treatment in hospital, or dogs that were stable enough to be managed as outpatients. [20] 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. [16]

The response of the pituitary- adrenal and pituitary- thyroidal axes to plasma glucose disturbances in dogs with *B. rossi* infections have also been evaluated. Basal and ACTH-stimulated cortisol concentrations were both significantly higher, whilst T4 and fT4 concentrations were significantly lower, in hypoglycaemic dogs in comparison to those with normoglycaemia and hyperglycaemia. [21] A further study went on to assess the adrenocortical function in 68 dogs with *B. rossi* infection. Infected dogs 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 hospitalised 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. [67]

These studies illustrate the significant and repeatable endocrine derangements that occur during critical illness and the value they may have with prognostication. 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.

Chapter 2: Methodology

2.1 Objectives

a) To compare endocrine parameters (cortisol, T4, T3 and TSH) in dogs with experimental *B. rossi* infection with healthy control dogs.
b) To compare endocrine parameters (cortisol, T4, T3 and TSH) in dogs with experimental *B. rossi* infection, infected with a low dose of parasite inoculum (10^4 parasitised red blood cells [RBCs]) with those infected with a high dose (10^8 parasitised RBCs).
c) To correlate cortisol, T4, T3 and TSH concentrations in dogs with experimental *B. rossi* infection.

2.2 Hypotheses

a) H_0: There will be no significant difference in endocrine parameters (cortisol, T4, T3 and TSH) between dogs with experimental *B. rossi* infection and healthy controls.
   H_1: There will be a significant difference in endocrine parameters (cortisol, T4, T3 and TSH) between dogs with experimental *B. rossi* infection and healthy controls.
b) H_0: There will be no significant difference in endocrine parameters (cortisol, T4, T3 and TSH) between dogs experimentally infected with a low dose of *B. rossi* parasite inoculum (10^4 parasitised RBCs) and those infected with a high dose (10^8 parasitised RBCs).
   H_1: There will be a significant difference in the endocrine parameters (cortisol, T4, T3 and TSH) between dogs experimentally infected with a low dose of *B. rossi* parasite inoculum (10^4 parasitised RBCs) and those infected with a high dose (10^8 parasitised RBCs).
c) H_0: There will be no correlation between cortisol, T4, T3 and TSH concentrations in dogs experimentally infected with *B. rossi* parasite inoculum.
   $H_{1:}$ There will be a negative correlation between cortisol and T4, T3 and TSH concentrations, and a positive correlation between T4, T3 and TSH concentrations in dogs experimentally infected with *B. rossi* parasite inoculum.

2.3 Benefits of the study

Canine babesiosis is a common disease that negatively impacts animal health across the globe. Furthermore, canine babesiosis has been proposed as a model for human falciparum malaria and may provide clinical insight into a variety of human and animal conditions that share a common inflammatory process.

An experimental *B. rossi* infection in dogs described here has provided insight into the pathophysiology and the endocrine changes associated with this disease from the onset of infection. This has hitherto been impossible to investigate in a clinical setting, because dogs typically presented at differing stages of disease with differing parasitaemia and may have been suffering from co-morbidities that complicate interpretation of data. Consequently, a study that can describe the magnitude and exact timings of the endocrine responses to a protozoal infection with a known inoculum size and time would provide insights into the body's response to infection and be of great translational value.

2.4 Study design

A prospective longitudinal observational study was performed using six purpose bred castrated male beagle dogs to assess endocrine parameters in healthy control dogs and those experimentally infected with *B. rossi*. One randomly selected dog was splenectomised and infected with a wild- type *B. rossi* cryopreservate for the creation of a *B. rossi* parasite inoculum. The remaining five experimental dogs had samples taken prior to experimental infection which were used as healthy control samples. The experimental dogs were randomly divided into two groups where two dogs were experimentally infected with a low dose (10^4 parasitised RBCs) of *B. rossi* parasite inoculum and three dogs were infected with a high dose (10^8 parasitised RBCs). Basal serum cortisol, T4, T3 and TSH concentrations were measured at 24, 72, 108, 144 and 192 hours post infection in all experimental dogs. The high dose group had additional samples taken at 96 and 120 hours, as clinical deterioration necessitated more intensive monitoring. Once the predetermined end points of infection were reached, all dogs were drug cured with diminazene aceturate (3.5mg/kg subcutaneously). The study was approved by the Faculty Research Ethics committee (REC050-19).

2.5 Study population

The population under study consisted of six purpose bred castrated male beagle dogs, identified by an implanted microchip (Back Home®, Virbac, South Africa). The dogs were housed at the Onderstepoort Veterinary Animal Research Unit (OVARU) from eight weeks of age until the end of the study period. All dogs were habituated to the process of examination and sample collection through daily training. All dogs were well socialized with people and the normal sounds of a domestic home. They were housed in a play-enriched environment as a group until the infection phase at which time they were housed individually but next to each other with visual and limited physical contact. 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 *B. rossi*, *B. vogeli* and *Ehrlichia canis* infection (confirmed by polymerase chain reaction- reverse line blot [PCR-RLB] prior to experimental infection).

One dog was randomly selected for splenectomy and infection with a wild-type *B. rossi* cryopreservate. The splenectomised dog was treated with diminazene aceturate (3.5mg/kg subcutaneously), excluded from further sampling and rehomed as a pet. The remaining five experimental dogs had baseline samples taken on two separate occasions prior to experimental infection. These samples provided control data. Thereafter, the experimental dogs were randomly divided into two groups where two dogs were experimentally infected with a low dose of *B. rossi* parasite inoculum (10^4 parasitised RBCs) and three dogs were infected with a high dose (10^8 parasitised RBCs).

2.6 Experimental design

Peripheral blood smear evaluation was used to identify a dog naturally infected with *B. rossi* presenting for veterinary care at the Outpatients clinic of the Onderstepoort Veterinary Academic Hospital (OVAH). PCR-RLB was performed to confirm the presence of a *B. rossi* infection and to exclude co- infection with *B. vogeli* and *Ehrlichia canis*. The dog was used to

isolate a pure *B. rossi* parasite cryopreservate. Owner consent was acquired for participation in the study.

Fifty millilitres of blood were collected and divided into 10 ml heparinised tubes from the naturally infected dog. The blood was stored at 4 °C for two hours prior to further processing. 45 ml of blood was decanted from the collection tubes into a 50ml flask and kept on melting ice. Using a 1 ml pipette, 5 ml of dimethyl sulfoxide (DMSO) was added one drop at a time, with a waiting period of 10 seconds between drops, whilst simultaneously swirling the flask kept on melting ice. Blood was divided into 4 ml cryotubes kept on melting ice. The cryotubes were transferred to a CooiCell container and stored in a -40 °C freezer overnight and then transferred to an appropriate container the next day in a -80 °C freezer.

One purpose bred laboratory beagle dog was randomly selected for splenectomy that was performed by a qualified veterinarian at the OVAH. Six weeks post splenectomy, 100ml of venous blood was collected into acid citrate from the splenectomised dog and refrigerated for future dilutions of the parasitised blood donated by this dog. The cryopreserved ampules from the naturally infected dog were thawed in a water bath set at 37 °C. The contents were then transferred to a syringe and intravenously inoculated within 15 minutes to the splenectomised dog.

