Adrenal response to the low dose ACTH stimulation test and the cortisol-to-adrenocorticotrophic hormone ratio in canine babesiosis

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With 1 table and 4 figures

Abstract
This prospective, interventional, case-controlled study sought to determine the association between adrenocortical function and mortality in dogs with naturally occurring *Babesia rossi* babesiosis. Sixty-eight dogs with canine babesiosis were studied and fifteen normal dogs were used as controls. Blood samples were obtained from the jugular vein in each dog prior to treatment, at admission to hospital, for the measurement of basal plasma ACTH (adrenocorticotrophic hormone) and serum cortisol concentrations. Immediately thereafter, each dog was injected intravenously with 5 µg/kg of ACTH (tetracosactrin). A second blood sample was taken 1 h later for serum ACTH-stimulated cortisol measurement and the resultant calculation of delta cortisol by subtracting basal from ACTH-stimulated cortisol. Diagnosis of babesiosis was confirmed by polymerase chain reaction (PCR) and reverse line blot (RLB). Three outcomes were defined: hospitalization with subsequent death (n = 4); hospitalization followed by recovery (n = 48); and treatment as an outpatient (n = 16). Basal cortisol, but not ACTH-stimulated cortisol, was significantly higher in patients compared to control dogs. Basal- and ACTH-stimulated serum cortisol concentrations were significantly higher in the dogs that died, compared to hospitalized dogs that survived and compared to dogs treated as outpatients. There was no significant difference in delta cortisol concentrations or cortisol to ACTH ratios across outcome groups in dogs suffering from *B. rossi* babesiosis However, dogs with delta cortisol concentrations below 83 nmol/l had significantly higher cortisol to ACTH ratios compared to dogs with delta cortisol concentrations above 83 nmol/l. These findings of increased basal- and ACTH-stimulated cortisol and increased cortisol to ACTH ratios confirm the absence of adrenal insufficiency and concur with those in human malaria.

Keywords: Canine babesiosis; Human critical illness; Malaria; Basal cortisol; Delta cortisol; ACTH-stimulated cortisol

1. Introduction
The adrenal response to critical illness and its attendant role in prognostication has been an area of intense study in human medicine over the last few decades. There is general consensus on the positive association of high basal cortisol with disease severity and mortality (Annane et al., 2000; Sam et al., 2004). The association of serum adrenocorticotrophic hormone (ACTH)-stimulated cortisol and delta cortisol concentrations with mortality is, however, in a state of equipoise (Annane et al., 2006). Various cut-off values for serum delta cortisol, as well as various definitions for relative adrenal insufficiency based on basal-, ACTH-stimulated and delta cortisol concentrations abound in the human literature (Annane, 2003). Equally confusing are the wide array of test protocols and doses of ACTH that should be used to assess adrenal function in the critical illness setting (Kozyra et al., 2005; Annane, 2005; Siraux et al., 2005).
In animal studies the association between basal cortisol concentrations and outcome have been less clear. A study investigating the role of adrenal function in canine critical illness found no difference in either basal- or ACTH-stimulated cortisol between survivors and non-survivors (Prittie et al., 2002). The dearth of information on ACTH-stimulated and delta cortisol concentrations in canine critical illness has been highlighted in a recent review (Martin and Groman, 2004). A more recent study purported to document relative adrenal insufficiency in a group of critically ill septic dogs based on a dichotomized delta cortisol cutoff of 83 nmol/l

