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Preliminary Reference Intervals for Capillary Zone Electrophoresis Fractions and an Examination of MRP-126 as a Potential Marker of Inflammation in the Aldabra Giant Tortoise (*Aldabrachelys gigantea*)

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Abstract: The diagnostic utility and reference intervals for blood studies in Aldabra giant tortoises (*Aldabrachelys gigantea*) are not well described. Capillary zone electrophoresis (CZE) has been evaluated in non-mammalian vertebrates and shows a higher fraction resolution and less overall variation in results than agarose gel electrophoresis. To date, the investigation of novel biomarkers has been limited in reptiles. MRP-126, a calgranulin homologue in reptiles, has not been evaluated for its diagnostic potential in tortoises. The goals of this study were to establish preliminary reference intervals for CZE protein electrophoresis and to examine MRP-126 as a potential biomarker of inflammation in Aldabra giant tortoises. In 27 clinically healthy tortoises, CZE resolved seven protein fractions. In tortoises with an inflammatory or infectious disease process (n = 4), MRP-126 concentrations and CZE fractions did not consistently increase or were abnormal. To strengthen the understanding of the diagnostic value of CZE and MRP-126 concentration in this species, future studies should evaluate a larger sample set inclusive of repeated measures of clinically abnormal tortoises as well as CZE and MRP-126 variations in regard to additional health conditions, age, sex, season, and geographic location.

Keywords: Aldabra giant tortoise; *Aldabrachelys gigantea*; biomarker; capillary zone electrophoresis; MRP-126; protein electrophoresis

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1. Introduction

The Aldabra giant tortoise (*Aldabrachelys gigantea*) is one of the largest species of tortoise and is classified as vulnerable to extinction [1]. Though relatively common in zoological collections, there is limited information on the plasma protein electrophoretogram of this species or its diagnostic value [2]. In addition, no studies have been conducted to evaluate the specific biomarkers of inflammation in this species.

Hematology has variable utility in the detection of inflammatory or infectious processes in many reptile species [3–5]. Studies suggest that plasma protein electrophoresis (EPH) may provide a more clinically useful monitoring tool than hematology in reptiles [6–9]. Agarose gel electrophoresis (AGE) and, to a lesser degree, capillary zone electrophoresis (CZE), are commonly performed in veterinary laboratories [6]. Typically, CZE has a higher fraction resolution than AGE, allowing for improved quantitation of

globulin fractions and less overall variation in results [6,10]. Previous studies have found plasma protein electrophoretic profiles to be species-specific, requiring the calculation of reference intervals (RI) by species for improved clinical application of EPH [7,9–18]. Additionally, the bromocresol green (BCG) method of albumin measurement, used in most chemistry analyzers, has been found to not be valid in chelonian species [19–21].

Acute phase proteins (APP) are part of the innate immune response and can be biomarkers of inflammation, infection, neoplasia, stress, and trauma [22]. Toll-like receptors (TLR) are a family of highly evolutionarily conserved transmembrane proteins involved in cell–cell interactions and signaling within the innate immune system [23–27]. Calgranulins, a class of S100 proteins, are expressed by mammalian white blood cells in response to TNFα and IL-1β and have high expression under infectious conditions [28,29]. Through the binding of Ca(II), these proteins allow intracellular regulation, facilitate extracellular cell-to-cell communication, and inhibited microbial growth through trace element sequestration [28,29]. Calgranulins are not present in birds and reptiles; rather, MRP-126 is the single calgranulin homologue in these species [27]. In domestic chickens (*Gallus domesticus*), MRP-126 has been identified as a homologue to mammalian TLR-4 that restricts microbial growth through calcium-dependent zinc sequestration [27,29,30]. Experimental *Salmonella enteritidis* infections in chickens resulted in increased MRP-126 concentrations [31–33]. MRP-126 has been identified in green turtles (*Chelonia mydas*), and its expression significantly decreased during the rehabilitation of debilitated animals, suggesting that its expression may be related to disease state [34,35]. A commercial assay is available for use in sea turtles, making it a readily accessible research tool for projects in other chelonian species when there is a demonstration of reagent cross reactivity.

The objective of this study was to establish preliminary CZE reference intervals and to evaluate MRP-126 as a potential biomarker of inflammation in Aldabra giant tortoises.

