

Research article

Effect of diet type on serum and faecal concentration of S100/calgranulins in the captive cheetah

Sarah Depauw¹, Romy M. Heilmann², Katherine Whitehouse-Tedd³, Myriam Hesta¹, Jörg M. Steiner², Jan S. Suchodolski² and Geert P.J. Janssens¹

¹Laboratory of Animal Nutrition, Faculty of Veterinary Medicine, Ghent University, Heidestraat 19, B-9820 Merelbeke, Belgium

²Gastrointestinal Laboratory, Department of Small Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843-4474, USA

³Cheetah Outreach, 209-211 Victoria Junction, Prestwich Street, Cape Town, Western Cape, South Africa

Correspondence: Sarah Depauw; e-mail: Sa.depauw@UGent.be

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Abstract

Gastrointestinal disease is omnipresent in captive cheetahs (*Acinonyx jubatus*), in contrast to its free-ranging populations. The current study aimed to evaluate the effect of diet type (meat-only whole prey) on gastrointestinal health in captive cheetahs by measuring faecal and serum concentrations of S100/calgranulins. This paper reports faecal S100A12 and calprotectin concentrations in 12 captive cheetahs fed supplemented beef versus whole rabbit for one month in a cross-over design. Also, serum S100A12 and calprotectin concentrations were determined in four cheetahs fed whole rabbit and six cheetahs fed supplemented beef, and these were compared to the faecal concentrations of the respective marker proteins. Both the immunoassay for canine calprotectin and canine S100A12 were precise and reproducible for use with serum samples and faecal extracts. Whereas the assay for S100A12 was linear and accurate, an inconsistent linearity of the canine calprotectin assay was observed and could be indicative of an insufficient cross-reactivity of the specific antibody used for this assay. Serum concentrations of S100A12 and calprotectin were not altered by diet type, and were not correlated with the respective concentrations in faeces. Numerically ($P=0.241$) greater calprotectin concentrations and greater ($P=0.041$) faecal S100A12 concentrations were detected in cheetahs fed supplemented beef compared with whole rabbit. These findings demonstrate that whole prey feeding may decrease intestinal inflammation in the captive cheetah. Consequently, the relation between diet type and intestinal inflammatory conditions in the captive cheetah warrants further investigation.

Introduction

The prevalence of gastrointestinal disease is high in the captive cheetah (*Acinonyx jubatus*) compared to the wild population (Munson 1993; Munson et al. 1999, 2005; Kotsch et al. 2002). The type of diet offered to captive cheetahs has been implicated as a potential factor in gastro-intestinal disease in this species (Whitehouse-Tedd 2012). Recently, we demonstrated that the type of a strict carnivorous diet modulates microbial fermentation processes in the cheetah (Depauw et al. 2011). Cheetahs fed a supplemented beef diet were observed to have significantly higher concentrations of faecal putrefactive compounds, accompanied by looser stool and a higher incidence of diarrhoea as compared to the same

cheetahs fed whole rabbit. In contrast, faecal concentrations of total short-chain fatty acids (SCFA) were unaltered by diet type (Depauw et al. 2011). These differences in fermentation were attributed to the relatively high intake of animal fibre (bone, fur, connective tissue), in combination with a reduced protein intake, of whole prey compared with meat-only diets (Depauw et al. 2011).

Whereas SCFAs are generally associated with important beneficial effects on intestinal function and metabolism (Wong et al. 2006), putrefactive compounds are commonly linked with chronic intestinal inflammatory diseases (Weber et al. 1987; Matsui et al. 1995; Pedersen et al. 2002; Tuohy et al. 2006). As such, Depauw et al. (2011) postulated that the feeding of whole prey modulates hindgut fermentation towards gastrointestinal

Table 1. Results of the analytical validation of the canine calprotectin and S100A12 assays for use with serum and faecal extracts from cheetahs.