(Burkitt et al., 2007). However, this study did not report the basal cortisol in non-survivors versus survivors and found no association between survival and delta cortisol when it was assessed as a continuous, non-dichotomized variable. The most recently reported study found that high basal serum cortisol concentrations were associated with mortality in canine parvoviral diarrhoea (Schoeman et al., 2007a).
The cortisol to ACTH ratio has recently been described as a highly reliable test in the diagnosis of primary hypocortisolism (Addison's disease) in dogs (Javadi et al., 2006). This ratio is a proximate measure of the integrity of the whole pituitary–adrenal axis and might provide more useful information than the ACTH stimulation test about the state of adrenocortical function in critical illness. To the authors' knowledge this ratio has not been used to assess adrenocortical function in canine critical illness.
Many factors including high parasitaemia and collapsed state at presentation (Böhm et al., 2006),= high serum lactate that fails to decrease after 24 h (Nel et al., 2004) and hypoglycaemia (Keller et al., 2004), high endogenous ACTH, high basal serum cortisol and low serum thyroxine and free thyroxine (Schoeman et al., 2007b) as well as cerebral, lung and renal involvement (Welzl et al., 2001) have previously been shown to be associated with mortality in the virulent form of canine babesiosis found in South Africa. This virulent form is caused by *Babesia rossi*, which is an acute, mainly haemolytic disease with a range of severe complications such as acute respiratory distress syndrome, acute renal failure, marked icterus and cerebral pathology amongst others (Welzl et al., 2001).
In contrast, babesiosis caused by the many other subspecies is generally a milder, more chronic disease (Irwin and Hutchinson, 1991; Furlanello et al., 2005).
Studies in human falciparum malaria, a disease in which pathophysiology similar to that of virulent canine babesiosis has been postulated (Jacobson and Clark, 1994; Clark and Jacobson, 1998; Schetters and Eling, 1999), have shown elevated serum basal cortisol concentrations in patients compared with controls, but showed no difference in basal cortisol concentrations between survivors and non-survivors (Davis et al., 1997; Wilson et al., 2001). Conflicting studies have shown either no evidence of pituitary–adrenal dysfunction (Brooks et al., 1969; Phillips et al., 1986; Taylor et al., 1988; Shwe et al., 1998; Wilson et al., 2001) or suggested a pituitary contribution to the relative adrenal insufficiency documented in malaria (Davis et al., 1997). Other studies have shown either markedly increased cortisol and lactate concentrations in malaria patients (van Thien et al., 2001) or evidence of impaired cortisol secretion in some patients (White et al., 1983). Increased serum basal cortisol concentrations have been associated with an increased susceptibility to *Plasmodium falciparum* infection, especially in primigravid women (Bouyou-Akotet et al., 2005) and basal serum cortisol concentrations were found to be significantly higher on day 0 versus day 7 of uncomplicated *P. falciparum* malaria in Brazil (Libonati et al., 2006).
The cortisol to ACTH ratio, ACTH-stimulated serum cortisol and delta cortisol concentrations and their association with disease severity and mortality in canine babesiosis or in canine critical illness in general are currently unknown or poorly described. The aims of this study were to describe adrenocortical function and its association with disease severity and mortality in virulent *B. rossi* babesiosis and to ascertain whether the study of adrenocortical function in canine babesiosis can shed light on the conservance of the stress response to parasitic disease and critical illness across species.

2. Materials and methods

This prospective study was performed on dogs with canine babesiosis presenting to the Onderstepoort Veterinary Academic Hospital of the University of Pretoria in South Africa. Initial diagnosis was made based upon the detection of large *Babesia* spp. Parasites on stained thin capillary blood smears. Dogs were excluded from the study if they had: a history of previous exogenous corticosteroid therapy; known malignancies which might have involved the adrenal glands; known concurrent disease; *Ehrlichia canis* morulae detected on blood smear; or if the owner refused permission for an ACTH stimulation test to be performed. After the dogs had been sampled, they were further excluded if concurrent disease was identified during their hospital stay or if their blood samples were positive for *B. vogeli* or *E. canis* by polymerase chain reaction (PCR) and reverse line blot (RLB) as previously described (Matjila et al., 2004). PCR was conducted with a set of primers that amplified a 460-540 base pair fragment of the 18S SSU rRNA spanning the V4 region, a region conserved for *Babesia* and *Theileria*. The Ehrlichia PCR amplified the V1 hypervariable region of the 16S SSU rRNA

(Schouls et al., 1999; Bekker et al., 2002). The membrane used for RLB included probes for *B. vogeli, B. rossi, B. canis and E. canis*. Fifteen healthy control dogs, admitted to the Onderstepoort Veterinary Academic Hospital for routine vaccination, underwent an ACTH stimulation test using the same protocol as the study subjects. They were considered healthy on the basis of a full physical examination and routine laboratory testing (i.e., CBC determination and serum biochemical analysis). The study was reviewed and approved by the Animal Use and Care Committee of the University of Pretoria.