2. Materials and Methods

Sample Information: Lithium heparinized plasma samples were obtained from clinically healthy Aldabra giant tortoises (n = 27) from 9 zoological institutions. The study population age ranged from 1 to 91 years of age with 3 tortoises of unknown age (median = 45 yr, 95% CI: 15.5–58.8 yr) and included 13 male, 11 female, and 3 tortoises of unknown sex. Animals were deemed clinically healthy by the attending veterinarian at each facility following a physical examination, complete blood count, and plasma biochemistry at the time of sample collection. Sample collection and processing were performed according to each institution's protocol. Heparinized plasma samples were analyzed prospectively on submission to the laboratory (University of Miami, Miami, FL 33136, USA) (n = 25) or shared as banked samples after storage at −80 °C for less than 4 years with no previous freeze–thaw cycles (n = 2). All samples were free from lymph contamination, hemolysis, and lipemia. Samples were collected between May 2020 and February 2024 with the majority of samples collected between May 2023 and October 2023. A total of 5 samples from tortoises with infectious or inflammatory disease processes were evaluated, representing 3 individuals with 1 sampled serially. The unhealthy animals had an age range of 30–90 years and included 2 males and 1 female. The represented disease processes were two localized infections, hepatic lipidosis, and follicular stasis. This small number of clinically unhealthy tortoises was used for a preliminary comparison of electrophoretic changes between diseased and healthy animals, as well as for the initial evaluation of MRP-126 as a potential marker of inflammation in this species.

Capillary Zone Protein Electrophoresis: Total protein was determined using the biuret method on the Vitros 5600 analyzer (Ortho Vitros, Rochester, NY 14626, USA). Samples were analyzed per manufacturer protocols for urine protein electrophoresis using a Sebia

Capillarys 2 Flex Piercing system (Sebia, Norcross, GA 30093, USA). Samples were diluted 1:8 with urine running buffer. The dilution was previously optimized by the laboratory for use in many animal species, including tortoises. The urine buffer was used as it aids in the fraction migration of avian and reptilian species by placing anodic and cathodic migration stopping points. Fractions were quantitated as the percentage of total protein, and absolute values were determined by multiplying these results by the total protein. Albumin was also determined by the BCG method on the Vitros 5600 analyzer. A coefficient of variation analysis was conducted for the CZE method. A single sample was run eight times within one run.

MRP-126 Testing: Samples were analyzed in duplicate using the turtle MRP-126 SPARCL assay (Life Diagnostics, West Chester, PA 19380, USA) per manufacturer recommendations. Analysis was conducted using a FLUOstar Omega reader (BMG LABTECH, Cary, NC 27513, USA). A coefficient of variation analysis was conducted on a single sample run eight times within one run.

Statistics: Reference intervals were generated per the American Society for Veterinary Clinical Pathology (ASVCP) Quality Assurance and Laboratory Standards guidelines for sample sizes of less than 40 using MedCalc software (version 22.009, MedCalc Software, 8400 Ostend, Belgium) [36]. This robust method was used to generate the intervals, and no outliers were removed. Spearman's correlation coefficient analyses were conducted to compare patient age versus protein fraction data. Method comparison analyses for albumin measured by CZE EPH and BCG were conducted using Passing and Bablok regression and Bland–Altman analysis [37].

3. Results

A representative plasma capillary zone electrophoretogram of a healthy tortoise is presented in Figure 1. Capillary zone electrophoresis consistently resolved a minimum of seven fractions in Aldabra giant tortoises: two prealbumin migrating fractions, albumin, α1-globulin, α2-globulin, β-globulin, and γ-globulin fractions (Figure 1A). In some clinically healthy and unhealthy tortoises, additional fractions were observed mostly as shoulders off the primary fractions for α1, β, and γ-globulins (Figure 1B). The intra-assay coefficient of variation ranged from 1.7 to 8.6% for albumin and globulin fractions and 18.3 to 29.0% for the prealbumin fractions. There was no significant correlation between tortoise age and any CZE measurand ($p > 0.05$) except for a moderate positive correlation between age and γ-globulins (r = 0.46, $p = 0.03$).

