Sub-lethal impacts of lead poisoning on blood biochemistry, immune function and delta-aminolevulinic acid dehydratase (δ-ALAD) activity in Cape (*Gyps coprotheres*) and white-backed (*G. africanus*) Vulture chicks

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Highlights

- A significant proportion of Cape and White-backed Vulture nestlings sampled experienced elevated blood lead levels.
- Delta-aminolevulinic dehydratase (δ-ALAD) activity were significantly and negatively correlated to blood lead concentration in both species.
- Cape and White-backed Vultures are predicted to experience 50% depression of the δ-ALAD enzyme at blood [Pb] = 18.8 and 52.8 µg/dL, respectively.
- δ-ALAD activity may serve as a sensitive biomarker in both species.
- In White-backed Vultures blood [Pb] > 100 µg/dL suggest hepatic abnormality.
- Lead's ability to disrupt haem synthesis and cause hepatic injury may compromise young birds' survival as newly-fledged juveniles.

Abstract

Although the prevalence of lead poisoning in southern Africa's *Gyps* vultures is now well-established, its finer physiological effects on these endangered species remain poorly characterised. We evaluated the sub-lethal impact of acute lead exposure on Cape and White-backed Vulture chicks from two breeding colonies in South Africa, by analysing its possible effects on key blood biochemistry parameters, immune function, packed cell volume and δ-aminolevulinic acid dehydratase (δ-ALAD) activity. All 37 White-backed Vulture nestlings sampled displayed elevated lead levels (>10 µg/dL), and seven had blood [Pb] >100 µg/dL. Eight of 28 Cape Vulture nestlings sampled had blood [Pb] exceeding background exposure, with one showing blood [Pb] >100 µg/dL. Delta-aminolevulinic acid dehydratase (δ-ALAD) activity was significantly and negatively related to blood [Pb] in nestlings from both species, with 50% inhibition of the enzyme predicted to occur at blood [Pb] = 52.8 µg/dL (White-backed Vulture) and 18.8 µg/dL (Cape Vulture). Although no significant relationship was found between % packed cell volume (PCV) and blood [Pb], the relatively lower mean PCV of 32.9% in White-backed Vulture chicks, combined with normal serum protein values, is likely indicative of depression or haemolytic anaemia. The leukogram was consistent in both

species, although the presence of immature heterophils suggested an inflammatory response in White-backed Vulture chicks with blood [Pb] >100 µg/dL. Values for cholesterol, triglycerides, total serum protein, albumin, globulin, albumin/globulin ratio, alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT) were consistent with values previously reported. Calcium and phosphorus concentrations suggested no adverse effects on bone metabolism. A significant decrease in urea: uric acid (U:UA) ratio at blood [Pb] >100 µg/dL in White-backed Vulture chicks, brought about by a decrease in urea production, raises the possibility of hepatic abnormality. These results suggest that δ-ALAD activity may serve as a sensitive biomarker of lead toxicity in both species, while highlighting the need to better understand the significant variability in sensitivity that is observed, even between closely related members of the same genus.

Keywords: Vultures; Lead; δ-ALAD; Biochemistry; Immune function

1. Introduction

Lead is a pervasive environmental contaminant that serves no known biological function in any living organism (Franson and Pain, 2011). It acts as a non-specific poison that impacts all organ systems in the body, causing a range of sub-lethal to lethal effects which may vary substantially both intra- and interspecifically (Carpenter et al., 2003). As a toxin, lead competes with essential metals such as calcium and zinc for binding sites on proteins, which may compromise their function; a problem compounded by many proteins displaying a higher affinity for lead than other metals (Knollmann-Ritschel and Markowitz, 2017, Krone, 2018, Skerfving and Bergdahl, 2014).

One major protein inhibited by lead is δ-aminolevulinic acid dehydratase (δ-ALAD) (Pain, 1989); an enzyme in the haem synthesis pathway (Gurer and Ercal, 2000) and thus essential for the regulation of haemoglobin content in erythrocytes (Hoffman et al., 1981). δ-ALAD is the second enzyme in the haem synthesis pathway, promoting the addition of two molecules of δ-ALA to form porphobilinogen (Skerfving and Bergdahl, 2014). Lead inhibits δ-ALAD by binding to the sulfhydryl groups that play an essential role in the catalytic activity of the enzyme (Gurer and Ercal, 2000, Martinez-Haro et al., 2011). Inhibition of δ-ALAD may lead to a corresponding decrease in the production of haem (the molecule that binds oxygen in haemoglobin), as well as the accumulation of excess δ-aminolevulinic acid (δ-ALA) (Gurer and Ercal, 2000). δ-ALA can be oxidised to produce reactive oxygen species (ROS) which, in turn, have been implicated in enhanced lipid peroxidation (and consequent weakening) of cell membranes, potentially damaging cell DNA (Espín et al., 2015) and shortening the lifespan of circulating erythrocytes (Krone, 2018).

One way of quantifying the subclinical impact of lead exposure in raptors and other birds involves evaluating clinical pathological changes in relevant blood biochemical and haematological parameters such as red cell counts, haemoglobin concentration and haematocrit over time (Villegas et al., 2002). While these tests are more readily available for field and laboratory diagnostics, changes in these parameters may only become evident at higher exposure levels (Black et al., 2011). Moreover, haematological parameters in raptors could be influenced by a variety of factors, including age, sex, nutritional status and diet (Black et al., 2011, Casado et al., 2002, Dell'omo and Cavallina, 1996, Dobado-Berrios and Tella,

1998, Hollamby et al., 2004). The interpretation of changes in measured parameters is also dependent on reference ranges being available for the species under investigation, which is frequently not the case. Often, interpretation is based on reference ranges characteristic for closely related taxa (Ferrer et al., 1987), making a more sensitive and predictive test desirable for determining the true susceptibility of species to lead toxicity.