Parasitaemia of the splenectomised dog was manually determined on venous blood collected twice daily starting one day post infection. The method used to determine the parasitaemia was successfully performed in a previous study. Blood smears were stained with Kyro-quick (Kyron Laboratories, Benrose, South Africa) and scored at 1000 times magnification using a digital image analysis program (Optimas 6 forWin 95/ NT 4.0, Media Cybernetics, distributed by Carl Zeiss Ltd., Randburg, South Africa). A total of at least 1950 erythrocytes were examined per smear and counted as either parasitised or unparasitised by the principal investigators and expressed as a percentage of parasitised erythrocytes. Each oil immersion field was completely counted to avoid bias. Approximately 650 erythrocytes were counted in the red cell area, the feathered edge, and along the sides of the smear, respectively. Free parasites were not counted. A score of < 0.05 % was given if no parasitised erythrocytes were counted in the randomly selected areas but were observed elsewhere on the smear. A score of 0 % was assigned if no parasitised erythrocytes were detected after 15 minutes of blood smear evaluation. [17]

The manually determined parasitaemia allowed for the creation of two different infectious doses (10^4 and 10^8 parasitised RBCs per 1ml of blood) for injection into the experimental animals. The number of parasitised erythrocytes in 1ml was calculated by multiplying the parasite percentage with the RBC count obtained from the complete blood count (CBC) analysis (Number of parasitised RBCs per 1ml = % parasitised RBCs x RBC count). The previously collected 100ml of plasma from the splenectomised dog prior to infection was used as the diluent to create serial dilutions of 10^4 and 10^8 parasitised RBCs per 1ml. 1 ml of the sample was centrifuged, and a parasitaemia count was performed on the pellet to confirm presence of parasitised erythrocytes, prior to injection of the infectious dose.

The splenectomised dog was drug cured with diminazene aceturate (3.5mg/kg subcutaneously). The experimental dogs were then injected with parasitised fresh whole blood, diluted to achieve the two required doses. The two dogs in the low dose group were injected intravenously with 1 ml containing 10^4 *B. rossi* parasitised RBCs. The three dogs in the high dose group were injected intravenously with 1 ml containing 10^8 *B. rossi* parasitised RBCs.

Clinical examinations were performed daily in all dogs. Blood smears were performed each day using a drop of peripheral blood from the ear. Parasitaemia was calculated using the same method employed for determination of parasitaemia in the splenectomised dog. [17] Blood was collected daily between 8:00 am and 10:00 am as part of a larger study until the predetermined end points of the infection were reached:

A haematocrit or packed cell volume [PCV] < 15 %

A habitus score of 1+ (defined as lethargic and non- responsive)

Neurological symptoms, whether due to neuroglycopaenia or not

Clinical evidence of lung pathology with arterial blood gas evidence of acute respiratory distress syndrome (defined as partial pressure of oxygen [PaO2] < 60mmHg)

Evidence of oliguria (defined as a urine production of < 1 ml/kg/hour) with a serum creatinine > 200mmol/L (normal < 140mmol/L)

Evidence of haemoconcentration (defined as a PCV > 55%) in the presence of haemolysis (macroscopically evident haemoglobinuria and/ or haemoglobinaemia)

A dog that lived to 20 days post infection.

Once the end points were reached, the dogs were cured with diminazene aceturate (3.5mg/kg subcutaneously) and provided with the supportive treatment required to return them to health. At the end of the experimental study the dogs were rehomed as pets.

2.7 Experimental procedures

All clinical examinations and sample collections were performed between 8:00 am and 10:00 am each day, with the exception of a single day where clinical deterioration necessitated additional monitoring and sampling at 8pm. Blood smear evaluation and sample collection was performed exclusively by the principal investigator and co-workers, according to the collection schedule provided in Appendix A. All blood samples were collected from the jugular vein with 21- gauge vacutainer needles (Precision Glide^{TM},UK). Serum samples were collected in serum vacutainer brand tubes (Beckton Dickinson Vacutainer Systems, UK), centrifuged, aliquoted and immediately frozen thereafter. Cortisol, T4, T3, and TSH concentrations were analysed as a single batch using a solid- phase, competitive chemiluminescent enzyme immunoassay (Immulite® 2000, Siemens) as described in previous studies. [82,92-94] The laboratory reference intervals for cortisol, T4 and TSH were 20 – 200 nmol/L, 13 - 41 nmol/L, and 0 - 0.45 ng/mL, respectively.

2.8 Data management

All data obtained from the study was recorded onto a spreadsheet (Microsoft Excel®, Microsoft Corporation, Redmond, WA, USA) which was stored on the principal investigator's laptop computer. A back-up copy was stored on an external hard drive, whilst another copy was stored online using Google Drive, with an updated copy sent daily to all parties involved in the project during the experimental period.

2.9 Statistical analysis

Linear mixed models with Bonferroni adjustment for multiple comparisons was used to compare results within each experimental group to the control samples collected at the beginning of the study period, and to compare results between the low and high dose groups at each timepoint. Correlations between the variables were assessed using Spearman's rank correlation with Bonferroni adjustment. Statistical analysis was performed using a commercial software package STATA 15 (Statacorp, College station, TX, USA). Data is presented as median and interquartile range (IQR). For all tests, significance was set at $P < 0.05$.

Chapter 3: Results

3.1 Study population

The experimental population under study consisted of five purpose bred castrated male beagle dogs of the same age. Pre-infection samples were used as controls on two separate occasions, creating ten control samples in total. One dog from the high dose group died unexpectedly on day four of the study, shortly after the 96-hour sampling timepoint.

3.2 Percentage parasitaemia

As shown in Figure 1, the high dose group had high parasitaemias, with a peak of 45.5% (40.3- 52.7), 96 hours post infection. Treatment was given to the high dose group at this 96- hour timepoint. The low- dose group had lower parasitaemias, often only reaching a tenth of the parasite density seen in the high dose group. A much lower peak of 5.8% (5.2- 6.3) occurred in the low dose group, 108 hours post infection. Treatment was given to the low dose group at this 108- hour timepoint. All dogs in the high dose group became depressed and this quickly evolved into collapse (defined as the inability to stand unaided), whilst none of the dogs in the low dose group were collapsed.

