AMYLOIDOSIS IN CAPTIVE EUROPEAN EASTERN BONGO (*TRAGELAPHUS EURYCERUS ISAACI*): PREVALENCE, PREDICTIVE FACTORS, ORGAN PREDILECTION AND SERUM AMYLOID A CONCENTRATIONS

Hannah Rowland, BVM BVS (Hons), MRCVS, Richard Blundell, BVetMed, MSc, PhD, Dipl.ECVP-CFVP, FHEA, MRCVS, Julian Chantrey, BVM&S, PhD, FRCPath, Dip ECZM (ZHM), MRCVS, Katie L. Edwards, PhD, Andrew Moss, PhD, Mark F. Stidworthy, MA, VetMB, PhD, FRCPath, MRCVS, Janet Gilbertson, CSci, FIBMS, Philip N Hawkins, FMedSci, Jane Hopper, MA, VetMB, CertZooMed, MRCVS, Gabby J. Drake, BSc(hons), BVSc, Cert(Zoo Med), MSc, MRCVS, and Javier Lopez, Ldo Vet, MSc, Dip ECZM (ZHM), MRCVS

From Chester Zoo, Caughall Road, Chester, CH2 1LH, UK (Rowland, Edwards, Moss, Drake, Lopez); Institute of Veterinary Science, University of Liverpool, Chester High Road, Neston, CH64 7TE, UK (Blundell, Chantrey); International Zoo Veterinary Group, Station House, Parkwood Street, Keighley, BD21 4NQ, UK (Stidworthy); University College, London, NW3 2PF, UK (Gilbertson. Hawkins); The Aspinall Foundation, Port Lympne Reserve, Lympne, Kent, CT21 4PD, UK (Hopper).

Corresponding author: Hannah Rowland, h.rowland@chesterzoo.org

Abstract: Amyloidosis is frequently identified during post-mortem examination of captive eastern bongo (*Tragelaphus eurycerus isaaci*) in the European Endangered Species Programme (EEP). However, its significance and etiopathogenesis are poorly understood. The objective of this study was to investigate the prevalence of amyloidosis within this population and identify potential predictive factors for the presence of disease. Necropsy reports obtained from 24 EEP institutions were analyzed and assessed for evidence of amyloidosis. Seventy-two individuals had histopathological assessment performed after gross necropsy and were included in the study. Further histopathological analysis was performed on Congo red stained slides from 26 individuals and organ predilection sites identified. Immunohistochemical analysis was performed in six individuals to identify the type of amyloid present. Serum amyloid A (SAA) analysis was performed on blood samples from 34 individuals and concentrations in affected and unaffected individuals were compared. Amyloidosis was reported in 26 animals (36%). There was no statistically significant association between the presence of amyloidosis and sex, age, or body condition. However, amyloidosis was not identified in any individuals under the age of 6 years. The presence of chronic inflammatory conditions was the only statistically significant predictive factor for the presence of amyloidosis ($p=0.03$). Chronic inflammatory conditions present included nephritis, enteritis, and pneumonia. The majority of affected animals presented with amyloid deposition in multiple organs, with the liver and kidneys being most commonly affected. Immunohistochemistry confirmed the presence of AA amyloid. There was no statistically significant association between the presence of amyloidosis and SAA values measured on a single occasion. This study identified a high prevalence of amyloidosis within the captive European eastern bongo population, associated with chronic inflammatory conditions. Ante-mortem diagnosis of amyloidosis remains challenging, and this study indicates that SAA protein concentrations are not a reliable indicator for the presence of amyloidosis.

INTRODUCTION

The Eastern bongo (*Tragelaphus eurycerus isaaci*) is a critically endangered bovid species endemic to the forested mountain zones of Kenya. The wild population has undergone a severe decline, with only an estimated 70-80 individuals remaining in as few as 2-5 isolated populations.[14] The species is held widely in zoological collections and the European population, managed by the European Endangered Species Programme (EEP), currently contains 167 individuals in 51 institutions. Due to the continued decline of the wild population, the captive population is becoming increasingly important as an ex-situ 'safety net' as well as a source of genetic diversity.[15] Consequently, it is important to identify and understand health issues that may threaten the viability of this population.