Blood δ-ALAD activity is a well-established and sensitive biomarker of sub-lethal lead toxicity, with its inhibition resulting in adverse health effects such as haemolytic anaemia (Finkelstein et al., 2012; Ray, 2016). Enzyme inhibition occurs within 24 h of lead exposure and may persist for three months thereafter (Dieter and Finley, 1978). Negative correlations between blood [Pb] and δ-ALAD activity have been demonstrated in numerous bird species (Hoffman et al., 1981; Work and Smith, 1996; Strom et al., 2002; Martinez-Haro et al., 2011; Finkelstein et al., 2012) and 50 % inhibition of avian δ-ALAD is regarded as the threshold value indicating biological injury (Vanparys et al., 2008; van der Merwe et al., 2011). Inhibition of 43–60 % has been linked with significantly lower haematocrit, brain mass and haemoglobin concentration (Grue et al., 1986). Sub-lethal effects, including the inhibition of δ-ALAD activity, may be induced by lead levels <15 μg/dL (Martínez-López et al., 2004; Gómez-Ramírez et al., 2011), although there is evidence for significant inter- and intraspecific variation (Dieter et al., 1977; Hoffman et al., 1981; Henny et al., 1991; Vanparys et al., 2008; Gómez-Ramírez et al., 2011; Finkelstein et al., 2012).

Whereas the prevalence of lead poisoning in Cape (*Gyps coprotheres*) and White-backed (*G. africanus*) vultures in southern Africa has been documented in several studies over the last decade (Kenny et al., 2015; Naidoo et al., 2017; Garbett et al., 2018; van den Heever et al., 2019), the physiological impact on these threatened species remains poorly characterised. Because lead toxicity has been associated with a variety of pathologies such as poor reproductive success and central nervous system damage (Krone, 2018), sublethal effects may be important, especially in species which raise only one chick a year. In light of these potential impacts, we aimed to a) quantify δ-ALAD activity in Cape and White-backed Vulture chicks displaying a range of different lead exposure levels under natural conditions, b) establish whether a correlation exists between δ-ALAD activity and blood Pb levels, c) determine the value at which Pb induces a 50% inhibition in δ-ALAD activity, d) quantify the effects of different Pb levels on haematocrit and leukocyte counts, and e) quantify a variety of blood biochemistry parameters in order to evaluate general liver, kidney and skeletal health.

2. Materials and methods

2.1. Study sites and sample collection

Blood samples were collected from White-backed Vulture nestlings (n = 37) at Dronfield Nature Reserve (28.618° S; 24.809° E) near Kimberley (Northern Cape Province, South Africa) in 2019, where previous studies have highlighted the prevalence of lead poisoning among a subset of chicks in the colony (van Wyk et al., 2001; van den Heever et al., 2019). Blood samples were collected from Cape Vulture nestlings (n = 28) in a breeding colony at Karnmelkspruit (30.832° S; 27.244° E, Eastern Cape Province) in 2020 and 2021.

Samples were collected in September (Cape Vultures) and October (White-backed Vultures), when most of the chicks were 60–115 days old and exceeded a minimum body mass requirement of 3 kg. Prior to sampling, each bird was examined externally to evaluate its demeanour and overall condition. Using a disposable needle and syringe, 4.5 mL of blood was collected from the brachial vein under the wing, following van den Heever et al. (2019). Each sample was transferred to a heparinised tube (4 mL) and an EDTA tube (0.5 mL), and carefully swirled to ensure mixing with the respective media. The EDTA sample was frozen for lead testing. Using a 2-mL disposable needle and syringe, 1.5 mL of the heparinised sample was extracted and transferred to a cryovial, which was then flash-frozen in liquid nitrogen in preparation for δ-ALAD analysis. A haematocrit tube was filled from the remaining heparinised sample. A droplet of blood was placed on a microscope slide to perform a blood smear before the haematocrit tube was spun down for 10 min in a microcentrifuge and the % packed cell volume (%PCV) recorded. The remaining heparinised sample was centrifuged at 6000 rpm for 10 min. The plasma portion of the sample (intended for biochemistry analysis) was collected using a glass pipette, and divided between two cryovials, which were frozen for subsequent analysis. The erythrocyte portion of the heparinised sample was discarded. The protocol described above was approved by the BirdLife South Africa Animal Research Ethics Committee (protocol 2019/05/B), the Animal Ethics Committee of the University of Pretoria (NAS215/2019) and the SANBI Research Ethics and Scientific Committee (P2020-21 A).

Although samples were collected from 37 White-backed Vulture nestlings, severe clotting prevented blood smears, PCV, δ-ALAD activity and leukocyte counts in three of the samples. The White-backed Vulture sample size for blood [Pb] and blood biochemistry was consequently n = 37, whereas for PCV, δ-ALAD activity and leukocyte counts, n = 34.

2.2. Sample analyses

Blood lead concentration was determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) at V&M Analytical Toxicology Services (Pty) Ltd, an ISO 17025-accredited laboratory in George, South Africa (SANAS accreditation no. T0610). Samples were digested with diluted nitric acid (1,5 % HNO_3). Fresh multi-point calibration standards were prepared for every new batch. Various internal standards were added to facilitate accurate quantification. Control samples were included in every batch to verify accuracy and precision as per ISO 17025 requirements. These include Recipe ClinCheck controls, consisting of lyophilized blood. The Octapole Reaction System (ORS3) of the 7700x ICP-MS was operated in helium collision mode (He mode) for all analytes and all samples. An Agilent 7700x ICP-MS with a Micromist nebulizer was used throughout. Standard operating conditions were applied during sample analysis (plasma mode: normal, robust; RF forward power: 1550 W; sampling depth: 8 mm; carrier gas flow: 1.07 L/min; dilution gas flow: 0 L/min; spray chamber temperature: 2 °C; extraction lens 1: 0 V; kinetic energy discrimination: 4.5 V; He cell gas flow: 4.3 mL min^{-1}).