2.1. *Study design*

History and physical examination, including patient age, sex and duration of illness was determined. Blood samples were obtained prior to treatment on the day of presentation. Blood was taken from the jugular vein by needle venipuncture in all cases and placed into precooled EDTA and serum tubes. EDTA tubes were kept on ice and spun down within 10 min in a refrigerated centrifuge at 4 °C. Immediately thereafter, all patients were injected intravenously with 5 μg/kg of ACTH (tetracosactrin, Synacthen®, Alliance pharmaceuticals). Blood samples for ACTH-stimulated cortisol were taken 1 h later. Serum samples were allowed to clot and the tubes were spun down within 1 h. The serum and plasma were harvested and placed in dedicated plastic storage tubes and stored at _80 °C until analysis. Dogs were retrospectively grouped into the following outcome groups as described previously (Böhm et al., 2006): dogs that were admitted, but *died* (Group D); dogs that were admitted, but *survived* (Group S) and dogs that were not sick enough to be admitted, but instead were treated as outpatients and sent *home* (Group H). Dogs were followed up to determine their outcome. EDTA blood was used for haematocrit and endogenous ACTH determination. Hormones were assayed in a single batch. Cortisol (Radioimmunoassay cortisol, Coat-A-Count, Diagnostic Products Corp, USA) and ACTH (Immunoradiometric ACTH assay, Coat-A-Count, Diagnostic Products Corp, USA) assays were performed in duplicate with kits previously validated for dogs. Sensitivity of the cortisol and ACTH assays were 5.5 nmol/l and 2 pmol/l, respectively. For statistical purposes, values below the limit of detection were taken as 5.4 nmol/l and 1.98 pmol/l. Delta cortisol was determined by subtracting basal cortisol from ACTHstimulated cortisol. Cortisol to ACTH ratio was determined by dividing cortisol in nmol/l by ACTH in pmol/l.

2.2. *Data analysis*

Parameters were tested for normal distribution using the one-sample Kolmogorov–Smirnov test. Differences in the median hormone concentrations between the three outcome groups (multiple comparisons) were analyzed for non-parametric data with the Kruskal-Wallis test on ranks. Only variables that showed significant difference were subjected to subsequent pair wise comparisons using the Mann–Whitney U-test to compare each of the groups separately with one another. For all comparisons, differences were considered significant when $p < 0.05$. Values in the text are given as median and inter quartile range (IQR). Statistical analysis was performed using a commercial software package (SPSS 14.0, 2005, SPSS Inc, Chicago, Illinois).

3. Results

Sixty-eight dogs were recruited in the study. They represent a subgroup (in which permission was obtained to perform an ACTH stimulation test) of the original 95 dogs in which basal ACTH, cortisol and thyroid hormones concentrations have been described (Schoeman et al., 2007b). Thirty-eight dogs (56%) were male and 32 dogs (44%) were female. The overall median patient age was 12 months (IQR 7.5–36 months), which did not differ significantly between the three outcome groups ($p = 0.2$). Control dogs had a mean age of 10 months (range 4–60 months). The median duration of illness prior to presentation was 2 days (IQR 2–4 days), which did not differ between the three outcome groups
($p = 0.65$).

3.1. *Serum cortisol concentrations in all patients and in control dogs*

The details of the serum cortisol concentrations in all patients and control dogs are displayed in Table 1. Patients had a significantly higher basal cortisol concentration than control dogs

($p < 0.05$). Serum ACTH-stimulated cortisol concentrations between patients and controls were very similar and failed to show a significant difference ($p = 0.282$). Patients had lower serum delta cortisol concentrations that control dogs, showing a trend towards significance ($p = 0.068$).

3.2. *Serum cortisol concentrations in the three babesiosis groups*

The details of all the hormone results in the outcome groups are displayed in Table 1. Median basal serum cortisol concentrations were significantly higher in Group D compared to Group S ($p < 0.01$) and compared to Group H ($p < 0.001$). The serum basal cortisol concentrations were not significantly different between Group S and Group H ($p = 0.1$) (Fig. 1).
Median ACTH-stimulated serum cortisol concentrations were significantly higher in Group D compared to Group S ($p = 0.038$) and compared to Group H ($p = 0.007$). The difference between Groups S and H was not significant ($p = 0.193$) (Fig. 1).
No significant difference was detected between the three outcome groups for delta cortisol concentrations, however, the median delta cortisol concentrations in Group D was markedly lower, approaching our preset significance level ($p = 0.076$) (Fig. 2).