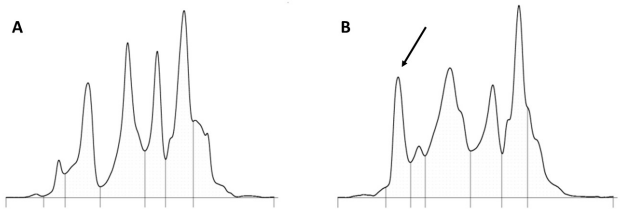

Figure 1. Respective plasma capillary zone electrophoretograms for clinically healthy (**A**) and unhealthy ((**B**); Case 3) Aldabra giant tortoises (*Aldabrachelys gigantea*). The fractions are prealbumin 1, prealbumin 2, albumin, α1-globulin, α2-globulin, β-globulin, and γ-globulin from left to right. In (**B**), note the marked decrease in albumin and increase in prealbumin 2 (arrow) when compared to (**A**).

The MRP-126 assay was validated for use in Aldabra giant tortoises. Linearity under dilution was found by Deming's regression; the slope included 1, and the y-intercept included 0. The Runs test did not show a significant deviation from linearity ($p = 0.67$). The

mean intra-assay coefficient of variation was 2.3%, and the minimum detection level was 0.1 mg/L.

Reference intervals were calculated using CZE fractional data and MRP-126 concentration from the 27 clinically normal tortoises (Table 1). MRP-126 had a RI of 0–14.4 mg/L; however, three of the reportedly healthy animals had concentrations outside of the calculated reference interval, measuring 18.1, 19.9, and 23.4 mg/L.

Table 1. Reference intervals of plasma capillary zone electrophoresis fractions and MRP-126 in healthy Aldabra giant tortoises (*Aldabrachelys gigantea*, n = 27). NG = non-Gaussian, G = Gaussian.

Protein	Unit	Mean	SD	Median	Min	Max	Normality	*p*-Value	Reference Interval	Lower CI 90%	Upper CI 90%
Total protein	g/dL	4.3	1.2	4.0	2.4	7.0	G	0.39	1.6–6.7	1.0–2.3	5.9–7.4
A/G Ratio		0.24	0.05	0.23	0.18	0.41	NG	0.001	0.12–0.34	0.09–0.16	0.3–0.38
Prealbumin 1	g/dL	0.03	0.02	0.03	0.01	0.08	NG	0.015	0–0.07	0-0	0.05–0.08
	%	0.7	0.5	0.6	0.2	2.1	NG	0.007	0–1.7	0-0	1.3–2.1
Prealbumin 2	g/dL	0.28	0.29	0.17	0.04	1.32	NG	<0.0001	0–0.83	0-0	0.51–1.08
	%	6.0	4.4	4.5	1.7	18.9	NG	0.004	0–14	0-0	10.5–17.6
Albumin	g/dL	0.53	0.13	0.53	0.3	0.82	G	0.59	0.23–0.79	0.18–0.32	0.72–0.88
	%	12.7	2.2	13	7.6	16.1	G	0.56	8.1–17.5	6.8–9.6	16.4–18.5
α1-globulins	g/dL	1.34	0.48	1.28	0.61	2.63	NG	0.004	0.18–2.19	0–0.56	1.85–2.55
	%	31	6	30.3	19.6	39.5	G	0.12	18.4–43.9	14.7–21.6	40.4–47.6
α2-globulins	g/dL	0.65	0.34	0.53	0.25	1.38	G	0.13	0–1.32	0–0.01	1.04–1.57
	%	15.0	5.9	14.0	4.1	28.8	G	0.45	1.9–27	0–5.1	22.8–30.6
β-globulins	g/dL	0.81	0.26	0.77	0.42	1.54	G	0.06	0.22–1.34	0.05–0.37	1.14–1.51
	%	19.3	4.8	19.3	11.1	26.9	G	0.31	9–29.6	6.7–12.1	27.2–32.2
γ-globulin	g/dL	0.65	0.23	0.63	0.31	1.21	G	0.33	0.15–1.1	0.04–0.28	0.96–1.25
	%	15.3	3.6	15.2	7.6	20.3	G	0.44	8.1–23	6–10.1	21.3–24.8
MRP-126	mg/L	3.45	6.33	0.86	0.3	23.4	NG	<0.0001	0–14.4	0-0	5.4–20

Albumin levels were compared between the BCG method and CZE. The median of the BCG method was 1.6 g/dL (95% CI: 1.5–1.8) compared to the CZE method median of 0.52 g/dL (95% CI: 0.48–0.56). The BCG results were found to positively but weakly correlate with the albumin determined by CZE (r = 0.41, $p = 0.0076$). The regression equation was y(BCG) = −0.17 + 3.3x (CZE). Using Passing and Bablok regression, a proportional error was observed as the slope differed from 1 (0.30, 95% CI: 0.18–0.0.48) (Figure 2). By Bland–Altman analysis, the BCG method was found to have a mean positive bias of 1.14 g/dL (Figure 2).