Blood smear and biochemistry analyses were conducted at the Clinical Pathology section of the Onderstepoort Veterinary Academic Hospital (Pretoria, South Africa). Automated staining of peripheral blood smears with Wright-Giemsa was performed using a Siemens Hema-Tek. Various morphological features were assessed semi-quantitatively by the assignment of

values 1+ to 4+ corresponding to relative fraction of the abnormality of the total cells assessed. Percentage ranges did vary between features. When used, ‘few’ related to 5–10% of total, ‘moderate’ to 11–30% and ‘many’ greater than 30%. Erythrocytes were examined in the red cell area, and abnormalities of shape, size, pigmentation, presence of inclusion bodies, immature erythrocytes and haemoparasites were quantified following (Campbell and Ellis, 2013).

Manual total leukocyte counts were performed by enumerating cells in 10 monolayer fields using the 40× objective, and then calculating the average per 40× field. The average was then multiplied by 2 to arrive at x109 L^{-1} units. This count was further corrected whenever haematocrit was less than 40% or greater than 50%. Differential white cell counts were performed by counting one hundred leukocytes and calculating percentages of cell types. Heterophil toxic change, monocyte activation and lymphocyte reactivity were assessed following (Campbell and Ellis, 2007) and Harvey (2001). Heterophils were semi-quantitatively graded for severity of toxic changes using mammalian ranges. In general, increasing signs of cytoplasmic basophilia, cytoplasmic vacuolation and presence of toxic granules were the main factors in assessment. Monocytes were assessed for signs of activation. Lymphocytes were graded for reactivity from 1+ to 4+ using an increase in cell size, increasing cytoplasmic basophilia, and a decrease in the nucleocytoplasmic ratio as indicators of reactivity.

A Cobas Integra 400 Plus automated chemistry analyser (Roche Products, Basel, Switzerland) was used to quantify the following parameters in plasma samples (method in parenthesis): albumin ($g.L^{-1}$, bromocresol green dye-binding method), alanine aminotransferase (ALT) activity ($U.L^{-1}$, kinetic with L-alanine and 2 oxoglutarate), cholesterol ($mmol.L^{-1}$, kinetic with cholesterol esterase and oxidase), gamma-glutamyl transferase, GGT ($U.L^{-1}$, kinetic with L-glutamul-3-carboxy-4-nitroanilide and glycylglycerine), globulins ($g.L^{-1}$), triglycerides ($mmol.L^{-1}$, kinetic with lipoprotein lipase and glycerol kinase), inorganic phosphates ($mmol.L^{-1}$, phosphomolybdate method), total calcium ($mmol.L^{-1}$, nitromethyl-BAPTA method), total serum protein ($g.L^{-1}$, biuret method), urea, U ($mmol.L^{-1}$, kinetic with urease), uric acid, UA ($mmol.L^{-1}$, kinetic with uricase).

δ-ALAD activity was determined at the Department of Biochemistry at the University of KwaZulu-Natal, based on a method recommended by the US Geological Survey Eastern Ecological Science Center at the Patuxent Research Refuge (Beyer et al. 2013, 2014, 2018). After samples were defrosted in an ice bath, 350 µL of distilled water (dH_2O) was pipetted into a microfuge tube followed by 25 µL of blood sample. The mixture was vortexed for 10 s before being placed back on ice. All samples were prepared in duplicate. Once all the samples were prepared, the tubes were incubated at 38 °C for 10 min. During this time, the ALA reagent (5-Aminolaevulinic acid hydrochloride Sigma-Aldrich A3785) was prepared by reconstituting 17.76 mg ALA with 10 mL of 0.1 M $NaHPO_4$ (pH = 6.65). Tubes were removed from the ice, two at a time. The ALA reagent (250 µL) was pipetted into each tube, before they were vortexed for 5 s and returned to the ice. Reagent for the blanks consisted of 0.1 M $NaHPO_4$ pH 6.65 only. All samples and blanks were incubated at 38 °C for 1 h. Tubes were placed back in an ice bath. The reaction was stopped by adding 10% (w/v) trichloracetic acid (TCA) to each tube, two tubes at a time. Samples were vortexed for 5 s and placed back on ice. Samples were subsequently centrifuged at 1500×*g* for 10 min. Supernatant (100 µL) was pipetted into a 96 well plate in triplicate. Under a fume hood and using a multi-pipette, 100 µL

of Ehrlich's reagent (0.63 g p-dimethylaminobenzaldehyde to which 12.5 mL glacial acetic acid and 6.125 mL perchroric acid were added slowly in a fume hood before the reagent is made up to 25 mL with glacial acetic acid) was added to each well, before it was subjected to gentle shaking for 20 s. The absorbance values of samples were read in a Versamax microplate reader (Molecular Devices, CA, USA) at 555 nm (A555) and at 630 nm (A630) every 90 s for 30 min. Peak absorbance was determined at 9 min.

To calculate the sample absorbance, each value was blank corrected, before A630 was subtracted from A555. The average, standard deviation and %CV (coefficient of variation) was calculated for each sample. If %CV was greater than 20%, the sample was repeated. δ-ALAD activity (µmol PBG/h/L RBC) was calculated as (mean absorbance x 1881 × 2)/haematocrit, using the method described by Berlin and Schaller (1974).