3.3. *Endogenous ACTH concentrations and cortisol to ACTH ratios*

Median plasma ACTH concentration was significantly lower in patients than in controls ($p < 0.001$), whereas it was not significantly different between the three babesiosis groups ($p = 0.143$). Median cortisol to ACTH ratio was significantly higher in patients than in control dogs ($p < 0.001$), but was not significantly different between the three outcome groups ($p = 0.428$). However, when the babesiosis dogs were dichotomized into those with a delta cortisol <83 nmol/l and those with delta cortisol >83 nmol/l, the former group showed a significantly higher median cortisol to ACTH ratio than the latter group ($p = 0.017$); Fig. 2.

3.4. *Correlations between endocrine variables*

There was a significant positive correlation between basal- and ACTH-stimulated serum cortisol concentrations ($r_s = 0.674$, $p < 0.001$, Fig. 3). However, despite this positive correlation there was a marked trend for delta cortisol concentrations to reduce as basal serum cortisol became elevated, resulting in a significant negative correlation between these two variables ($r_s = -0.516$, $p < 0.001$, Fig. 4). No evidence was found for a significant positive correlation between delta cortisol concentrations and ACTH-stimulated cortisol concentrations ($r_s = 0.155$, $p = 0.2$).

4. Discussion

The results of this study demonstrated significantly higher serum basal, but lower delta cortisol concentrations in dogs with canine babesiosis compared to control dogs. Serum ACTH-stimulated cortisol concentrations were, however, very similar between the babesiosis and control groups. Adrenocortical function (as indicated by basal- and ACTH-stimulated cortisol concentrations as well as cortisol to ACTH ratios) was upregulated in this model of canine critical illness. Basal- and ACTH-stimulated cortisol concentrations as well as cortisol to ACTH ratios were the lowest in Group H dogs (110, 301 nmol/l and 44), higher in Group S dogs (161, 347 nmol/l and 49) and the highest in the sickest dogs belonging to Group D that died (481, 510 nmol/l and 90). Elevated ACTH-stimulated cortisol concentrations were positively associated with disease severity and mortality. In this subgroup of 68 cases, ACTH-stimulated serum cortisol showed the same association with mortality that serum basal cortisol concentrations showed in the formerly described larger babesiosis group (Schoeman et al., 2007b), as well as in parvoviral diarrhoea (Schoeman et al., 2007a). This paired association of basal- and ACTH-stimulated cortisol with disease severity and mortality, which has not been reported before, is entirely explained by the positive correlation that was demonstrated in this study between the two hormone concentrations. The correlation between basal- and ACTH-stimulated cortisol is, however, not linear across the whole range (as demonstrated by the diminishing delta cortisol concentration from Group H to Group D), because ACTHstimulated cortisol has a maximum concentration beyond which the adrenal glands cannot be stimulated.

This has been illustrated in canine studies which produced similar 60 min ACTH-stimulated cortisol concentrations, despite using ACTH doses ranging from 1 μg/kg to 250 μg/dog (Kerl et al., 1999; Frank et al., 2000). It therefore follows that delta cortisol concentrations would be reduced to a greater extent in situations of high basal cortisol concentrations. The findings of our study demonstrate this phenomenon of lower delta cortisol concentrations, despite clearly elevated ACTH-stimulated cortisol concentrations, especially in our group of dogs that died. This phenomenon has also been illustrated in normal cats, where delta cortisol concentrations in cats presenting with high basal cortisol concentrations were markedly lower compared to other feline studies, despite similarly elevated ACTH-stimulated cortisol concentrations (Schoeman et al., 2000). More importantly, these similar ACTH-stimulated cortisol concentrations were achieved despite using an ACTH dose of 250 μg/cat, which is 50 times higher than doses of 1 μg/kg (±5 μg/ cat) that have been shown to produce comparable ACTH-stimulated concentrations (Peterson and Kemppainen, 1993). Human studies have also corroborated this maximum adrenal stimulation phenomenon, when it was found that doses as low as 1mg/patient achieve peak cortisol concentrations comparable to doses which were 250 times higher (Abdu et al., 1999; Beishuizen et al., 2000). Overall, data in all three species concur with our assertion that ACTH-stimulated cortisol has a maximum beyond which it cannot be stimulated