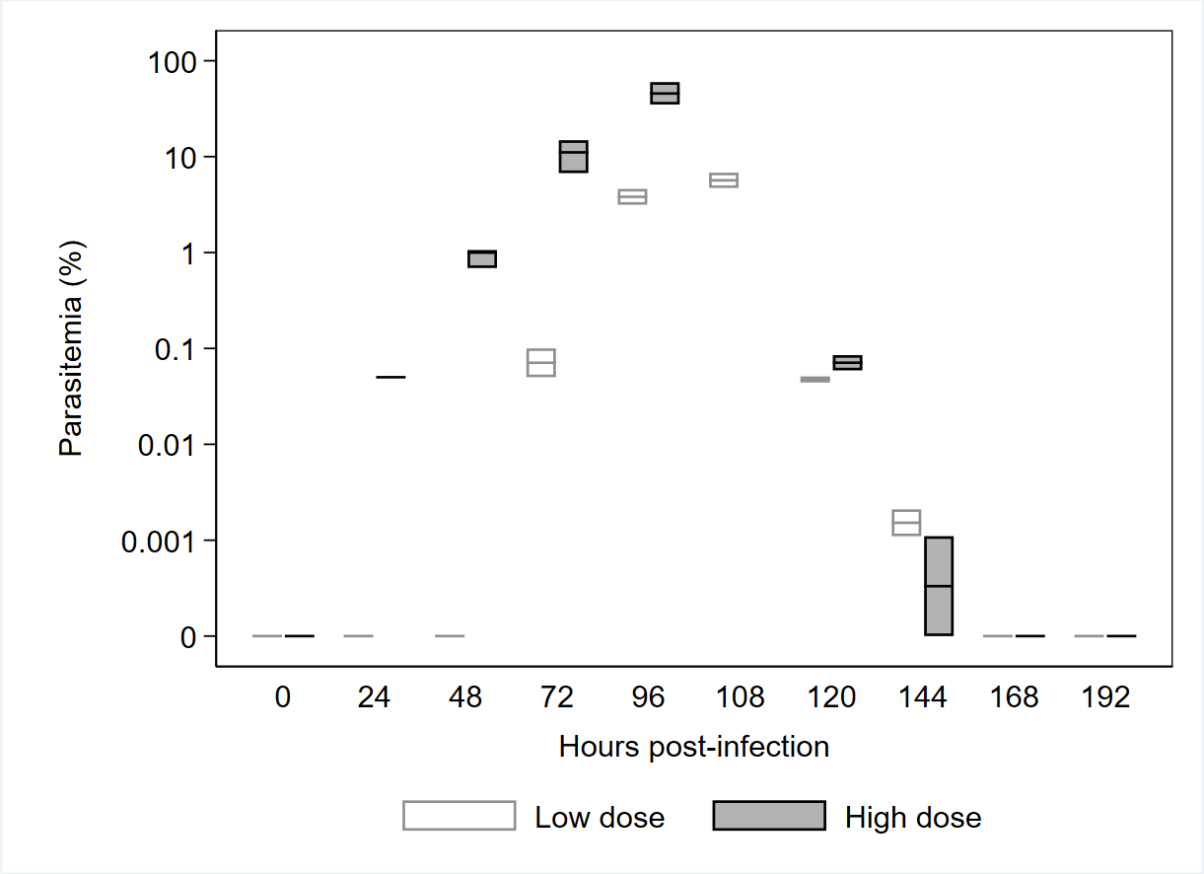

Figure 1: Box plots illustrating the temporal changes in parasitaemia percentage with IQR after experimental B. rossi infection. Boxes illustrate the IQR and the central line within each box represents the median.

3.3 HPA axis in response to experimental *B. rossi* infection

The natural response of the HPA to experimental *B. rossi* infection at different infectious doses is illustrated in Figure 2. The control group's cortisol concentration prior to infection was 27.5 nmol/L (27.5- 35.7). The high dose group's cortisol concentration increased rapidly to reach a peak of 315 nmol/L (269- 462.5) prior to treatment at 96 hours. The low dose group's cortisol concentration demonstrated a more protracted increase to 52.15 nmol/L (43.32- 60.98) prior to treatment at 108 hours. After treatment, the cortisol concentration in the high dose group rapidly declined to a nadir of 31 nmol/L (29.25- 32.75), 192 hours post infection. The cortisol concentration in the low dose group declined gradually after treatment and returned to the pre-infection cortisol concentration of 27.5 nmol/L (27.5- 27.5), 192 hours post infection.

When compared to control samples, a significantly higher cortisol concentration was seen at 108 hours ($P < 0.001$) and 144 hours ($P = 0.028$) in the low dose group; and at 72 hours ($P < 0.001$), 96 hours ($P < 0.001$), 108 hours ($P < 0.001$), 120 hours ($P < 0.001$) and 144 hours ($P < 0.001$) in the high dose group. No samples were collected from the low dose group at the 96- and 120-hour timepoints due to ethical concerns related to excessive sampling. A more rapid increase with a significantly higher cortisol concentration was seen in the high dose group when compared to the low dose group at 72 hours ($P < 0.001$), 108 hours ($P < 0.001$) and 144 hours ($P = 0.001$) post infection (Figure 2). One dog from the high dose group died during the study. This dog showed the highest cortisol concentration of 610 nmol/L at 96 hours post infection, shortly before death.

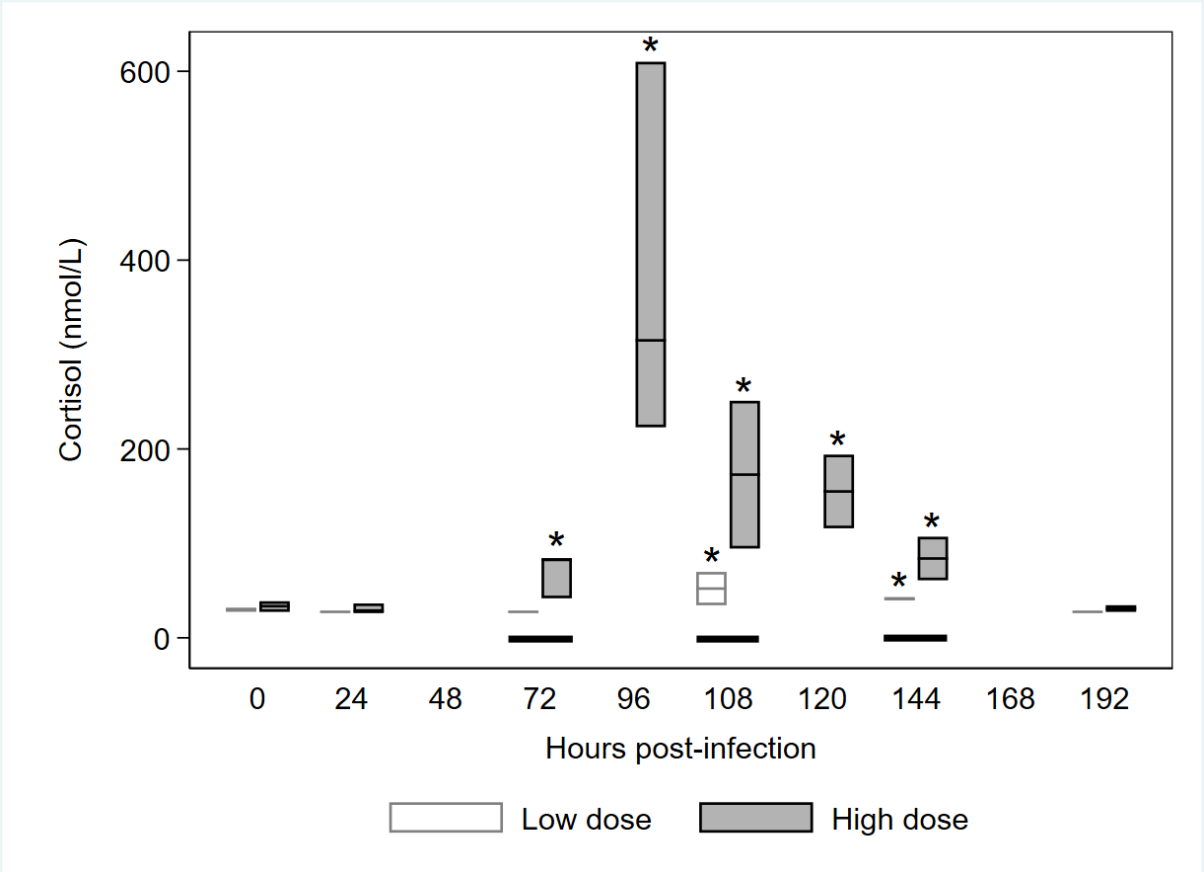

Figure 2: Box plots illustrating the temporal changes in median cortisol concentration with IQR after experimental B. rossi infection. Boxes illustrate the IQR and the central line within each box represents the median. Horizontal black bars indicate the timepoints at which the high

and the low dose groups differ significantly from each other ($P < 0.05$); asterisks indicate significant differences compared to the controls at time 0 ($P < 0.05$).