Sample number	Dilutional parallelism				Spiking recovery (canine protein)			
	Calprotectin		S100A12		Calprotectin		S100A12	
	Mean (µg/L or ng/g)	O/E (%) ± SD	Mean (µg/L or ng/g)	O/E (%) ± SD	Mean (µg/L or ng/g)	O/E (%) ± SD	Mean (µg/L or ng/g)	O/E (%) ± SD
S1	2918	120.5 ± 15.8	65	123.1 ± 6.4	1613	133.3 ± 32.3	46	88.9 ± 5.5
S2	1536	113.6 ± 7.8	93	103.9 ± 16.3	2154	114.9 ± 15.9	73	96.7 ± 9.0
S3	2023	101.6 ± 6.2	115	108.5 ± 16.5	3190	112.0 ± 14.3	126	90.4 ± 3.5
S4	2953	114.8 ± 20.5	184	104.2 ± 21.2	2103	113.3 ± 14.8	199	92.9 ± 3.8
F1	5197	103.6 ± 7.5	155	118.1 ± 5.7	2949	178.9 ± 73.5	505	94.7 ± 4.1
F2	5881	114.2 ± 9.8	146	104.6 ± 4.6	6498	130.2 ± 35.3	166	88.8 ± 3.2
F3	5009	115.2 ± 13.9	826	111.6 ± 1.8	5572	150.7 ± 42.7	825	94.6 ± 4.8
F4	2256	104.3 ± 16.8	181	108.2 ± 1.0	12260	116.3 ± 20.7	1589	95.4 ± 5.6

O/E(%) = percentage recovery calculated as [(observed concentration × 100)/expected concentration]; SD = standard deviation.

Sample number	Intra-assay variability				Inter-assay variability			
	Calprotectin		S100A12		Calprotectin		S100A12	
	Mean (µg/L or ng/g)	CV (%)	Mean (µg/L or ng/g)	CV (%)	Mean (µg/L or ng/g)	CV (%)	Mean (µg/L or ng/g)	CV (%)
S1	1007	5.5	51	8.4	1118	7.1	53	3.4
S2	2272	3.8	66	2.4	1491	6.4	71	3.5
S3	2892	8.5	104	3.4	2271	9.3	111	3.7
S4	1693	5.1	150	4.5	1523	8.0	191	4.3
F1	2752	5.0	503	2.7	1376	28.5	544	4.5
F2	13368	5.6	129	4.1	10726	12.8	186	8.2
F3	5573	3.0	694	2.8	4232	5.8	737	5.3
F4	5847	4.6	1291	2.8	3627	14.0	1261	4.3

CV = coefficient of variation

health in the captive cheetah. However, there is a dearth of data on the functionality of fermentation products in felids (Rondeau et al. 2003) and many other mammals, with current research typically restricted to studies in rats, humans or in vitro. Therefore, the relevance of changes in fermentation pattern to gastrointestinal health of cheetahs is still unknown.

In exotic felids, faecal consistency is often used to assess intestinal health. However, scientific evidence of an association between faecal consistency measurements and gastrointestinal disease is still lacking, and the sensitivity of faecal scoring as a marker for gastrointestinal health is questionable.

The proteins calprotectin (S100A8/S100A9) and S100A12 are secreted by activated granulocytes and monocytes during times of acute intestinal inflammation, and calprotectin can also be produced in epithelial cells at sites of inflammation (Johne et al. 1997; Roth et al. 2003; Foell et al. 2009). Both faecal proteins have been successfully used as markers of gastrointestinal inflammation in humans (Fagerhol 2000; Konikoff et al. 2006; Foell et al. 2009) and their validity is currently being evaluated in dogs (Grellet et al. 2011; Heilmann et al. 2012a; Otoni et al. 2012). The current study was therefore designed to evaluate faecal as well as serum calprotectin and S100A12 as potential biomarkers of gastrointestinal health in captive cheetahs fed either a whole prey or a meat-only diet.