In 2006, a case of generalized AA amyloidosis was reported in an eastern bongo in the UK,[31] and later, results from an EEP survey in 2014 indicated that this may be a significant condition within the population (Gilbert, European Endangered Species Programme bongo health and veterinary care questionnaire, 2014).In a North American eastern bongo mortality study, it was identified as one of the most common chronic conditions present post-mortem.[3] Amyloidosis results from the abnormal folding of proteins into highly stable β-pleated sheets which are then deposited as amyloid fibrils in extracellular tissues.[17] Over 20 different proteins can be involved, including immunoglobulin light chains, transporter proteins, and acute phase proteins. The most common form of amyloidosis in domestic animals is AA amyloidosis[32] which results from the misfolding of serum amyloid A (SAA), an acute phase protein produced by the liver. Sustained or periodic elevations of SAA, typically as a result of chronic inflammation, result in amyloid formation and amyloidosis in some individuals.[17] A genetic component has been identified in some domestic species, in which familial amyloidosis results in AA amyloid deposition.[6,24] A mortality study of captive bongos in North America reported 32% of individuals to be

affected by amyloidosis,[3] making it a potentially significant health issue for the population as a whole. Identifying the prevalence of this condition within the EEP is required to understand the relevance of this condition. Determining risk factors for the development of disease, and identifying a method for ante-mortem diagnosis will allow for improved management of this condition within the population.

This study aimed to assess the prevalence of amyloidosis in the European captive eastern bongo population as well as characterize the condition in this species by assessing the organs affected, and severity of the disease. Furthermore, it aimed to assess SAA protein levels as a predictor for the presence of amyloidosis in captive bongos in the European population.

MATERIALS AND METHODS

Pathology reports, stored serum, and histopathology blocks were requested from all collections participating in the Eastern Bongo EEP for individuals that died during the period 1st January 2009 to 31st July 2021.

Retrospective pathology review

Post-mortem reports were assessed for completeness. Individuals were excluded from the study if histopathological examination was not performed. Cases where less than four organs were assessed and no amyloidosis was identified were also excluded as the presence of amyloidosis could not be confidently ruled out. Individuals in which amyloid deposition was identified in any organ were considered amyloidosis positive and used in the prevalence calculations. Information relating to the individual's sex, age, body condition, institution at death, weight at death, presence of chronic inflammatory conditions, and evidence of

amyloidosis, as well as other gross and histopathological findings were recorded from the clinical information provided.

Histopathological analysis

All formalin-fixed paraffin embedded histopathology blocks that were available were processed for examination to identify organ predilection sites and severity of disease. All blocks were microtome cut to 6µm before being mounted onto glass slides. Sections were stained with hematoxylin and eosin (H&E) and Congo red before examination. The examination was performed blind by a board-certified veterinary pathologist. The presence of amyloidosis was identified and the severity of amyloid deposition was assessed using a scale of zero to five (0-negative, 1-mild, 2-mild to moderate, 3-moderate, 4-moderate to severe, 5-severe) based on the comparative assessment of the amount of Congo red staining present in the tissue (Figure 1). The levels of amyloid deposition varied between organs, therefore, the amyloidosis severity for an individual was determined using an average severity grade (sum of severity scores of affected organs divided by the number of organs affected). In cases where amyloidosis was present in individuals previously classed as negative from the post-mortem report, the amyloidosis status was updated to positive.

Immunohistochemistry staining was performed on 2µm formalin fixed deparaffinised tissue sections using an in-house polyclonal antibody against SAA protein, on a manual platform and Impress™ detection kits, followed by a metal enhanced DAB substrate kit for visualizing the immuno compound to determine the amyloid fibril type. For all staining, positive and negative controls were used in parallel.

Serum analysis

Individuals for which there was an available serum sample, collected within the six months prior to death, were included for SAA analysis. A single sample was assessed from

each individual. Blood samples were collected by the holding institution and allowed to clot for at least one hour before centrifugation for serum collection. Serum was stored at -20°C before transportation and all samples were transported with cold packaging to prevent thawing. Upon arrival, samples were stored at -80°C until analyzed at Chester Zoo's Wildlife Endocrinology laboratory.

SAA concentrations were analyzed by enzyme immunoassay using a commercially available sandwich ELISA assay (phase multispecies SAA ELISA kit, Tridelta Development Ltd, Kildare, Ireland) according to manufacturer instructions. Serum samples were initially diluted at 1:500 with calibrator diluent; any serum samples exceeding the highest calibrator were further diluted (up to 1:100,000) until they were within range of the standard curve. SAA concentrations are expressed as bovine SAA equivalent (mg/l). The assay was validated for this species via linearity (96.4%) and recovery (105.2%) assessment within the range of dilutions used, and the inter-assay coefficient of variation for a control pool run in every assay (n=3) was 10%.