The infectious or inflammatory disease processes in the unhealthy tortoises are described in Table 2. The sample size was insufficient for statistical analysis by health status. Cases 1 and 2 were diagnosed on examination, while hepatic lipidosis and follicular stasis were identified on necropsy of Case 3. There was a mild increase in α2-globulin with a carapace infection (Case 2). The increase in prealbumin 2 measured at the start of clinical signs in Case 3 (Case 3B) decreased to within the reference interval in 9 days (Case 3C). Over 9 days of clinical signs, the electrophoretogram had a 20% decrease in total protein with a 12.8-fold increase in MRP-126. The electrophoretogram for sample 3C is shown in Figure 1B. There were no consistent electrophoretic abnormalities in these tortoises, although mild changes were observed. MRP-126 was increased outside of the RI in two out of three tortoises.

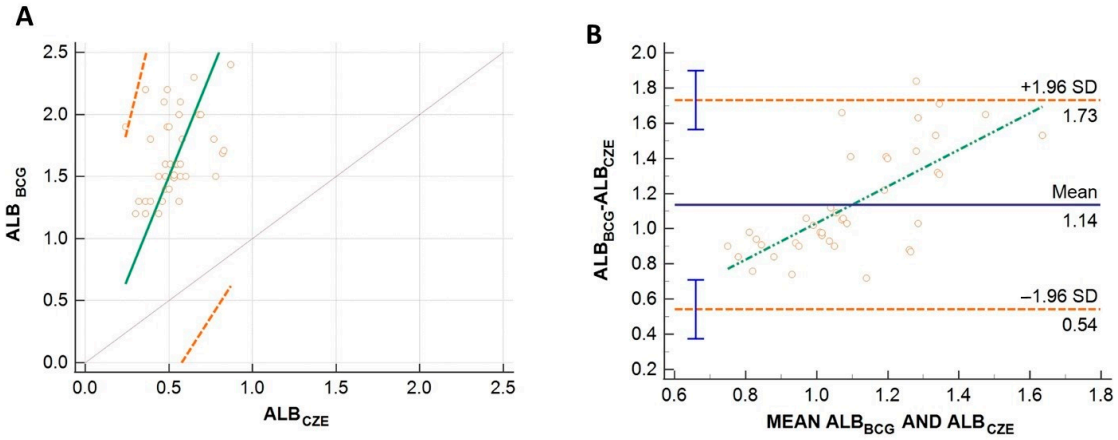

Figure 2. Passing–Bablok regression analysis (**A**) and Bland–Altman plot (**B**) for the comparison of albumin measured by CZE and BCG methods in Aldabra giant tortoises (*Aldabrachelys gigantea*). In panel (**A**), the green line is the regression line, and the dotted gray line is the line of identity. The two dashed lines show the 95% limits of agreement. In panel (**B**), the mean percentage and limits of agreement are shown. The green dashed line is the regression line of the differences between the methods.

Table 2. Case presentations of Aldabra giant tortoises (*Aldabrachelys gigantea*) with infectious or inflammatory disease processes. Bolded values fall outside of the reference interval (RI) calculated in this study. M = male, F = female.

Protein	Unit	Case 1	Case 2	Case 3A	Case 3B	Case 3C	Reference Interval
Age/sex	Y	90/M	56/M	30/F	33/F	33/F	
Clinical finding		Chronic limb infection	Carapace infection	Clinically normal	Hepatic lipidosis, follicular stasis		
Total protein	g/dL	4.8	5.4	6.2	6	4.6	1.6–6.7
A/G ratio		0.18	0.18	0.19	0.3	0.23	0.12–0.34
Prealbumin 1	g/dL	0.01	0.02	0.03	0.07	0.05	0–0.07
Prealbumin 2	g/dL	0.17	0.29	0.1	**0.96**	0.58	0–0.83
Albumin	g/dL	0.56	0.49	**0.87**	0.36	0.24	0.23–0.79
α1-globulins	g/dL	1.05	1.33	**2.98**	1.96	1.39	0.18–2.19
α2-globulins	g/dL	1.17	**1.52**	0.61	1.03	0.81	0–1.32
β-globulins	g/dL	1.33	0.94	1.05	0.79	1.06	0.22–1.34
γ-globulin	g/dL	0.51	0.8	0.55	0.83	0.47	0.15–1.1
MRP-126	mg/L	0.7	**19.3**	1.5	2.8	**33.3**	0–14.4