Methods

Experimental design

This experiment was part of a study on the effect of diet type on microbial fermentation in the captive cheetah. As such, the detailed methodology and diet composition has previously been reported (Depauw et al. 2011). In brief, a cross-over study including 12 clinically healthy cheetahs (4 male, 10 female, 3.0 ± 1.6 years old) housed at Ree Park Ebeltoft Safari (Ebeltoft, Denmark) was conducted and faecal samples were collected from cheetahs consuming two alternative diets. The test diets consisted of either unsupplemented whole rabbit (2.5–3 kg diet/animal daily, Kiezebrink Putten B.V., Hoge Eng Oost, the Netherlands) without additional supplementation, or beef (1.2–1.6 kg diet/animal daily, Kiezebrink Putten B.V., Hoge Eng Oost, The Netherlands) supplemented with a vitamin and mineral premix (10 g/kg meat (as fed), Carnizoo®, Twilmij B.V., Stroe, The Netherlands).

Before the onset of the trial, all cheetahs were fed mainly on supplemented beef. A three-week adaptation period was used prior to collection of faeces during the fourth week of each testing period. One fresh faecal sample was collected for each individual within 15 min of voiding and was immediately frozen and stored at -20°C. During the trial, 10 cheetahs were anaesthetised as part of the zoo's routine veterinary plan, and a subsample of serum was

Table 2. Recovery of calprotectin and S100A12 in serum samples and faecal extracts from cheetahs, spiked among each other.

Sample number	Spiking recovery (samples)			
	Calprotectin		S100A12	
	Concentration (µg/L or ng/g)	O/E(%)	Concentration (µg/L or ng/g)	O/E(%)
S1+S2	4773	98.4	121	106.2
S1+S3	3887	94.4	103	109.4
S1+S4	3655	94.9	95	104.9
S1+S5	3410	88.9	101	109.7
S2+S3	3123	95.4	70	102.3
S2+S4	2708	90.2	66	102.6
S2+S5	2927	97.9	74	111.5
S3+S4	2099	92.4	47	106.0
S3+S5	2113	93.6	47	102.2
S4+S5	1821	91.7	42	100.4
All		93.8 ± 3.1		105.5 ± 3.7
F1+F2	38566	89.3	2241	101.9
F1+F3	36468	95.8	1864	105.8
F1+F4	31044	90.1	1828	109.3
F1+F5	28307	87.4	1819	95.8
F2+F3	17268	85.8	654	101.1
F2+F4	15065	91.1	552	98.9
F2+F5	12725	87.9	737	93.9
F3+F4	11926	104.6	149	123.5
F3+F5	11450	122.4	350	101.1
F4+F5	5896	102.5	284	109.9
All		95.7 ± 11.4		104.1 ± 8.6

O/E (%) = percentage recovery calculated as [(observed concentration × 100) / expected concentration]

donated to this study. As a result, serum was collected from four cheetahs fed whole rabbit and six cheetahs fed supplemented beef. The samples were centrifuged and serum was immediately frozen and stored at -20°C until analysis.

Faecal/serum calprotectin and S100A12 analyses

Faecal samples were thawed and 1 g was diluted 1:5 in faecal extraction buffer (20 mM CH₃CO₂ and 3 mM CaCl₂ [pH 7.6]) containing an EDTA-free proteinase inhibitor cocktail (1 tablet/25 mL, Roche Diagnostics GmbH, Mannheim, Germany). The samples were then vigorously shaken (30 min, 23°C) and centrifuged (20 min, 5°C, 2100 × g). Finally, the supernatant was collected by use of a serum filter (Fisherbrand serum filter system [IB model], Fisher Scientific Inc., Pittsburgh, USA) and again centrifuged (30 min, 23°C, 10,600 × g). Calprotectin and S100A12 concentrations in faecal extracts and serum were measured by in-house ELISAs according to Heilmann et al. (2011b; 2012a). The assays were analytically validated for the quantification of both markers in serum and faecal samples from cheetahs by determination of dilutional parallelism, spiking recovery, and intra- and inter-assay variability using four different sera and four faecal extracts from cheetahs for each. Dilutional parallelism was determined by evaluating sera and faecal extracts at a serial 2-fold dilution from 1:50–1:400 and