3.4 HPT axis in response to experimental *B. rossi* infection

The changes in T4 concentration after experimental *B. rossi* infection at different infectious doses is illustrated in Figure 3. The control group's T4 concentration was 40.3 nmol/L (36- 52). The high dose group's T4 concentration declined rapidly from 51.6 nmol/L (48.65- 53.2) upon infection, to 7.14 nmol/L (6.79- 7.94), 96 hours post infection. The low dose group's T4 concentration declined slowly from 47.45 nmol/L (46.12- 48.78) upon infection, to 26.1 nmol/L (23.75- 28.45), prior to treatment at 108 hours post infection. The T4 concentration in the high dose group continued to show a mild decline after treatment with a concentration of 6.43 nmol/L (6.43- 6.43) seen at 108 hours post infection. Thereafter, the T4 concentration increased to reach 34.4 nmol/L (34.4- 34.4) by 192 hours post infection. The T4 concentration in the low dose group followed a similar pattern with a decline after treatment to 16.66 nmol/L (12.68- 20.63), 144 hours post infection; before increasing rapidly to 36.4 nmol/L (35.7- 37.1), 192 hours after infection.

When compared to control samples, a significantly lower T4 concentration was seen at 24 hours ($P < 0.001$), 108 hours ($P < 0.001$) and 144 hours ($P < 0.001$) in the low dose group. The T4 concentration in the high dose group was significantly lower than control samples at 72 hours ($P < 0.001$), 96 hours ($P < 0.001$), 108 hours ($P < 0.001$), 120 hours ($P < 0.001$), 144 hours ($P < 0.001$) and 192 hours ($P < 0.001$). This study showed a more rapid decline and lower T4 concentration in the high dose group when compared to the low dose group with a significant difference seen at 108 hours ($P < 0.001$) post infection (Figure 3). The single dog that died during this study had a T4 concentration below the limit of detection at 96 hours post infection, shortly before death refer to the comment on Cortisol.

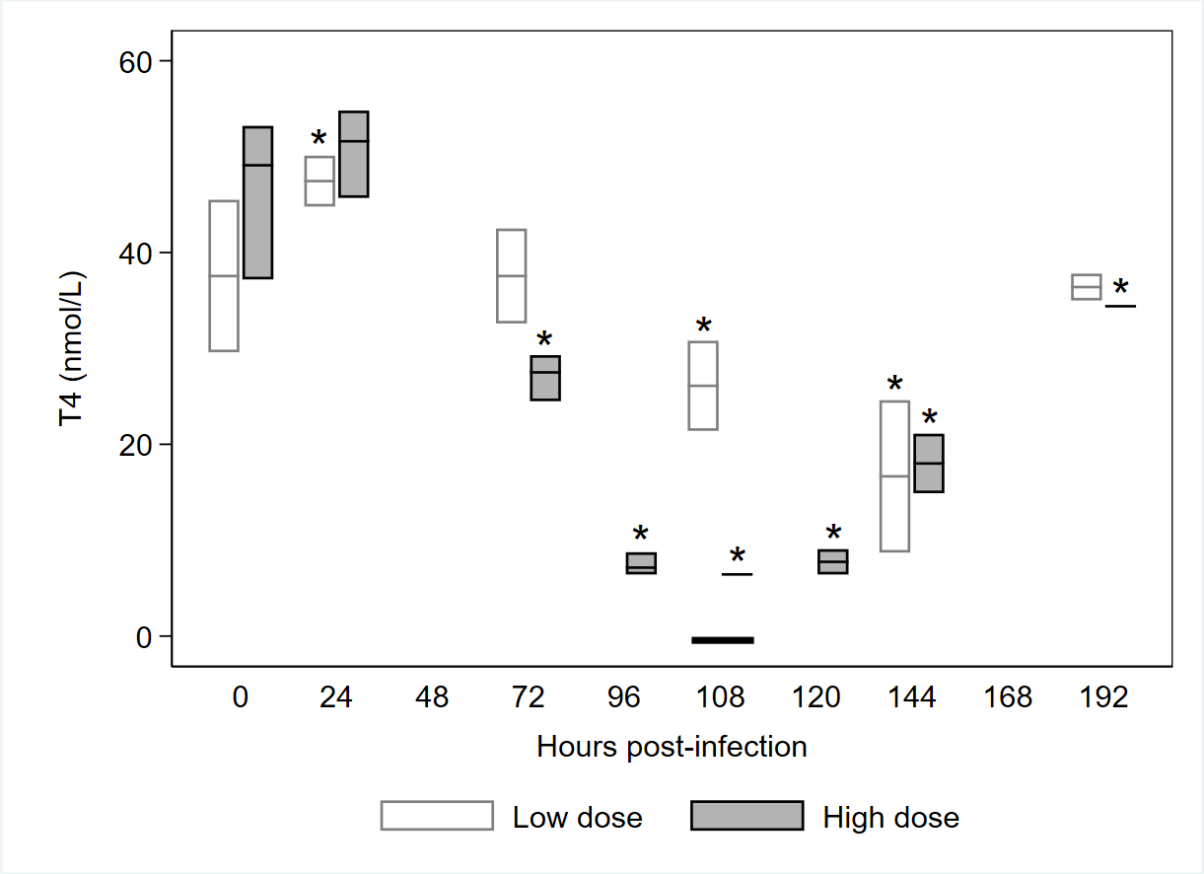

Figure 3: Box plots illustrating the temporal changes in median T4 concentration with IQR after experimental B. rossi infection. Boxes illustrate the IQR and the central line within each box represents the median. Horizontal black bars indicate the timepoints at which the high and the low dose groups differ significantly from each other ($P < 0.05$); asterisks indicate significant differences compared to the controls at time 0 ($P< 0.05$).

The changes in T3 concentration after experimental *B. rossi* infection at different infectious doses is illustrated in Figure 4. The control group's T3 concentration was 0.69 nmol/L (0.62- 0.84). The high dose group's median T3 concentration declined from 0.665 nmol/L (0.638- 0.736) to 0.61 nmol/L (0.61- 0.61), below the assay's limit of detection, 72 hours post infection, and remained at this value for the period of the study. The low dose group's T3 concentration declined from 0.909 nmol/L (0.858- 0.959) to 0.71 nmol/L (0.66- 0.76), prior to treatment at 108 hours post infection. Similar to the T4 results, the T3 concentration in the low dose group continued to decline after treatment with a T3 concentration of 0.61 nmol/L (0.61- 0.61) at 144 hours; before increasing to 0.819 nmol/L (0.809- 0.828), 192 hours post infection.

Although the study showed a decline in T3 concentration in both groups, a significant difference was only seen between the control and low dose group at 144 hours ($P = 0.011$) post infection. The T3 concentration in the high dose group was often lower than that of the low dose group but a significant difference was only seen at 24 hours ($P = 0.005$) and 192 hours ($P = 0.008$) post infection. The T3 concentrations in the high dose group measured below the limit of detection at all time points taken after experimental infection therefore, it is impossible to determine the true value of these concentrations.