The significant negative correlation between serum basal cortisol and delta cortisol concentrations demonstrated in this study adds further substantiating evidence to this argument of the confounding effect brought about by basal cortisol, when interpreting delta cortisol concentrations in severe illness. The most convincing evidence of the flawed nature of using the formerly described delta cortisol of <83 nmol/l as a sole measure of adrenocortical insufficiency is provided by the cortisol to ACTH ratios in these patients. Here it was clearly demonstrated that serum cortisol showed a marked response to ACTH, resulting in significantly higher cortisol to ACTH ratios in these so-called ‘‘adrenal-insufficient’’ patients (delta cortisol <83 nmol/l) compared to the patients with ‘‘normal’’ adrenal reserve (delta cortisol >83 nmol/l).

The issue of lower delta cortisol concentrations is not merely a peripheral one, as this is the central tenet of the argument for diagnosing so-called relative adrenal insufficiency and the resultant justification for corticosteroid supplementation in human critical illness (Hatherill et al., 1999; Pizarro et al., 2005). Our findings demonstrate that delta cortisol concentrations cannot be used as an isolated parameter when evaluating the ‘‘reserve’’ of the adrenal gland in acute canine illness. We would therefore argue that studies which ignored the basal cortisol concentrations when according patients the status of ‘‘relative adrenal insufficiency’’, should be viewed with caution (Burkitt et al., 2007). After many years of negating this fact and not taking basal cortisol concentrations into account when determining adrenal status, a recent publication in human critical care has shown that non-surviving patients had a lower delta cortisol than survivors. This was, however, partly caused by the elevated basal serum cortisol concentrations in non-surviving patients (Lipiner-Friedman et al., 2007). Yet another recent human study noted the marked inter-dependence of basal and delta cortisol and the negative correlation between the two parameters, similar to what we have demonstrated in our dogs (Doi et al., 2006).

We initially postulated that adrenal failure (indicated by a low basal or post-ACTH serum cortisol or low cortisol to ACTH ratios) might be associated with poor outcome in this acute haemoparasitic disease model. This does not seem to have been the case, because the pituitary–adrenal axis responded appropriately under conditions of severe illness and inflammation. It has been shown that cytokines such as IL-1, IL-6 and TNF alpha are responsible for pituitary stimulation and cortisol release in malaria and other models of non-specific immune system activation (Sapolsky et al., 1987; Bernardini et al., 1990; Davis et al., 1997; Tilg et al., 1997; Bethin et al., 2000; Paltrinieri, 2007). In turn, the hypercortisolism elicited by acute illness results in immunoregulatory feedback which inhibits various cytokines and other components of the immune system, thus protecting the organism against the deleterious effects of a pro-inflammatory over-response (Munck et al., 1984; Besedovsky et al., 1986). Our findings thus concur with most of the malaria studies which showed increased cortisol secretion and no indication of adrenal insufficiency (Brooks et al., 1969; Phillips et al., 1993; Shwe et al., 1998; van Thien et al., 2001; Wilson et al., 2001; Libonati et al., 2006). Our findings would thus suggest that canine patients with acute critical illness are unlikely to present with adrenal insufficiency. This agrees with a human study which showed that older patients with prolonged ICU stays are at the greatest risk of adrenal insufficiency (Barquist

and Kirton, 1997). A review of the human literature concurred that the neuroendocrine paradigms in acute and chronic critical illness are very different (van den Berghe et al., 1998). Indications are that the same might be true for dogs and that this virulent form of canine babesiosis may be a heuristic tool to provide insight into the adrenal response to critical illness across species, particularly in conceptualising the pathogenesis of critical illness-associated relative adrenal insufficiency.