1:100–1:800, respectively. The remaining validation parameters were determined with serum samples diluted 1:100 and a final dilution of 1:500 for faecal extracts. Spiking recovery was determined by adding purified canine calprotectin (0, 0.5, 1, 2, 5, 10 and 25 ng/mL) or canine S100A12 (0, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.5 ng/mL) to each of the samples and calculating the percentage of antigen recovery. Further, five different serum samples and five different faecal extracts were spiked with each other. Calprotectin and S100A12 were measured in each of the spiked and unspiked samples and the percentage of recovery was calculated. Intra-assay variability was evaluated by assaying each sample 10 times within the same assay and calculating the intra-assay coefficients of variation (%CV) for each sample. Inter-assay variability of the assay was determined by analysing each sample in 10 consecutive assay runs and calculating inter-assay %CVs.

Data analysis

Data were analysed using IBM SPSS version 20 (IBM SPSS Statistics, IBM Inc, Armonk, NY, USA). Following distribution analysis (using a Shapiro-Wilk test), median values (lower quartile Q₁, upper quartile Q₃) were reported for the nonparametric data.

Spearman correlation coefficients were used to evaluate the associations among faecal as well as serum calprotectin and S100A12 concentrations. Further, concentrations of both calprotectin and S100A12 in faeces and in serum were compared within individual animals using a Spearman's test.

A Wilcoxon signed rank test was performed to evaluate the effects of test diet on faecal calprotectin and S100A12 concentrations. Differences in serum concentrations of calprotectin and S100A12 between diets were evaluated using a Mann-Whitney U Test. Statistical significance was set at $P \leq 0.05$.

Results

Both the assay for canine calprotectin and for canine S100A12 detected the respective analytes in serum and faecal samples collected from cheetahs. The dilutional parallelism, spiking recovery, intra-assay and inter-assay variability are summarised in Tables 1 and 2. Of note, faecal calprotectin concentrations did not consistently parallel the standard curve of the calprotectin assay in some of the samples from cheetahs, especially for samples with a high concentration of calprotectin. Likewise, an over-recovery was detected in faecal samples from cheetahs spiked with canine calprotectin, especially samples with a low concentration of calprotectin.

Faecal calprotectin and S100A12 were positively correlated ($r = 0.918$, $P < 0.001$). Likewise, serum calprotectin and S100A12 correlated positively ($r = 0.967$, $P < 0.001$). However, faecal

Table 3. Calprotectin and S100A12 concentrations in faeces and serum from cheetahs fed whole rabbit or supplemented beef for a period of 1 month. Values presented as median (Q1, Q3); Q1 = lower quartile, Q3 = upper quartile.

	Whole rabbit	Supplemented beef	P
<i>n</i>	12	12	
Faecal calprotectin (µg/g)	1.3 (0.04, 7.8)	8.8 (2.8, 24.4)	0.241
Faecal S100A12 (ng/g)	301 (223, 1185)	1671 (494, 4415)	0.041
<i>n</i>	4	6	
Serum calprotectin (µg/L)	2755 (1798, 4093)	2104 (1580, 2184)	0.257
Serum S100A12 (µg/L)	117 (85, 207)	96 (76, 107)	0.476

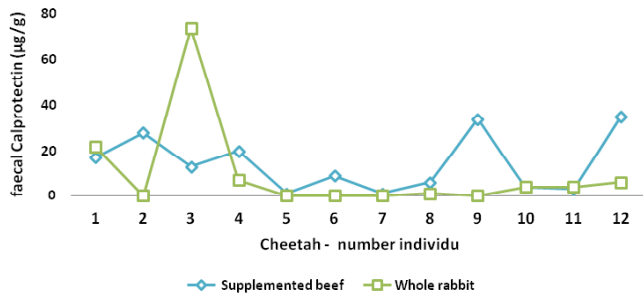


Figure 1. Individual faecal calprotectin concentrations in faeces from cheetahs fed whole rabbit or supplemented beef for a period of 1 month.