Inbreeding coefficient (F) and mean kinship values

To investigate the potential heritability of amyloidosis in this species, inbreeding coefficient (F) and mean kinship analysis were performed. The inbreeding coefficient or F is the probability that two alleles present at a given locus in an animal are identical by descent. It measures how inbred an animal is. The mean kinship of an individual is the average kinship coefficient between that animal and all the animals in the population, including itself. The kinship coefficient is defined as the the probability that any two alleles from two individuals are identical by descent.[13] Mean kinship measures the relatedness of an animal to the population. F numbers and mean kinship values were available from the EEP studbook and were collated and compared between individuals with and without amyloidosis.

Mean kinship values can be affected by the number of descendants within the population. Fewer descendants can falsely lower the mean kinship value of an individual. To investigate the effect of the number of descendants on the mean kinship values of the study population, data on number of descendants was collated and compared between individuals with and without amyloidosis.

Statistical analysis

Sex, age, body condition, and the presence of chronic inflammatory conditions were assessed as potential predictive factors for the presence of amyloidosis. Age was analyzed as a continuous variable. Body condition data was divided into two categories; good/fair/moderate and emaciated/poor/thin. Descriptive statistics were calculated for these variables, including mean, standard deviation, and median for continuous variables and frequency and percent for categorical variables. A binomial logistic regression model was performed to investigate the significance of these potential predictive factors.

To evaluate the histopathological findings, descriptive statistics were performed to compare severity of disease with an individual's age, as well as the number of organs affected. The number of organs affected were also compared with an individual's age. Due to the small sample size, further statistical analysis was not performed.

The non-parametric Kruskal-Wallis test was used to investigate any differences in SAA concentrations in individuals with and without amyloidosis. The Kruskal-Wallis test was also used to compare differences in SAA between individuals that presented with and without any inflammatory disease as well as comparing between individuals with acute, chronic and both acute and chronic inflammatory disease processes.

A paired t-test was used to compare F and mean kinship values between amyloidosis positive and negative individuals. The number of descendants was not normally distributed,

therefore, a Mann-Whitney test was used to compare the number of descendants between amyloidosis positive and negative individuals. All analyses were conducted in the Jamovi statistical programme (www.jamovi.org) with statistical significance set at 0.05.

RESULTS

Retrospective pathology review

Seventy two of the 86 reports received met the inclusion criteria, including 24 males (33%) and 48 females (67%) which died between the ages of six months and 21 years. Twenty six individuals (36%) were identified as amyloidosis positive, of which 12 were male and 14 female. A higher percentage of males (50%) than females (29%) were reported as positive, however, there was no statistically significant association between sex and the presence of amyloidosis. A diagnosis of amyloidosis was only reported in individuals between the ages of six and 18 years, with no amyloidosis observed in younger animals (between six months and six years of age). There was no statistical difference between the age of affected (11.65 +/-3.02yr) and unaffected animals (9.66 +/- 5.31yr).

Body condition data was only available for 57 individuals. Twenty one of these were positive for amyloidosis, of which, 76% (n = 16) were recorded as poor, thin or emaciated at necropsy. The percentage of cases recorded as poor, thin, or emaciated was 56% (n = 20) in unaffected animals, but again, there was no statistical significant correlation between low body condition (poor, thin, or emaciated at necropsy) and presence or absence of amyloidosis.

Forty four (61%) individuals in the study presented evidence of one or more ongoing chronic inflammatory conditions at necropsy. These included nephritis (31/44), enteritis (7/44), pneumonia (9/44), abomasitis (5/44), rumenitis (3/44), hepatitis (2/44), colitis (2/44),

endometritis (1/44), and lymphadenitis (1/44). There was a statistically significant association between the presence of a chronic inflammatory condition and the presence of amyloidosis (p=0.03), with 22 (84.6%) of the 26 individuals diagnosed with amyloidosis presenting one or more concurrent chronic inflammatory conditions compared to 22 (47.8%) of the 46 non-affected cases.

Histopathological analysis

Of the 72 individuals included in the study, tissues from 15 individuals reported as positive for amyloidosis and 11 individuals reported as negative, were available for histopathological analysis. All tissues available from these animals (n=26) were examined which varied from five to 17 per individual and with the liver and/or kidney being available in all individuals. Following Congo red histological assessment, amyloidosis was confirmed in all positive cases but was also identified in a further three individuals for a total of 18 positive cases. In these instances, amyloidosis severity was limited to mild or moderate.

No association between age and amyloidosis severity or number of organs affected and amyloidosis severity could be identified. However, a pattern of association between increasing age and decreasing number of organs affected was apparent. Due to the small sample size, statistical testing was not performed to confirm these.