4. Discussion

This study provides the first description of the CZE method of electrophoresis in Aldabra giant tortoises, which consistently resolved seven fractions. The CZE method with a different analyzer and protein buffer in spur-thighed tortoises (*Testudo graeca*) resolved five fractions, while samples from Hermann's tortoises (*Testudo hermanni*), red-eared sliders (*Trachemys scripta elegans*), and map turtles (*Graptemys* spp.) consistently resolved five fractions often with a split albumin peak (Table 3) [13]. In green turtles, CZE resolved more fractions than Aldabra tortoises with nine, including three prealbumin migrating fractions and two γ-globulin fractions [10]. While prealbumin migrating fractions could be resolved, the utility of these measurements may be limited by the high intra-assay coefficient of variation in these fractions. The latter finding is common to protein electrophoresis where

fractions with lower composition show higher variation [6]. The variation observed in the quantitation of the other fractions is consistent with that commonly reported with CZE in animal species [6]. While these fractions are labeled as prealbumin because of their migration characteristics, the composition of the fractions has yet to be determined [6]. Notably, in addition to differences in fraction resolution, the Aldabra tortoise species exhibits a consistently lower A/G ratio versus other chelonian species (Table 3). This is related to a > 50% lower albumin fraction and increased globulin expression. As additional CZE studies are completed in tortoises, it will be of interest to see if the Aldabra tortoise is unique in this plasma protein composition. The higher globulin component may reflect the presence of lipoproteins and other acute phase reactants which are present at higher levels with a clinically normal status. A wide variation in EPH globulin composition is a characteristic across different taxonomic classes and orders [6]. Phylogenetic studies have suggested that the tortoise species inhabiting Madagascar and the surrounding islands, including the Aldabra giant tortoise, split from *Geochelone* about 14.5–9.5 million years ago [38]. This evolutionary separation may also contribute to the different electrophoretic fractions seen in Aldabra giant tortoises.

Table 3. Mean (and standard deviation) values reported as reference intervals for total protein, A/G ratio, and albumin in chelonian species. Albumin was determined and A/G ratio calculated from capillary zone electrophoresis (CZE) methodology. Fall season data were selected for consistency of data presentation; see reference for additional data on spring and summer seasons.

Species [Ref.]	Number of Total CZE Fractions/Globulin Fractions	Experimental Design	Total Protein, g/dL	A/G Ratio	Albumin, g/dL
Aldabra giant tortoise (*Aldabrachelys gigantea*) [current study]	7/4	Various seasons and sexes, n = 27	4.3 (1.2)	0.24 (0.05)	0.53 (0.13)
Spur-thighed tortoise (*Testudo graeca*) [13]	5/3	Fall season, male	3.6 (0.9)	0.67 (0.24)	1.44 (0.55)
Spur-thighed tortoise (*T. graeca*) [13]	5/3	Fall season, female	3.7 (0.8)	0.88 (0.27)	1.71 (0.48)
Hermann's tortoise (*Testudo hermanni*) [14]	5/3	Fall season, male, n = 80	2.9 (0.9)	0.57 (0.12)	1.01 (0.50)
Hermann's tortoise (*T. hermanni*) [14]	5/3	Fall season, female, n = 39	3.3 (1.3)	0.91 (0.22)	1.41 (0.64)
Red-eared slider (*Trachemys scripta elegans*) [12]	5/3	Fall season, male, n = 23	4.5 (1.1)	0.38 (0.09)	1.21 (0.31)
Red-eared slider (*T. scripta elegans*) [12]	5/3	Fall season, female, n = 56	4.6 (1.1)	0.49 (0.14)	1.49 (0.51)
Map turtle (*Graptemys* spp.) [12]	5/3	Fall season, male, n = 7	4.4 (0.7)	0.59 (0.19)	1.63 (0.53)
Map turtle (*Graptemys* spp.) [12]	5/3	Fall season, female, n = 22	4.3 (1.3)	0.54 (0.13)	1.50 (0.53)
Green turtles (*Chelonia mydas*) [10]	9/5	Various seasons and sex, n = 21	n/a	n/a	n/a