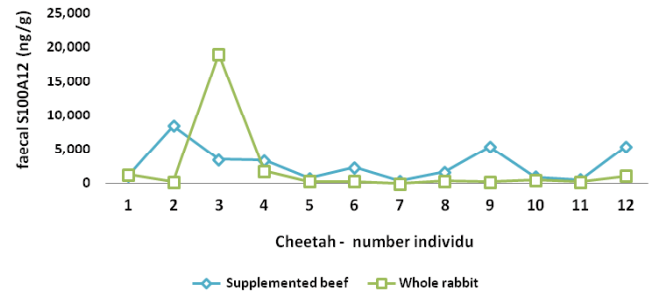


Figure 2. Individual faecal S100A12 concentrations in faeces from cheetahs fed whole rabbit or supplemented beef for a period of 1 month.

concentrations of both calprotectin and S100A12 did not correlate with the concentrations of these proteins in serum.

Concentrations of calprotectin and S100A12 in faeces and serum collected from captive cheetahs are presented in Table 3. The marked numerical increase in faecal calprotectin concentration in cheetahs when fed supplemented beef failed to reach the level of significance. Cheetahs fed whole rabbit exhibited significantly lower faecal S100A12 concentrations (median: 301 ng/g, Q_1 223, Q_3 1185) compared to the same cheetahs fed supplemented beef (median: 1671 ng/g, Q_1 494, Q_3 4415, $P=0.041$). The individual changes of faecal calprotectin and S100A12 are presented in Figure 1, respectively Figure 2.

No significant differences were detected between the diet groups in serum calprotectin or S100A12.

Discussion

The pro-inflammatory proteins calprotectin (S100A8/S100A9) and S100A12 have been extensively studied for their use as faecal biomarkers in inflammatory bowel disease (IBD) in humans (Røseth et al. 1992; Costa et al., 2003; Foell et al., 2009). Given their value as sensitive markers for clinical as well as subclinical intestinal inflammation (Foell et al. 2003; D'Inca et al. 2008), the utility of calprotectin and S100A12 in the diagnosis and/or monitoring of canine IBD is currently under investigation (Grellet et al. 2011; Heilmann et al. 2012a,b; Otoni et al. 2012). However, faecal calprotectin and S100A12 are non-specific markers of intestinal inflammation, and as such, these markers in isolation may not differentiate IBD from other intestinal inflammatory conditions (Summerton et al. 2002).

Both the immunoassay for canine calprotectin and canine S100A12 were precise and reproducible for use with serum samples and faecal extracts from cheetahs, and the assay for S100A12 was also linear and accurate for serum samples and faecal extracts from cheetahs. However, the canine calprotectin assay showed an inconsistent linearity together with an apparent over-recovery of spiked canine calprotectin in samples with a low analyte concentration. However, these deviations were not present in samples that were spiked with other serum or faecal samples from cheetahs, and therefore, these results are consistent with a lower avidity of the antibodies generated against canine calprotectin for the homologous protein in the cheetah. Whilst for the generation of these initial data, the use of the canine calprotectin assay for serum and faecal samples from cheetahs was deemed appropriate, further studies into calprotectin in cheetahs may require a species-specific assay.

Faecal concentrations of calprotectin and S100A12 were highly variable among cheetahs, paralleling findings in dogs (Heilmann et al. 2008, 2011a,b). Ideally, faecal concentrations of these biomarkers should be quantified in 3-day sample collections

(Heilmann et al. 2008), however, only 1-day sample collections were available for this study. Cheetah 3 showed, when fed whole rabbit, markedly higher faecal concentrations of both markers compared to the other cheetahs, without any clinical signs of compromised gut health. Although an analytical error could not be totally ruled out, cheetah 3 was not excluded from the dataset in this early stage of evaluating S100/calgranulins in an exotic felid.