In conclusion, the results of our study indicate that the cortisol to ACTH ratio (which assesses the whole pituitary–adrenal axis), demonstrates upregulation of adrenocortical function in acute canine critical illness, in contrast to the delta cortisol parameter which tends to show the reverse. Furthermore, we would argue that testing only the adrenocortical component of the axis, especially in situations of high baseline cortisol concentrations, could lead to erroneous inferences and the unnecessary and gratuitous use of corticosteroid supplementation. In addition, the cortisol to ACTH ratio has the advantage of being determined on a single blood sample.

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Table 1
Endogenous ACTH, cortisol to ACTH ratios and cortisol data in the control groups, all patient group and the three outcome groups of dogs with *B. rossi* babesiosis; Group H = dogs treated as outpatients and sent home; Group S = dogs admitted for treatment that survived to hospital discharge; Group D = dogs admitted for treatment that died; IQR = interquartile range

Parameter	Median (IQR)				
	Control group (n = 15)	All patients (n = 68)	Group H (n = 16)	Group S (n = 48)	Group D (n = 4)
Endogenous ACTH (pmol/l)	7.01 (5.6–8.6)	2.86 (1.98–4.11)	1.98 (1.98–3.51)	2.86 (1.98–4.34)	4.48 (2.3917.58)
Cortisol to ACTH ratio	11.9 (7.6–15.7)	49 (28.3–64.7)	44 (26.9-57.6)	49 (27.7–71.3)	90.7 (34.4-215.4)
Basal Cortisol (nmol/l)	83 nmol/l (43–154)	156 nmol/l (71–268)	110 nmol/l (63–168)	161 nmol/l (70–274)	481 nmol/l (316-601)
Delta Cortisol (nmol/l)	239 nmol/l (185–259)	185 nmol/l (96–254)	223 nmol/l (128–275)	185 nmol/l (99–254)	92 nmol/l (2.5–139)
ACTH-stimulated Cortisol (nmol/l)	322 nmol/l (290–365)	346 nmol/l (281–423)	301 nmol/l (270–368)	347 nmol/l (288–423)	510 nmol/l (389–734)

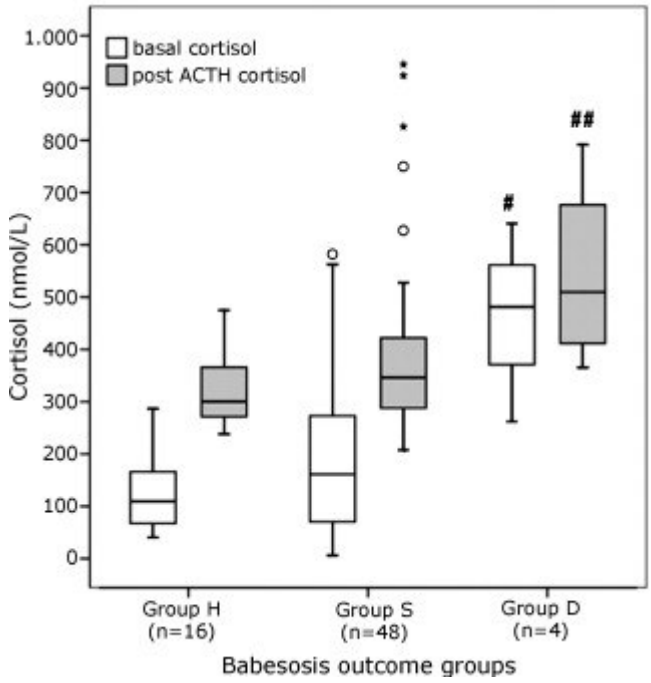

Fig. 1. Box plots of the basal serum cortisol and ACTH-stimulated cortisol concentrations of the three outcome groups in 68 dogs with canine babesiosis. For each box plot the box represents the inter quartile range (i.e., the 25th–75th percentile range or the middle half

of the data). The horizontal bar in the box is the median and the T-bars represent the main body of the data, which in most instances is equal to the range. Outliers are indicated by open circles and asterisks. # = significantly ($p < 0.05$) higher than the median basal cortisol concentration of the other two groups. ## = significantly ($p < 0.01$) higher than the median ACTH-stimulated cortisol concentration of the other two groups.