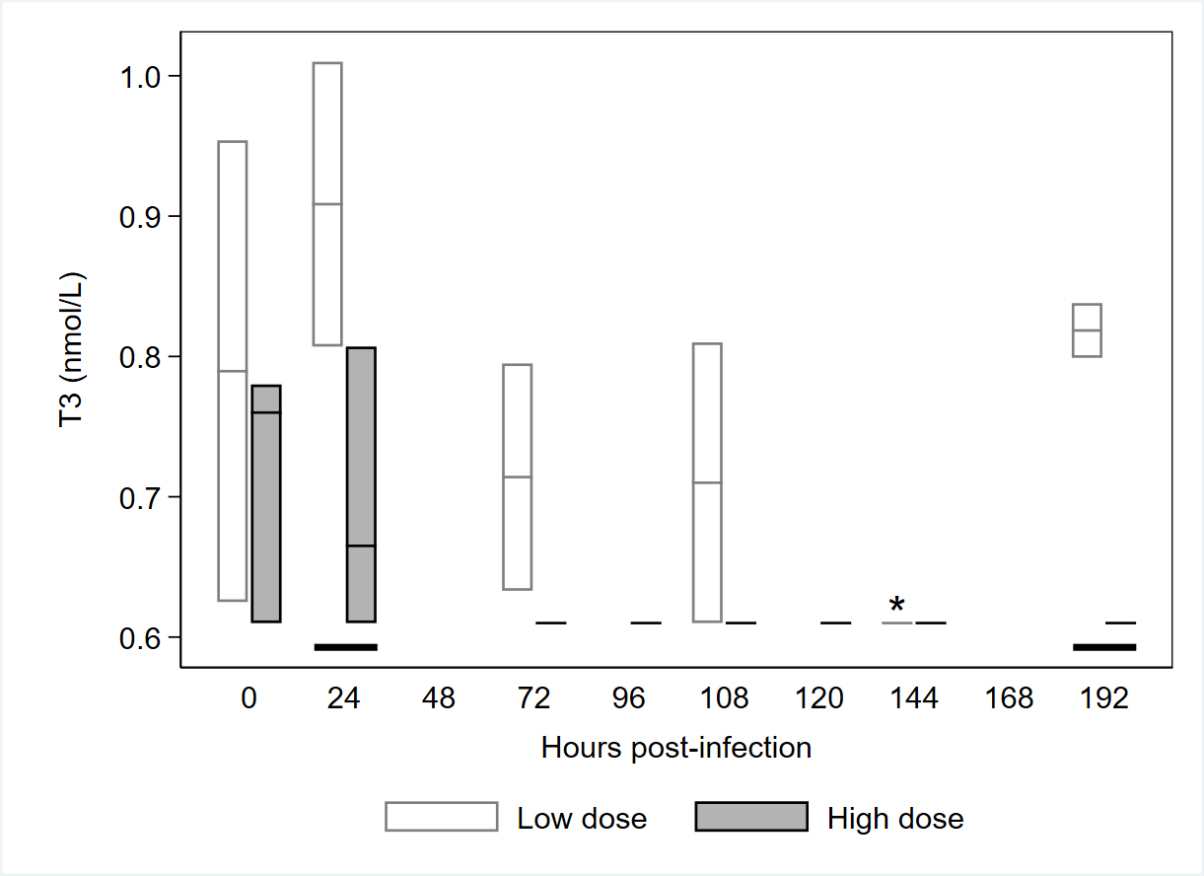

Figure 4: Box plots illustrating the temporal changes in median T3 concentration with IQR after experimental B. rossi infection. Boxes illustrate the IQR and the central line within each box represents the median. Horizontal black bars indicate the timepoints at which the high and the low dose groups differ significantly from each other ($P < 0.05$); asterisks indicate significant differences compared to the controls at time 0 ($P < 0.05$).

The changes in TSH concentration after experimental *B. rossi* infection at different infectious doses is illustrated in Figure 5. The control group's TSH concentration was 0.074 ng/dL (0.055- 0.098). Both the high and low dose group showed an initial decline in TSH concentration 72 hours post infection, but a divergence between the two groups was seen thereafter. The TSH concentration in the high dose group continued to decline to 0.029 ng/dL (0.029- 0.029), 108 hours post infection, whilst the low dose group's TSH concentration increased dramatically to 0.228 ng/dL (0.209- 0.246), 108 hours post infection (point of treatment). As mentioned, the TSH concentration in the high dose group continued to show a mild decline after treatment with a TSH concentration of 0.029 ng/dL (0.029- 0.029) at 108 hours post infection. Thereafter, the TSH concentration increased to reach 0.107 ng/dL (0.1- 0.112) at 192 hours post infection. The TSH concentration in the low dose group showed a rapid decline after treatment to 0.060 ng/dL (0.047- 0.072), 144 hours post infection, before increasing to 0.082 ng/dL (0.076- 0.087), 192 hours post infection.

A significant difference was seen between the control and the high dose group at 96 hours ($P < 0.001$), 108 hours ($P < 0.001$), 120 hours ($P = 0.001$), and 144 hours ($P = 0.049$) post infection. A significant difference was only seen between the control and the low dose group at 108 hours ($P= 0.011$) post infection. Although the TSH concentration in the high dose group was often lower than that of the low dose group, a significant difference between the two groups was only seen at 108 hours ($P < 0.001$). Despite these variations, the TSH concentrations remained within reference intervals throughout the study period for both groups.