Faecal calprotectin concentrations were not significantly different between diet groups. However, whereas calprotectin concentrations in cheetahs fed supplemented beef were within the same range as reported for healthy dogs using the same assay (3.2–65.4 µg/g, Heilmann et al. 2011b), the whole rabbit diet resulted in markedly lower calprotectin values as compared to previously reported concentrations in dogs. Compared to calprotectin, faecal S100A12 did differ between diet groups. Cheetahs fed supplemented beef had significantly higher faecal S100A12 concentrations compared to when fed whole rabbit. In addition, faecal S100A12 concentrations in cheetahs fed beef exceeded the central 95th percentile of a single day faecal collection in healthy dogs (2–1575 ng/g, Heilmann et al. 2012a), which may indicate on-going inflammatory conditions.

Simultaneously with the present study, the intestinal fermentation profile of these cheetahs on both diet types was examined (Depauw et al. 2011). Cheetahs fed a supplemented beef diet were observed to have significantly higher concentrations of putrefactive compounds, accompanied by looser stool and a higher incidence of diarrhoea compared to the same cheetahs fed whole rabbit. This difference is probably due to a combination of a higher protein intake and lower animal fibre content in meat-only diets compared with whole prey (Depauw et al. 2011, 2012). The significantly higher faecal S100A12 concentrations in cheetahs fed beef, compared with whole rabbit, strengthen the assumption that the higher incidence of putrefactive compounds and looser stool is indicative of compromised gut health in the cheetah.

To further evaluate the association between putrefactive compounds and intestinal inflammation, and to broaden our insight into gut health assessment, we examined the possible correlation between faecal S100A12 and calprotectin, faecal scores (Depauw et al. 2011) and faecal fermentation metabolites (Depauw et al. 2011). No significant correlations (data not shown) between these parameters were present, which is possibly due to the small number of studied cheetahs.

Recent studies revealed that faecal S100A12 has an improved diagnostic accuracy and a higher correlation to intestinal mucosal inflammation compared to faecal calprotectin in humans (Kaiser et al. 2007; Leach et al. 2007; Sidler et al. 2008). This might explain why faecal S100A12 concentrations, but not calprotectin concentrations, were significantly elevated in cheetahs fed a meat-only diet compared to whole rabbit diet for only one month.

Given the limitations of the calprotectin assay, however, the lack of a significant difference in faecal calprotectin concentrations between the two dietary groups needs to be interpreted with caution.

Although blood concentrations of calprotectin and S100A12 have been reported as useful markers of intestinal inflammation (Foell et al. 2003; Heilmann et al. 2012b), increased serum concentrations occur in several disease conditions, particularly in inflammatory diseases, certain microbial infections, and neoplastic conditions (Johne et al. 1997; Striz and Trebichavsky 2004). At present, no studies have evaluated the relationship between serum and faecal concentrations of calprotectin or S100A12. In the current study, serum concentrations of neither marker correlated with their faecal concentrations. Moreover, serum concentrations were unaffected by diet and comparable with concentrations detected in healthy dogs (969–11,873 µg/L, Heilmann et al. 2011b and 57–326 µg/L, respectively, Heilmann et al. unpublished). It is possible that the feeding trial was too short to elicit changes in blood concentrations of these markers or that the sample size was inadequate. However, it is likely that serum calprotectin and S100A12 are less specific markers for intestinal inflammation, which warrants further investigation and the establishment of normal serum calprotectin and S100A12 concentrations in a larger sample of healthy cheetahs.

Conclusion

In the cheetah, faecal calprotectin and S100A12 concentrations were measured using ELISA assays that were previously analytically validated for dogs. These proteins are promising markers for future research on intestinal inflammation in the cheetah. Our results indicate that the type of diet affects faecal but not serum concentrations of S100/calgranulins in the captive cheetah. Significantly lower faecal S100A12 and numerically lower calprotectin concentrations were detected when cheetahs were fed whole rabbit compared with supplemented beef. Consequently, the diet type provided (whole prey or meat-only) can potentially affect intestinal inflammatory conditions in the captive cheetah. In light of the disproportionately high prevalence of gastrointestinal disease in cheetahs held in captivity, these findings warrant further investigation.

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