2.3. Statistical analysis

Blood [Pb] was interpreted as previously described by van den Heever et al. (2019), with <10 µg/dL regarded as background exposure; 10–20 µg/dL as mild to moderate sub-clinical effects; 20–50 µg/dL representing moderate to severe sub-clinical effects; 50–100 µg/dL equivalent to overt clinical poisoning and >100 µg/dL representing severe clinical poisoning. All statistical analyses were carried out in the R 4.1.0 (R Core Team, 2021) environment, using R Studio 1.4.1717 (RStudio, Inc.). The mean, median, standard deviation (SD), range and 90% confidence interval were calculated for each parameter. Reference intervals were calculated as mean ± 2SD. Data were tested for normality using the Shapiro-Wilk test. Where required, $\log_{10}$ or square root transformations were employed to establish normality. Spearman rank correlation coefficients were used to test for significant relationships between blood [Pb] and PCV. Linear regression models were fitted to describe δ-ALAD activity as functions of blood [Pb] in both species. Because δ-ALAD activity had not previously been established for Cape or White-backed Vultures, the 100% δ-ALAD activity reference value was calculated by substituting zero blood lead in the regression equation. The blood [Pb] at which a 50% reduction in δ-ALAD activity is likely to occur was subsequently estimated by using 50% of this reference value. Means and standard deviations were calculated for all parameters classified according to the blood lead level groups. One-way ANOVA was used to determine any significant differences between groups. Where normality could not be established, the non-parametric Kruskal-Wallace test was used to test for significance. The level of significance was set at $p < 0.05$.

Throughout the discussion, results are compared to previous studies where baseline haematology and blood biochemistry parameters were evaluated in White-backed Vulture chicks (van Wyk et al., 1998) and captive and wild White-backed Vulture adults (Naidoo et al., 2008), respectively. Neither study evaluated lead toxicity as a possible confounding factor in the parameters measured. The latter is particularly significant in the case of van Wyk et al. (2001), which also focused on the breeding colony at Dronfield Nature Reserve. Since lead toxicity was shown to be prevalent in the colony by the same authors in a subsequent study (van Wyk et al., 2001), it is reasonable to assume that lead toxicity was present when the baseline parameters were established.

3. Results

Lead concentrations varied from values indicative of background to severe clinical poisoning in both Cape and White-backed Vulture nestlings. Eight (28%) Cape Vulture nestlings sampled had blood [Pb] exceeding background exposure, of which one had blood [Pb] >100 μg/dL (Table 1). No samples were below the limits of detection, i.e. 0.10 μg/dL. All White-backed Vulture nestlings sampled had blood [Pb] exceeding background exposure (i.e. >10 μg/dL), while 19% (n = 7) of the birds had blood [Pb] exceeding 100 μg/dL (Table 1). A significant negative relationship ($p < 0.0001$) was found between blood [Pb] and δ-ALAD activity in both species (Fig. 1), with 100% δ-ALAD activity calculated as 4057 nmol PBG/h/L RBC and 4444.3 nmol PBG/h/L RBC in Cape and White-backed Vultures, respectively. The regression equations predict a 50% inhibition in δ-ALAD activity at blood [Pb] > 18.8 μg/dL in Cape Vulture nestlings (log10 δ-ALAD = 3.608–0.016 [Pb]) and [Pb] > 52.8 μg/dL in White-backed Vulture nestlings ($\log_{10}$δ-ALAD = 3.648–0.006 [Pb]). No relationship was found between PCV and blood [Pb] in Cape ($r = 0.080$, $p = 0.687$) or White-backed ($r = 0.217$, $p = 0.217$) Vulture nestlings. Immature heterophils were present in 10 of 25 White-backed Vulture nestlings with blood [Pb] <50 μg/dL, of which six also had leukocytozoon parasites present. Immature heterophils were present in three of the White-backed Vulture chicks with blood [Pb] >100 μg/dL, despite the absence of any parasites. Immature heterophils were present in all 27 Cape Vulture nestlings with blood [Pb] <50 μg/dL, and in no birds with blood [Pb] >100 μg/dL. No parasites were present in any of the Cape Vulture nestlings. No significant differences were found between the leukocyte parameters for the different lead exposure groups in either species (Table 1).

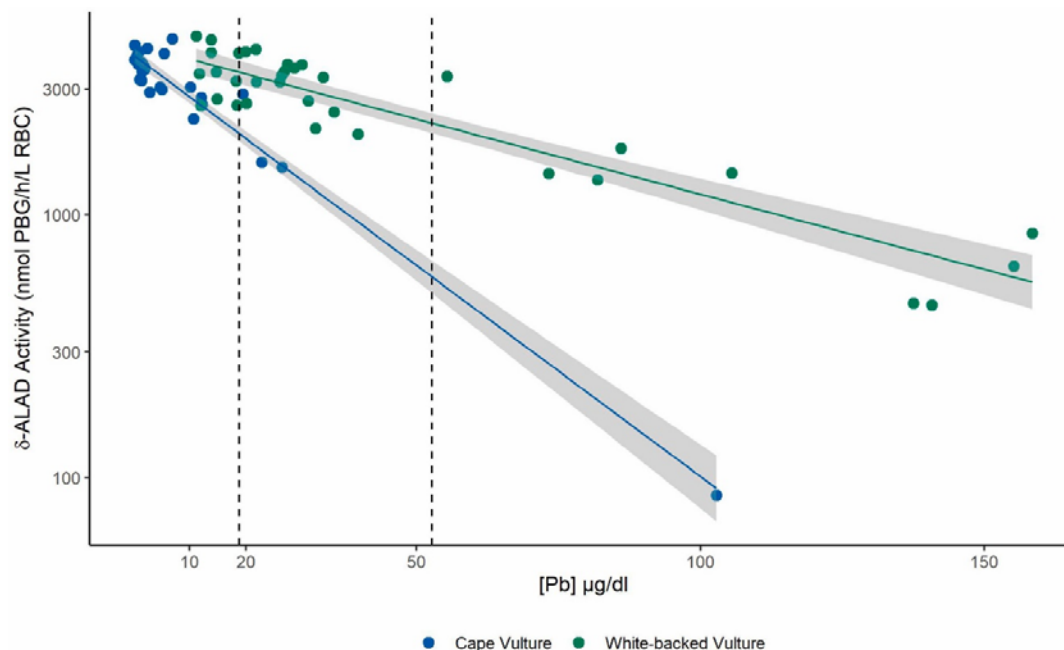

Fig. 1. Relationship between blood [Pb] and δ-ALAD activity in Cape ($r^2 = 0.96$, $p < 0.0001$, $\log_{10}$ALAD = 3.6082–0.0160 [Pb]) and White-backed ($r^2 = 0.85$, $p < 0.0001$, $\log_{10}$ALAD = 3.6478–0.0057 [Pb]) vulture nestlings. A 50% inhibition of enzyme activity is predicted to occur at blood [Pb] >18.8 μg/dL (Cape Vulture) and 52.8 μg/dL (White-backed Vulture). Grey shaded areas represent the 95% confidence intervals of the regression lines.