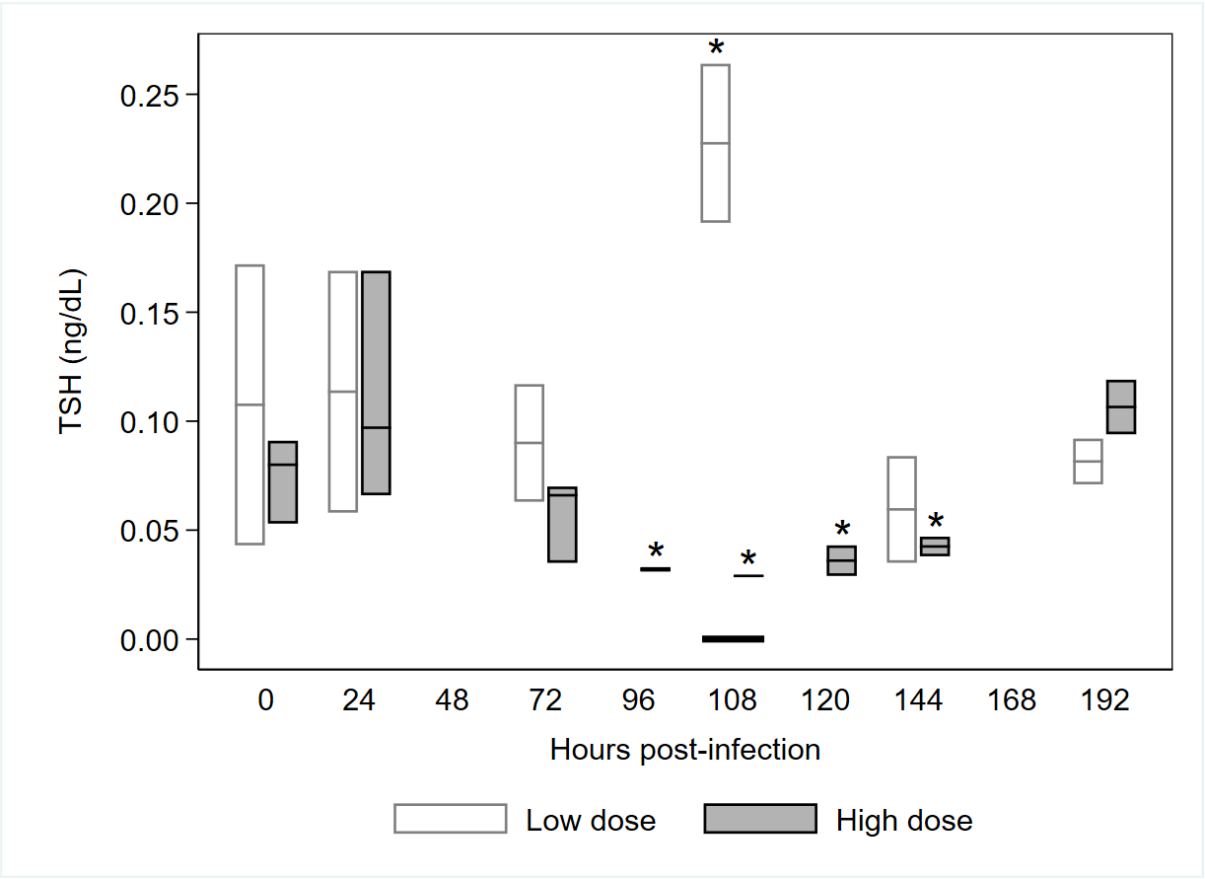

Figure 5: Box plots illustrating the temporal changes in median TSH concentration with IQR after experimental B. rossi infection. Boxes illustrate the IQR and the central line within each box represents the median. Horizontal black bars indicate the timepoints at which the high and the low dose groups differ significantly from each other ($P < 0.05$); asterisks indicate significant differences compared to the controls at time 0 ($P < 0.05$).

3.5 Correlations between parameters

A significant negative correlation was detected between cortisol, and T4, T3 and TSH respectively (r_s = -0.856, -0.651 and -0.698 respectively, $P < 0.05$). Both T4 and T3 showed a positive correlation with each other (r_s = 0.700, $P < 0.05$) and with TSH respectively (r_s= 0.732 and 0.558, $P < 0.05$).

Chapter 4: Discussion

1.1 General discussion

This study was the first to illustrate the temporal changes in endocrine parameters during an experimental *B. rossi* infection. Up until now, endocrine changes in canine babesiosis have only been described in natural infections [16,20,21,67,82] where differing stages of disease, differing parasitaemia, and comorbidities were likely to occur and obscure interpretation of data. 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.

The cortisol concentrations increased significantly in the experimental groups when compared to the control samples, with a significant increase seen for both groups at 108 hours post infection. This increase may have been earlier based on the changes seen at the 96- hour timepoint in the high dose group. Unfortunately, a 96- hour sample was not taken from the low dose group to investigate this possibility. A decline in cortisol concentration was seen in both groups after treatment. Similar changes in cortisol concentrations have been demonstrated in experimental canine staphylococcal pneumonia but a faster increase in cortisol concentration was seen in this study. [56] This faster increase in cortisol concentration compared to the current study may have been related to a shorter incubation period of bacterial pneumonia before the development of clinical disease, when compared to canine babesiosis. Although a different timeline was seen between the two studies, a similar pattern in cortisol kinetics still emerged.

Clinical severity was related to inoculum size illustrated by the higher peripheral parasitaemia [12,17] and clinical collapse [12,16] seen in the high dose group when compared to the low dose group. The cortisol concentration increased rapidly in the high dose group, compared to the low dose group, with a return to reference intervals seen within 24 hours post treatment. The cortisol concentration in the low dose group remained within reference intervals throughout the study period. Similar to our study, puppies that survived parvovirus infection showed a decrease in cortisol concentration back to reference intervals by 24 hours after admission and initiation of treatment. [52]

One dog from the high dose group died during the current study. This dog showed the highest cortisol concentration (610 nmol/L at 96 hours post infection) shortly before death. The highest recorded cortisol concentration of 529 nmol/L in a group of puppies with parvoviral diarrhoea was also seen in a puppy that died. Furthermore, none of the puppies that survived had a cortisol concentration above 224 nmol/L at 48 hours after admission using a laboratory reference interval of 10- 160 nmol/L. [52] In a clinical study of canine babesiosis, the highest recorded cortisol concentration of 562 nmol/L was again seen in a dog that died. [20] When cortisol concentrations were evaluated on admission and again 24 hours later in dogs hospitalised for a variety of diseases, cortisol concentration measured 24 hours after hospitalisation was considered a better predictor of mortality than the cortisol concentration on admission, with a cut-off of 182 nmol/L predicting mortality with a sensitivity of 89.5 % and a specificity of 61.9 %.[58] In a retrospective study evaluating dogs hospitalized for critical and emergency care, a higher cortisol cut- off of 210 nmol/L was proposed to predict mortality with a sensitivity of 58 % and a specificity of 80 % in the patient population. [57] It is important to note that the values mentioned in the latter study based their results on cortisol values tested anywhere within the first 3 days of hospitalisation, [57] whilst the cortisol concentrations mentioned in the study of parvoviral diarrhoea were based on samples obtained on the third day of hospitalisation, in addition to using a different assay to attain these measurements. [52] Since these were clinical studies, it is likely that the dogs presented at differing stages of disease which further limits direct comparisons, as cortisol concentrations change with the evolution of disease. This emphasises the value of an experimental study where the stage of disease can be accurately defined and the changes in cortisol concentration can be monitored during the progression of the disease process. Our findings were echoed in the largest cohort of natural *B. rossi* infections published to date, where high cortisol concentration was identified as one of the four parameters that was most predictive of outcome. This study proposed a cut- off value of 388 nmol/L on admission to predict mortality with a sensitivity of 87.5 % and a specificity of 82.9 %. [16]

The mechanism underlying increased cortisol concentrations during critical illness is thought to be multifactorial and results from a combination of increased glucocorticoid production secondary to stimulation of the HPA axis, [25,28,29] along with impaired metabolism resulting in a prolonged cortisol half-life. [30-32]

The T4 concentration decreased following infection with a more rapid and significant decline seen in the group with more severe disease that received the higher dose of parasite inoculum. The T4 concentrations gradually increased again after initiation of treatment. In a study that administered intravenous endotoxin to healthy experimental dogs, the T4 concentration was also shown to decrease post infection. [85] The single dog that died in our study had the lowest T4 concentration at the 96-hour timepoint, shortly before death. This value was below the limit of detection. Similar to our study, the T4 concentrations recorded in puppies with parvoviral diarrhoea that died were all below the limit of detection at 24 hours after admission, [52] whilst 6 out of 7 dogs that died from a natural *B. rossi* infection had T4 concentrations below the limit of detection on admission. [20] This emphasises the need for a more sensitive assay for future studies. The median T4 concentration on admission in puppies with parvoviral diarrhoea was significantly lower than control values, with gradual increases in T4 concentration seen over the subsequent 2 days in the dogs that survived, whilst those that died continued to show a decline in T4 concentration. [52] Additional studies in parvoviral diarrhoea also found a lower T4 concentration to be associated with mortality [90] whilst the largest cohort of *B. rossi* infections found similar results and calculated a cut- off value below 4.8 nmol/L on admission to predict mortality with a sensitivity of 75 % and a specificity of 81.6 %. [16] Systemic illness in both human and veterinary literature has been associated with decreased serum thyroid hormone concentrations [35,76-79,82,85,89,95,96] and a lower T4 concentration often reflected severity of disease. [80,81,86,90,97]