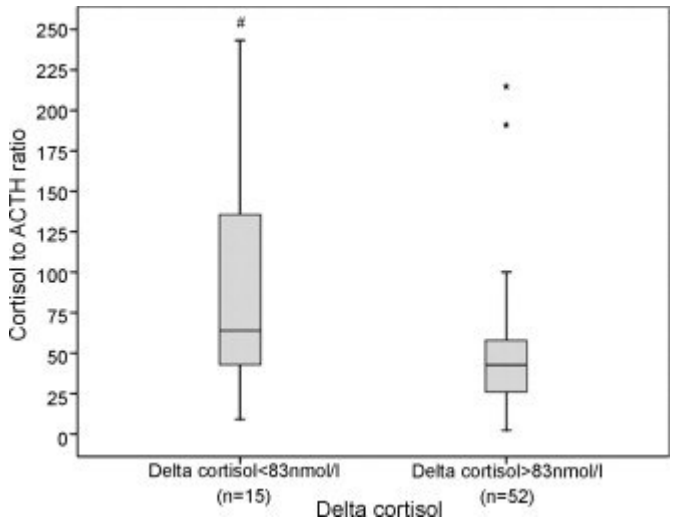

Fig. 2. Box plots of the cortisol to ACTH ratios in two groups of dogs with canine babesiosis categorized as having delta cortisol concentrations of <83 and >83 nmol/l, respectively. For the interpretation of box plot characteristics see legend to Fig. 1. The outliers in the >83 nmol/l group are indicated by asterisks*. # = significantly ($p < 0.05$) higher median cortisol to ACTH ratio than the other group.

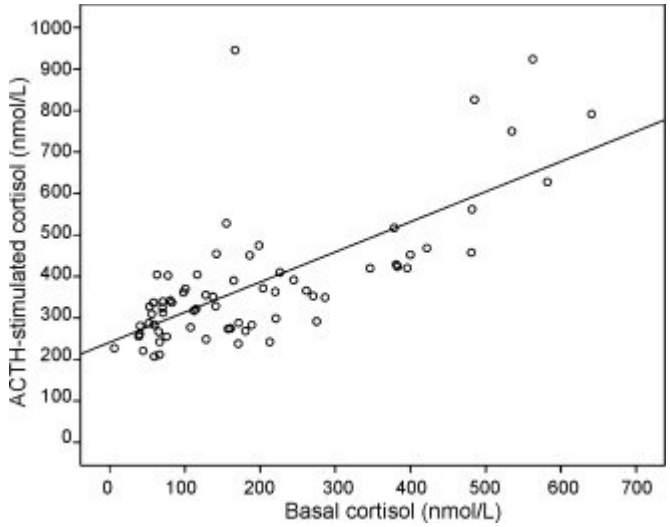

Fig. 3. Scatter plot demonstrating the significant positive correlation ($r_s = 0.674$, $p < 0.001$) between serum basal cortisol and ACTH-stimulated cortisol concentrations in 68 dogs with *B. rossi* babesiosis.

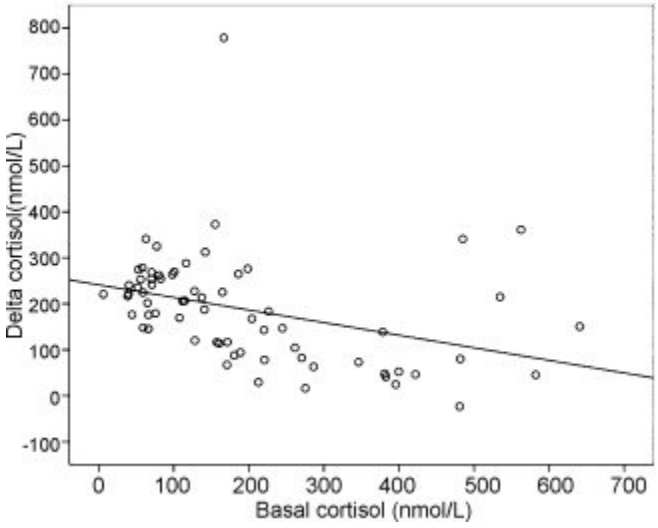

Fig. 4. Scatter plot demonstrating the significant negative correlation ($r_s = -0.516$, $p < 0.001$) between serum basal and delta cortisol concentrations in 68 dogs with *B. rossi* babesiosis.