Table 1. Blood [Pb], PCV, δ-ALAD activity and leukocyte counts measured in Cape (*Gyps coprotheres*) and White-backed (*G. africanus*) Vulture nestlings, grouped by lead exposure level (µg/dL). No significant differences were found between the leukocyte parameters for the different lead exposure groups in either species.

Parameter	**Cape Vulture**					**White-backed Vulture**				
	Pb < 10	**10 ≤ Pb < 20**	**20 ≥ Pb < 50**	**Pb ≥ 100**	**P value**	**10 ≤ Pb < 20**	**20 ≤ Pb < 50**	**50 ≤ Pb < 100**	**Pb ≥ 100**	**P value**
n	20	5	2	1		11	14	4	5	
Pb (µg/dL)										
Mean ± SD	2.3 ± 1.9	12.9 ± 3.7	24.6	102.8		15.2 ± 3.1	28.4 ± 5.4	74.1 ± 13.6	146.5 ± 23.0	
Range	0.4–7.0	10.2–19.4	22.8–26.4	–		11.2–19.9	20.1–39.7	55.4–86.0	105.5–180.4	
δ-ALAD (nmol PBG/h/L RBC)										
Mean ± SD	3714.2 ± 473.8	2723.3 ± 278.0	1543.8	85.6		1809.4 ± 392.2	1564.4 ± 326.7	989.3 ± 466.5	383.4 ± 204.2	
Range	2913.1–4649.1	2306.3–3040.3	1509.0–1578.7	–		1292.6–2378.3	1007.9–2124.9	676.3–1674.9	225.9–718.5	
PCV (%)										
Mean ± SD	34.7 ± 3.9	35.2 ± 2.2	31.5	40.0		31.9 ± 3.5	33.4 ± 2.3	32.6 ± 3.2	34.0 ± 2.1	
Range	23.0–39.0	32.0–38.0	27.0–36.0	–		28.0–38.0	30.0–39.0	30.0–37.0	32.0–37.0	
Mean ± SD (10^3/µL)										
Total leukocytes	22.8 ± 5.8	24.5 ± 3.5	22.0	20.0	0.622	15.9 ± 8.6	14.9 ± 4.7	11.0 ± 1.0	12.7 ± 2.1	0.547
Heterophils	15.0 ± 4.1	15.2 ± 3.8	12.2	12.8	0.953	9.7 ± 6.3	9.1 ± 3.0	7.5 ± 1.2	6.8 ± 1.2	0.531
Immature heterophils	0.1 ± 0.2	0.2 ± 0.2	0.1	0.0	0.605	0.11 ± 0.13	0.14 ± 0.25	0.0 ± 0.0	0.12 ± 0.12	0.335
Lymphocytes	6.5 ± 2.5	7.5 ± 1.3	8.4 ± 2.9	5.6	0.351	4.1 ± 1.8	4.4 ± 1.6	2.9 ± 1.9	4.9 ± 1.2	0.298
Monocytes	0.8 ± 0.6	1.3 ± 0.6	0.8 ± 0.2	1.4	0.132	1.5 ± 1.1	0.9 ± 0.5	0.5 ± 0.4	0.7 ± 0.5	0.089
Eosinophils	0.2 ± 0.2	0.2 ± 0.1	0.1 ± 0.1	0.0	0.410	0.2 ± 0.2	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.398
Basophils	0.1 ± 0.2	0.0 ± 0.1	0.5 ± 0.7	0.2	0.497	0.2 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.1	0.634

Table 2. Selected blood biochemistry parameters measured in Cape and White-backed Vulture nestlings, grouped by lead exposure level (µg/dL). The mean U:UA (urea:uric acid) ratio of White-backed Vulture chicks with blood [Pb] > 100 µg/dL was significantly lower than conspecifics with blood [Pb] in all three other lead exposure groups.