Similar to the changes recorded in T4 concentration, the T3 concentration decreased in the experimental groups, but a significant difference was only seen between the control and low dose group at 144 hours. Unfortunately, the low dose group did not have samples taken at the 96- and 120-hour time point due to ethical concerns related to unnecessary sampling. The T3 concentration in the high dose group was often lower than that of the low dose group, but a significant difference was only seen at 24 hours and 192 hours post infection. The T3 concentration in the high dose group often measured below the limit of detection; therefore, it is impossible to determine the true value of these samples. These differences might have been more pronounced with the use of a more sensitive assay. In agreement with our study, dogs with *B. canis* infection showed T3 concentrations on admission that were significantly lower when compared to control samples, often measuring below the limit of detection. [82] When thyroid hormones were evaluated in dogs with non- thyroidal illness, T3 concentrations were significantly lower in dogs that were euthanized compared to those that recovered. The latter

study utilised a T3 assay with a lower limit of detection which may have been preferred for our trial. [97] A low T3 concentration has often been associated with disease and has been used as an indicator of severity in multiple human and animal studies. [78,97-101]

An initial decline in TSH concentration occurred in both groups but significant differences were only seen between the control and the high dose group. Although the TSH concentrations in the high dose group were often lower than that of the low dose group, a significant difference was only seen at 108 hours and the TSH concentrations remained within reference intervals 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 ICU patients [102,103] but similar to the current study, the TSH concentrations often remained within reference intervals. When dogs with non- thyroidal disease were compared to clinically normal controls, the serum TSH concentration remained within the reference interval regardless of disease severity. [86] Similarly, TSH concentrations remained within the reference intervals in dogs with natural *B. canis* infection, [82] and TSH concentration was not associated with outcome in dogs with natural *B. rossi* infection. [20] Furthermore, TSH was considered a less sensitive indicator of outcome than T4 concentration in puppies with parvoviral diarrhoea. [90]

In the high dose group, TSH concentrations mimicked the changes in T4 concentrations with a decline in TSH post infection that gradually increased again post treatment. The median TSH concentration for this group often fell below the limit of detection therefore, the true TSH nadir could not be fully appreciated. A similar problem occurred when endotoxin was experimentally administered to healthy dogs. The authors of that study postulated that the poor sensitivity of the TSH assay may have contributed to the lack of significant findings [85] and this may very well have been the same scenario in the current study. The TSH concentrations in the low dose group made an unexpected increase at the 108- hour timepoint and declined again thereafter. These samples were repeated to investigate the possibility of sampling error, but the results remained consistent. This short- lived increase in TSH in the low dose group is difficult to explain but may be due to a transient loss in negative feedback inhibition associated with lower thyroid hormone concentrations. Further experimental studies with a larger population size would be required to illuminate whether this transient change in TSH concentration is a consistent finding.

Systemic illness in both human and veterinary literature has been associated with decreased serum thyroid hormone concentrations and is believed to result from a variety of different mechanisms. These include inhibition of the 1 and 2 deiodinase enzymes resulting in decreased conversion from T4 to T3; cytokine-induced inhibition of TSH synthesis; or the presence of circulating inhibitors to receptor binding. Decreased production of TSH in severe illness secondary to cytokines and reactive oxygen species has also been reported. [35,76-80,82]

A negative correlation was seen between cortisol and all thyroid hormones tested. Elevated cortisol has long been associated with suppressed thyroid hormone concentrations. Previous investigations have shown that pharmacological doses of dexamethasone and physiological concentrations of cortisol were both with suppressed TSH secretion. [104] When patients were injected with IL-6, a rise in cortisol preceded a decline in TSH concentration. [105] Cytokine production with severe illness typically leads to increased ACTH and suppressed TSH synthesis. [35,80] Similar to our findings, an inverse relationship between cortisol and thyroid hormones was documented in dogs with natural *B. rossi* infection [20] as has been shown in puppies with parvoviral diarrhoea. [52]

T4, T3 and TSH concentrations showed a positive correlation with each other. This is to be expected since TSH stimulates the release of T4 and T3 under physiological conditions. [76,78,83] During the recovery phase of disease, TSH has been found to increase and precede the rise in T4 and T3 concentration. [102] This preceding rise was not clearly seen in our study but may have been masked by the lower sampling frequency.

1.2 Limitations

This study had several limitations, the most notable being the small sample size. 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 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 low dose and high dose groups each received an identical

dose of parasite inoculum at the same time as the other dogs in their respective groups. Furthermore, the control samples were taken from the experimental dogs prior to infection to ensure that the control samples matched the study population as closely as possible.

Another limitation was that total cortisol, and not free cortisol, was measured in this study. In healthy individuals, 95% of circulating cortisol is protein bound to cortisol binding globulin (CBG) whilst only 5% is free and biologically active. In critical illness there is a marked reduction in CBG concentrations along with a decreased affinity for this protein, resulting in an increased fraction of free cortisol. [65,106] As a result, this method may be unreliable in estimating adrenal activity during critical illness since changes in CBG and albumin could potentially result in inaccurate estimations of free cortisol concentrations and adrenal function. [37,107,108] Despite this, total cortisol concentrations were markedly elevated in our sickest dogs and some studies have found a good correlation between total and free cortisol, suggesting that total concentrations can be considered representative. [37,68,109] This same limitation can be applied to our use of total T4 and T3 in this study, versus the use of fT4 and fT3. Total T4 and T3 concentrations often decrease sooner in disease states compared to fT4 and fT3 concentrations and is considered a more sensitive indicator of nonthyroidal illness. This earlier identification of endocrine perturbations provides a valuable benefit when trying to identify alterations early in the disease course and justifies its use in this scenario. [110]

Serum samples were collected at 24, 72, 108, 144 and 192 hours post infection in all experimental dogs based on ethical approval. Due to severe clinical deterioration, the high dose group had additional serum samples taken at 96 and 120 hours post infection to provide more intensive monitoring. This additional sampling could not be justified in the low dose 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 prioritised in this instance.

Finally, many of the T4, T3 and TSH concentrations were below the limit of detection, hampering true interpretation of the results. An alternative next generation assay with a lower limit of detection may have provided more meaningful information, especially with reference to positive predictive values for non-survival.

Chapter 5: Conclusion

This study illustrated the changes in endocrine variables after an experimental infection with *B. rossi* in beagle dogs. In both groups, the cortisol concentration increased and the T4 and T3 concentrations decreased after infection, with a return towards baseline concentration post treatment. Furthermore, a higher cortisol concentration with a more rapid increase and a lower T4 concentration with a more rapid decline, was associated with a higher dose of parasite inoculum and disease severity.

Changes in endocrine parameters have been consistently identified in inflammatory processes. Experimental infection appears to show a predictable and homogenous change in cortisol, T4 and T3 concentrations over time, related to infectious dose and disease severity. TSH concentrations remained within reference intervals throughout the study and appeared to be of less clinical and prognostic value. This dog model of a severe infectious disease offers the opportunity to explore the endocrine responses of critical illness more fully. Larger experimental studies would be beneficial to add to the knowledge gained from this study.