In clinically abnormal tortoises, there were no consistent changes in CZE fractions. Additional repeated measures would have been beneficial for this study, but the preliminary finding that no single fraction was consistently increased or decreased in tortoises with an inflammatory disease process suggests variation in protein expression by disease processes in Aldabra giant tortoises. An analysis of additional abnormal samples with repeated and additional disease processes is needed to identify any trends. A previous study

evaluating plasma proteomics of green turtles found a large number of proteins with variable expression between debilitated and recovered animals [35], and similar variation in protein expression may be present in Aldabra giant tortoises.

Studies in other chelonian species have found that EPH fractions can vary with sex, season, age, geographic location, health status, and reproductive status [4,7,9,11–18,20]. Although the sample size across ages was limited in the present study, γ-globulins were found to increase with age; this finding is consistent with reports in other mammalian and non-mammalian species [6]. An evaluation for the effects of season, sex, reproductive status, and geographic location on CZE fractions was outside the scope of this preliminary study. These factors and the relatively low sample set of 27 individuals may have had a role in the wide preliminary CZE RI which were calculated for this species. This preliminary CZE data in Aldabra giant tortoises should provide a foundation for future studies that investigate the effects of these variables on RI to improve the diagnostic utility of CZE in this species.

Studies in other non-traditional species, including chelonians, have found that the BCG method of albumin determination to be invalid and less accurate than EPH due to the reaction of the BCG dye with globulins [6,19–21]. One study in Hermann's tortoises found agreement between albumin concentrations measured using BCG and AGE EPH [16]; however, the BCG method was found to be inaccurate in five species of chelonians, including Hermann's tortoises, and particularly in diseased animals [20,21]. In Aldabra giant tortoises, the BCG method had a positive bias compared to CZE, as has been documented in other reptile species [19–21]. Given the BCG reactivity with globulins and the possible increase in globulins in unhealthy animals, CZE is recommended for albumin measurement in Aldabra giant tortoises.

This study validated the use of the commercially available MRP-126 assay in Aldabra giant tortoises and reported reference intervals for this protein. Acute phase proteins may be positive/increasing or negative/decreasing in response to activation of the innate immune system [22]. MRP-126 has been found to be a positive APP in domestic chickens and green turtles [31–35]. Three clinically healthy tortoises had MRP-126 concentrations several folds higher than the upper limit of the reference interval, but undiagnosed inflammatory conditions remain a possibility in these individuals. These values were not removed from RI calculations per ASVCP guidelines and thus may have skewed these preliminary values. Despite all clinically abnormal tortoises having been diagnosed with disease processes expected to involve inflammation, MRP-126 was not consistently increased; however, a 12.8-fold increase over the course of 9 days was documented in one tortoise (Table 2, Case 3). This suggests that MRP-126 expression can change over the course of a disease process in Aldabra giant tortoises. Furthermore, the variation in MRP-126 concentrations between the clinically abnormal tortoises and the three clinically normal tortoises with increased MRP-126 concentrations suggests that the expression of MRP-126 is not uniform across inflammatory disease processes in this species and may not be useful as a diagnostic tool in this species. Ongoing therapies may have affected both EPH and MRP-126 results; however, this was outside of the scope of this preliminary study. Additional prospective studies measuring MRP-126 repeatedly across multiple inflammatory disease processes, both acute and chronic, may help determine the association between MRP-126 concentration, specific inflammatory disease processes, and the severity of clinical disease.

The small sample size of clinically abnormal tortoises in this study limited the interpretation of MRP-126 levels in this species. Our preliminary findings suggest that MRP-126 concentration may be a marker of inflammation for some disease processes; however, the evaluation of additional disease processes, a larger number of samples, and the repeated sampling of clinically abnormal individuals is required to guide an interpretation of MRP-

126 concentrations in Aldabra giant tortoises and to better determine its use as a prognostic indicator. Additional studies should be undertaken to identify and develop reagents for other possible markers that may have clinical or research applications in tortoise species.

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