Parameter	**Cape Vulture**					**White-backed Vulture**				
	Pb < 10	**10 ≤ Pb < 20**	**20 ≥ Pb < 50**	**Pb ≥ 100**	**P value**	**10 ≤ Pb < 20**	**20 ≤ Pb < 50**	**50 ≤ Pb < 100**	**Pb ≥ 100**	**P value**
n	20	5	2	1		11	15	4	7	
Mean ± SD										
Cholestrol (mmol/L)	5.1 ± 0.9	5.8 ± 0.8	4.7	5.2	0.469	4.6 ± 1.2	4.4 ± 0.9	4.2 ± 0.8	4.4 ± 1.0	0.914
Trig (mmol/L)	0.8 ± 0.2	1.2 ± 0.7	0.8	0.6	0.539	1.0 ± 0.5	0.8 ± 0.5	1.1 ± 0.4	0.8 ± 0.3	0.234
Total protein (g/L)	31.3 ± 3.1	32.0 ± 1.7	29.5	33.7	0.566	31.2 ± 2.2	31.8 ± 2.2	32.8 ± 1.9	30.8 ± 1.5	0.374
Albumin (g/L)	14.1 ± 1.4	14.5 ± 0.4	13.2	14.6	0.839	13.6 ± 1.0	13.9 ± 0.9	14.0 ± 0.4	13.7 ± 0.6	0.628
Globulin (g/L)	17.2 ± 2.0	17.6 ± 1.6	16.3	19.1	0.576	17.6 ± 1.5	18.0 ± 1.5	18.8 ± 1.7	17.1 ± 1.0	0.324
Albumin:Globulin	0.8 ± 0.1	0.8 ± 0.1	0.8	0.8	0.632	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.507
ALT (u/L)	24.2 ± 8.0	23.4 ± 3.2	37.6	31.7	0.243	27.8 ± 13.6	26.6 ± 7.9	32.5 ± 12.6	28.5 ± 5.5	0.644
GGT (u/L)	2.9 ± 2.0	3.0 ± 2.5	3.1	5.6	0.361	2.8 ± 1.6	2.5 ± 1.4	2.1 ± 1.5	1.0 ± 1.0	0.102
Urea (U) (mmol/L)	0.6 ± 0.1	0.7 ± 0.2	0.5	0.4	0.342	1.1 ± 0.6	0.8 ± 0.3	0.8 ± 0.5	0.6 ± 0.3	0.177
Uric acid (UA) (mmol/L)	0.3 ± 0.1	0.4 ± 0.1	0.3	0.3	0.204	0.4 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.845
U:UA	2.0 ± 0.6	1.8 ± 0.3	1.5	1.3	0.288	2.6 ± 1.3	2.0 ± 0.8	1.9 ± 0.9	1.3 ± 0.5	<0.05
Phosphorus (mmol/L)	2.2 ± 0.2	2.3 ± 0.2	2.1	2.3	0.922	2.1 ± 0.3	2.1 ± 0.2	2.0 ± 0.2	1.9 ± 0.3	0.127
Calcium (mmol/L)	2.4 ± 0.2	2.6 ± 0.1	2.3	2.5	0.324	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.2	0.769

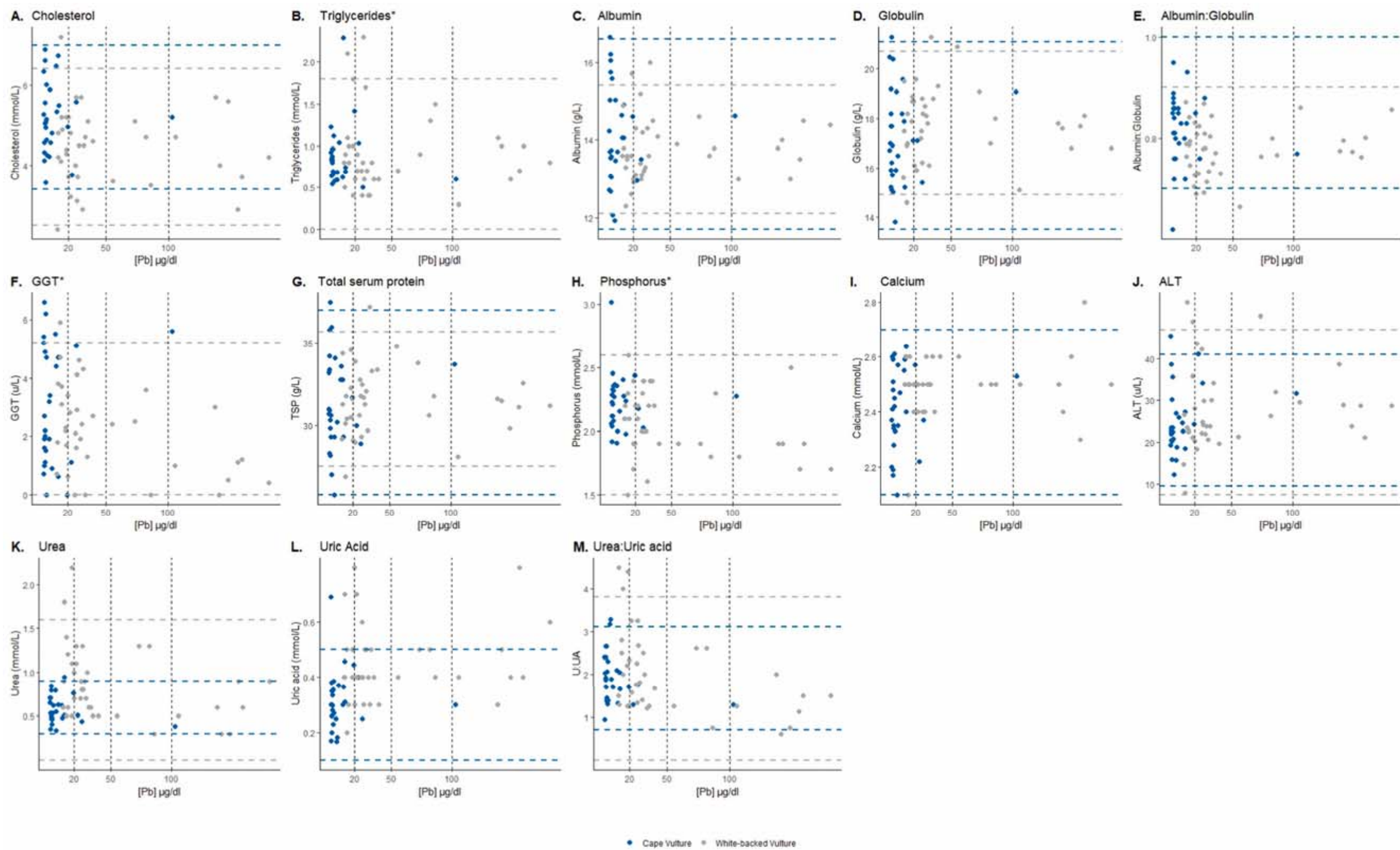

Fig. 2. Selected blood biochemistry parameters of Cape (n = 28, *Gyps coprotheres*) and White-backed (n = 37, *G. africanus*) vulture nestlings in relation to blood lead concentration. Where normality could be established, dashed lines represent the upper and lower limits of the reference interval, i.e. mean ± 2 x standard deviation.