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Appendices

Appendix A: Blood Collection Schedule Sheet

Parameter to be measured	**Schedule**	**Test to be run**
Habitus, appetite, temperature, pulse, respiratory rate, mucous membrane colour, blood pressure	Daily on all dogs	General health status and wellbeing of all dogs.
Blood smear	Daily on small volume of peripheral blood from the ears of all dogs and from the EDTA sample below.	Parasitaemia determination.
0.5 ml of whole blood in EDTA tube from jugular collection	Daily for CBC from all dogs from day 0 until end points are reached and infected dogs are treated.	Complete blood count and cytokine concentration determination.
3ml of whole blood in serum tube from jugular collection	Every second day from all dogs from day 0 (i.e., day 0 and then 2/4/6 etc) until end points are reached and infected dogs are treated.	Biochemistry (Total serum protein, glucose, albumin, creatinine, urea, C-reactive protein, ALT, ALP, bilirubin, potassium, and chloride) and endocrine parameters (T4, T3, TSH and cortisol).
0.5 ml of whole blood in heparinized syringe from jugular collection	Every second day from all dogs from day 0 (i.e., day 0 and then 2/4/6 etc) until end points are reached and infected dogs are treated.	Venous blood gas and lactate.
11 ml of whole blood in heparin[1]	Daily	NTBI, plasma Hb, CFHb, Transferrin/TBI, haptoglobin.
2.5 ml of whole blood in RNALater[2]	Every second day from all dogs from day 0 (i.e., day 0 and then 2/4/6 etc) until end points are reached and infected dogs are treated.	Transcriptomics.

[1] Samples collected for the Dog Model of Haemolysis study by Prof Andrew Leisewitz

[2] Samples collected for the Dog Model of Haemolysis study by Prof Andrew Leisewitz

Appendix B: Data collection

	Experimental group	Control 1	Control 2	24 hr	48 hr	72 hr	96 hr	108 hr	120 hr	144 hr	168 hr	192 hr
		2019/01/31	*2019/02/13*	*2019/03/01*	*2019/03/02*	*2019/03/03*	*2019/03/04*	*2019/03/04*	*2019/03/05*	*2019/03/06*	*2019/03/07*	*2019/03/08*
Patient 1	**Splenectomy**											
Cortisol (nmol/L)		**27.5**										
TT4 (nmol/L)		**38,9**										
T3 (nmol/L)		**0,648**										
TSH (ng/ml)		**0,084**										
Habitus		**4**										
Temp		**38,2**										
Pulse		**116**										
Resp		**20**										
MM		**3**										
CRT		**1**										
Patient 2	**Low dose**											
Cortisol (nmol/L)		**36,4**	**27,50**	**27,50**		**27,50**		**34,5**		**42,5**		**27,5**
TT4 (nmol/L)		**25,4**	**33,8**	**44,8**		**32,6**		**21,4**		**8,71**		**35,0**
T3 (nmol/L)		**0,61**	**0,639**	**0,807**		**0,633**		**0,61**		**0,61**		**0,838**
TSH (ng/ml)		**0,032**	**0,053**	**0,058**		**0,063**		**0,191**		**0,035**		**0,071**
Habitus		**4**	**4**	**4**	**4**	**4**	**3**	**4**	**4**	**4**	**3**	**4**
Temp		**38**	**38,5**	**37,8**	**38,6**	**38,7**	**39**	**39,6**	**39,9**	**39**	**38,5**	**38,6**
Pulse		**116**	**108**	**136**	**128**	**148**	**128**	**116**	**156**	**136**	**116**	**128**
Resp		**24**	**40**	**24**	**32**	**36**	**20**	**20**	**16**	**48**	**32**	**40**
MM		**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**1**	**3**	**3**
CRT		**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**
Parasitaemia (%)				**0**	**0**	**0,5**	**3,15**	**4,71**		**0**		

Patient 3	Low dose											
Cortisol (nmol/L)		27,50	27,50	27,50		27,50		69,8		40,3		27,5
TT4 (nmol/L)		36,3	54,7	50,1		42,5		30,8		24,6		37,8
T3 (nmol/L)		0,838	1,07	1,01		0,795		0,810		0,61		0,799
TSH (ng/ml)		0,166	0,178	0,169		0,117		0,264		0,084		0,092
Habitus		4	4	4	4	4	3	3	4	4	3	3
Temp		37,1	38,1	38,2	38,6	37,9	39,9	40,2	39,6	39,6	38,8	38,4
Pulse		120	120	148	132	128	120	140	160	188	132	136
Resp		32	32	20	24	24	28	32	24	32	16	20
MM		3	3	3	3	3	3	3	1	1	3	3
CRT		1	1	1	2	2	1	2	2	1,5	1	1
Parasitaemia (%)				0	0	0,1	4,61	6,81		0		
Patient 4	High dose											
Cortisol (nmol/L)		27,50	27,50	27,5		41,9	223	94,6	116	61,0		27,5
TT4 (nmol/L)		44,8	61,6	54,8		29,3	8,74	6,43	9,06	21,1		34,4
T3 (nmol/L)		0,679	0,841	0,807		0,61	0,61	0,61	0,61	0,61		0,61
TSH (ng/ml)		0,061	0,098	0,169		0,035	0,31	0,029	0,043	0,047		0,119
Habitus		4	4	4	3	2	2		2	2	3	3
Temp		37,6	38,5	38,1	39,2	39,6	40,7		39,7	38,5	38,6	38,1
Pulse		116	124	140	96	96	132		168	136	128	128
Resp		20	28	20	24	28	40		24	20	24	20
MM		3	3	3	3	3	3		3	1	3	3
CRT		1	1	1	2	2	1		1,5	1	1	1
Parasitaemia (%)				0,05	0,69	6,74	34,95			0		

Patient 5	High dose											
Cortisol (nmol/L)		33,7	43,6	27,5		83,0	315	251	194	107		34,5
TT4 (nmol/L)		42,2	56,0	51,6		27,5	7,14	6,43	6,43	14,9		34,4
T3 (nmol/L)		0,695	0,865	0,61		0,61	0,61	0,61	0,61	0,61		0,61
TSH (ng/ml)		0,096	0,086	0,097		0,070	0,032	0,029	0,029	0,038		0,094
Habitus		4	4	4	3	2	2		2	2	3	3
Temp		37,4	38	37,1	38,7	39,4	39,7		39,3	38,8	38,6	38,5
Pulse		132	136	132	124	92	168		160	140	128	132
Resp		24	24	24	24	20	48		36	20	20	24
MM		3	3	3	3	3	3		2	2	3	3
CRT		1	1	1	2	2	1		1,5	1	1,5	2
Parasitaemia (%)				0,05	1,06	11,08	59,8			0		
Patient 6	High dose											
Cortisol (nmol/L)		27,50	39,5	36,4		83,9	610					
TT4 (nmol/L)		35,9	38,5	45,7		24,5	6,43					
T3 (nmol/L)		0,61	0,6	0,665		0,61	0,61					
TSH (ng/ml)		0,059	0,047	0,066		0,066	0,033					
Habitus		4	4	4	4	2	1					
Temp		38,5	37,7	38	39	39,2	36,4					
Pulse		136	134	128	116	88	128					
Resp		40	36	20	32	20	40					
MM		3	3	3	3	3	3					
CRT		1	1	1	1,5	2	1					
Parasitaemia (%)				0,05	0,998	14,81	45,54					

Habitus: 1+ Lethargic and non-responsive, 2+ Lethargic but responsive, 3+ Alert and responsive, 4+ Bright alert and responsive

Mucous membranes: 1+ Pale, 2+ Icteric, 3+ Normal, 4+ Congested