Values for calcium, phosphorous, cholesterol, triglycerides, total serum protein, albumin, globulin, albumin/globulin ratio, alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT) were consistent with values previously reported (Table 2, Fig. 2). The mean U:UA (urea:uric acid) ratio of White-backed Vulture chicks with blood [Pb] > 100 µg/dL was significantly lower than conspecifics with blood [Pb] <100 µg/dL ($F (3,33) = 2.955$, $p < 0.05$, Table 2). Group means of the remaining biochemistry parameters did not vary significantly. No significant differences were found in Cape Vulture nestlings for any biochemistry parameters.

Descriptive statistics (including reference intervals from this and previous studies) are presented in Appendix A.

4. Discussion

The mean blood [Pb] of White-backed Vulture chicks at Dronfield Nature Reserve measured in the present study (27.3 µg/dL) was nearly double the corresponding value recorded in chicks from the same colony in 2016/2017 (14.4 g/dL, µvan den Heever et al., 2019). Concerningly, 19% (n = 7) of individuals sampled were found to have blood [Pb] >100 µg/dL, consistent with severe clinical poisoning and well above the maximum of 84.9 µg/dL previously recorded in the colony (van den Heever et al., 2019). Lead poisoning in the Cape Vulture nestlings sampled at Karnmelkspruit was less pronounced. Only 28% of chicks exceeded background exposure (>10 µg/dL). All but one of these individuals had blood [Pb] < 25 µg/dL. The lack of samples for birds with intermediate blood [Pb] (25–100 µg/dL) limited our ability to evaluate the effect of Pb on immune response and other biochemistry parameters at these levels., although Naidoo et al. (2012) suggested compromised reproductive potential at these intermediate levels based on effects in a captive breeding colony.

The significant negative correlation between blood [Pb] and δ-ALAD activity in Cape and White-backed Vulture chicks reveals increasingly compromised haem synthesis and erythropoiesis as total exposure to lead increases (Berglund et al., 2010). It is concerning that a significant proportion of Cape (14%, n = 4) and White-backed (26%, n = 9) Vulture nestlings displayed blood [Pb] above the species' respective thresholds for 50% inhibition of the δ-ALAD enzyme. Since δ-ALAD activity also remains depressed long after the initial lead exposure event (Dieter et al. 1978), the chicks may still suffer depressed haem production for their first couple of months as free-flying juveniles, even in the absence of subsequent lead exposure events. The resultant side-effects, such as anaemia, may further compromise the welfare of these birds during their first year of life, a period when raptors are particularly vulnerable to mortality (De Pascalis et al., 2020). However, the regression equation for Cape Vultures was strongly influenced by a single datum above 26.4 µg/dL, lowering the confidence for the blood lead level at which 50% inhibition of δ-ALAD activity occurs. Additional data at higher blood lead concentrations could alter the regression equation for δ-ALAD activity in Cape Vultures, highlighting the need for further research. Furthermore, although 50% reduction in δ-ALAD activity is considered the threshold beyond which severe physiological stress occurs (Finley et al., 1976), this is at best a broad estimate, given the intra-specific sensitivities to lead observed even in this study. When evaluating lead exposure in Cape and White-backed Vultures, it

would consequently be more prudent to consider a range of blood lead reference values, e.g. 10–20 µg/dL (Cape Vultures) and 50–60 µg/dL (White-backed Vultures).

Our results suggest a difference in lead tolerance between Cape and White-backed Vultures. Similar studies on other *Gyps* species are limited to the work of Espín et al. (2015), who observed a 90% inhibition in δ-ALAD activity at blood [Pb] >30 µg/dL in the Griffon Vulture (*Gyps fulvus*, Table 3), which is more in keeping with those observed in Cape Vultures in this study. The California Condor, considered to be highly sensitive to the impacts of lead toxicity, displays a 60% inhibition of the enzyme at blood [Pb] >20 µg/dL (Finkelstein et al., 2012). Although previous experimental studies support inter-specific differences in sensitivity to lead toxicity, the effects of lead can be impacted by a variety of factors (such as diet, sex and physical condition) that may influence absorption, retention and elimination of lead (Pain et al., 2019). Furthermore, comparison with δ-ALAD activity values measured in other studies could be problematic, as different methods and units are used to express δ-ALAD activity and % inhibition (Espín et al., 2015).

Table 3. Percentage δ -ALAD inhibition calculated in previous studies for raptor species.

Species	% Inhibition	Blood [Pb]	Reference
California Condor (Gymnogyps californianus)	≥60%	≥20 µg/dL	Finkelstein et al. (2012)
Eurasian Eagle Owl (*Bubo*)	55%	15 µg/dL	Gómez-Ramírez et al. (2010)
Osprey (Pandion haliaetus)	52%	9 µg/dL	Henny et al. (1991)
Bald Eagle (Haliaeetus leucocephalus)	80%	80 µg/dL	Hoffman et al. (1981)
Griffon Vulture (*Gyps fulvus*)	90%	30 µg/dL	Espín et al. (2015)

The mean PCV of 32.9% recorded in White-backed Vulture chicks falls below the means previously reported for chicks (41.8 ± 4.1%, van Wyk et al., 1998) and adults (42.0 ± 4.1%, Naidoo et al., 2008). Low PCV may be due to a lack of physical activity, as flight drives higher oxygen demand (Villegas et al., 2002), but this cannot explain the significantly higher PCV previously reported for the chicks at Dronfield by van Wyk et al. (1998). The low mean PCV, along with normal serum protein values, may be indicative of depression or haemolytic anaemia. Another noteworthy finding was the lack of correlation between PCV and blood [Pb], despite the existence of a significant negative correlation between blood [Pb] and δ-ALAD activity, as observed in other species (Pain, 1989; van der Merwe et al., 2011).

Increased susceptibility to infectious disease is thought to be one of the sublethal effects of lead poisoning in birds (Krone, 2018, Rocke and Samuel, 1991). Although mean total leukocyte counts in the present study were much lower than previously reported for White-backed Vulture nestlings, the range was similar to those previously reported for the species (van Wyk et al., 1998; Naidoo et al., 2008), Griffon Vultures (Polo et al., 1992) and raptors in general (Cooper, 2002) (Table A2). The leukogram for Cape Vulture nestlings is consistent with values previously reported (Table A1). A decline in circulating leukocytes was observed in lead-exposed waterbirds (Rocke and Samuel, 1991). Unlike the results reported for captive individuals by Naidoo et al. (2008), the leukogram of the White-backed Vulture chicks was characterised by the presence of immature heterophils in 40% (n = 10) of the birds with blood [Pb] <50 µg/dL, and in 43% (n = 3) of the birds with blood [Pb] >100 µg/dL. The presence of immature heterophils in birds with blood [Pb] >100 µg/dL, in the absence of leukocytozoons, could imply an inflammatory response to extreme lead toxicity. The absence of

leukocytozoons in all but one of the birds with blood [Pb] >50 µg/dL may imply that lead is adversely affecting pathogens, as suggested previously (Rocke and Samuel, 1991).

Values for cholesterol, triglycerides, total serum protein, albumin, globulin, albumin/globulin ratio, GGT and ALT were consistent with values previously reported (Tables A3 and A4). Although the U:UA ratio recorded in White-backed Vulture chicks fell within the range predicted by Naidoo et al. (2008), it decreased significantly at blood [Pb] >100 µg/dL. Changes in either urea or uric acid are best evaluated by the ratio of urea to uric acid (U:UA), with decreases usually indicative of renal damage (Naidoo et al., 2008). However, this decrease, caused by a lower production of urea without a corresponding decrease in uric acid (as observed here), is more consistent with hepatic injury (rather than renal damage), as has been observed in cockatoos (Harr, 2005). The latter however cannot be confirmed without pathological evaluation, which was not possible for the specific case. We are unaware of other data on U:UA at intermediate to high [Pb] for Cape Vulture chicks, although the one chick with blood [Pb] > 100 µg/dL displayed a U:UA ratio consistent with that recorded in White-backed Vulture chicks. Further research is necessary to determine if Cape Vulture chicks also display potential hepatic injury at blood [Pb] > 100 µg/dL.

5. Conclusion

The significant negative correlations between blood lead concentration and δ-ALAD activity in Cape and White-backed Vulture nestlings suggest δ-ALAD activity may serve as a sensitive biomarker in both species. Conclusions regarding the effect of lead poisoning on the health of Cape Vulture chicks were limited by the small number of samples with blood [Pb] > 25 µg/dL, which warrants further research. The difference in sensitivity to lead displayed by these two closely related members of the same genus, as well as the variation observed between individuals of the same species, highlights the need for species-specific investigations of the effect of Pb on δ-ALAD activity, and cautions against extrapolating lead-sensitivity data from one species to another. The presence of high lead levels in the nest-bound chicks of these highly threatened species is concerning. Lead's ability to disrupt haem synthesis and cause hepatic injury may compromise their ability to thrive as newly fledged juveniles. This is especially critical for young birds that need to be in optimal health to deal with the myriad of anthropogenic threats they may face during their first year of life.

Funding

The research was supported by the Isdell Family Foundation and the Mary Oppenheimer and Daughters Foundation.

Ethical approval

The research protocol described herein was approved by the BirdLife South Africa Animal Research Ethics Committee (protocol 2019/05/B), the Animal Ethics Committee of the University of Pretoria (protocol NAS215/2019) and the Research Ethics and Scientific Committee of the South African National Biodiversity Institute (SANBI, protocol P2020-21 A).

CRediT authorship contribution statement

Linda van den Heever: Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing. **Vinny Naidoo:** Methodology, Supervision, Writing - review & editing. **Theresa Coetzer:** Methodology. **Lauren Eyssen:** Methodology. **Jennie Hewlett:** Methodology. **Hanneline A. Smit-Robinson:** Supervision. **Andrew E. McKechnie:** Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Dr Barnett Rattner (US Geological Survey) for his assistance with the δ-ALAD methodology. We thank the De Beers Group for allowing us to collect samples from the White-backed Vulture breeding colony at Dronfield Nature Reserve, as well as Ian Cloete (owner of farm Karnmelkspruit) and René Feyth (owner of farm Nooitgedacht) for unrestricted access to their properties during sampling at the Karnmelkspruit Cape Vulture breeding colony. We thank Angus Anthony, Mark D. Anderson (BirdLife South Africa), Tania Anderson, Charles Hall (Dronfield Nature Reserve), Dr Stanley Harvey, Beryl Wilson (McGregor Museum), Julius Koen, Thierry Bouchet (Puy du Fou France), Heidi Fölscher (McGregor Museum), Andy Hinton (The Hawk Conservancy Trust), Graham Bessant (Gauntlet Bird of Prey, Eagle and Vulture Park), Melissa Groenewald (Airports Company South Africa) and many other volunteers for their invaluable assistance during sample collection at Dronfield Nature Reserve. A special word of thanks also goes to Craig Nattrass, Sunika Nattrass, Dr Caroline Howes, Neil Govender, Sarel Petrus, Dr Bronwyn Thomen and Kent Jennings for the collection of samples under challenging conditions at Karnmelkspruit.

Data availability

Data will be made available on request.

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