The organs most commonly affected by amyloidosis were the liver (14/22, 63.6%), kidney (14/24, 58.3%), and small intestine (12/23, 52.2%) respectively (Table 1). The liver and/or kidneys were affected in 17/18 individuals. Where multiple organs were affected, the liver and/or kidney were the most severely affected in 80% of individuals. Amyloid deposition in the liver was evident multifocally within the sinusoids. Where the small intestine was affected, amyloid was present within the lamina propria. In all cases of renal

amyloidosis, amyloid deposition was concentrated within the medullary interstitial tissue. Perivascular deposition was less frequent but was noted in all three of these organs.

Imuunohistochemistry (IHC) was performed on tissues from six of the 18 amyloidosis positive individuals (33%) for which blocks were available. These tissues had been previously confirmed amyloid positive using Congo red staining. IHC confirmed the presence of AA amyloid in all cases.

Serum analysis

Serum samples were available from 34 animals of which 14 had a diagnosis of amyloidosis in the necropsy report. Serum SAA values ranged from 99.1 - 7980 mg/l (953 +/- 2050) in amyloidosis positive individuals and from 12.8 – 10754 mg/l (487 +/- 3343) in negative individuals, with no statistically significant difference between the two groups. Average SAA concentrations were higher in cases suffering from acute inflammatory conditions but there was no statistically significant difference in SAA concentrations between individuals with and without the presence of inflammatory disease or between individuals with the presence of acute inflammatory disease, chronic inflammatory disease or both acute and chronic inflammatory disease processes.

F number and mean kinship values

F number and mean kinship values were available for 67 individuals. 24 of these were amyloidosis positive and 43 amyloidosis negative. The F number was not significantly different between amyloidosis positive and negative individuals. However, the mean kinship values of amyloidosis positive animals was significantly lower than amyloidosis negative individuals ($p=0.03$). The number of descendants produced by the amyloidosis positive individuals was significantly higher than the amyloidosis negative individuals.

DISCUSSION

In this study, a high prevalence of amyloidosis at post-mortem examination (36%) was observed in the eastern bongo EEP population, held in captivity in zoological collections in Europe, highlighting the importance of this disease in the species. The observed prevalence of amyloidosis post-mortem is similar to that reported in the North American captive population where amyloidosis was identified in 32% of animals necropsied at a diagnostic institution.[3] The prevalences observed in eastern bongos are much higher than those reported in similar multi-institutional studies in domestic cattle (6%),[9] and greater kudu ((*Tragelaphus strepsiceros*) (0.8%).[18] Although a higher prevalence has been reported in dorcas gazelles (*Gazella dorcas*) (53%)[27] and pronghorn antelope (*Antilocapra Americana*) (77%),[20] these studies were performed in small populations housed at single institutions. In these instances, the effects of management may have led to figures being magnified so resulting in bias and a seemingly more profound effect on prevalence. Due to the small sample size and fragmentation of the European eastern bongo population, the effect of management practices at different institutions could not be assessed and further investigation is required with a larger sample size.

The majority (75%) of amyloidosis positive individuals in this study had evidence of a reduction in body condition (poor, thin, or emaciated), however, there was no significant association between this and the presence of amyloidosis. This is unusual as body condition loss has been reported in domestic cattle with amyloidosis.[16]Clinical signs of amyloidosis in all species can be highly variable and dependent on the organs affected, lesion severity and the presence of underlying diseases.[32] Amyloidosis may be suspected in bongo with poor body condition. However, other chronic diseases may contribute to poor condition and some amyloid affected bongo show normal body indices.

In this study, amyloidosis was only observed in animals above 6 years of age, which suggests that this is a disease of older animals. This is consistent with findings in humans,

and domestic cattle, as well as bongos in the North American population, where bongos that died with amyloidosis were significantly older than those that died without amyloidosis.[2,3,34] However, there was no statistically significant association between the presence of amyloidosis and age at death in our study. The reasons for these differences are not readily apparent. It is possible that age plays a role in the development of amyloidosis, but with the presence of other chronic conditions in older animals, and 87% of the study population over six years of age, the sample size in this study may not be sufficiently large enough to confirm this.

An association between amyloidosis and chronic inflammatory conditions was identified in this study. This is a typical presentation in other species,[9,25,33] in which chronic inflammation is the most common cause of amyloidosis and the result of AA amyloid deposition following elevations of SAA during inflammation. AA amyloidosis results in systemic disease and has been reported in other exotic ungulate species including Arabian and mountain gazelle (*Gazella gazelle sp.*),[10,19] beira antelope (*Dorcatragus megalotis*),[13] and pronghorn antelope (*Antilocapra americana*).[20] Immunohistochemistry performed in this study confirmed the presence of AA amyloidosis in all tissues evaluated suggesting this is the typical form present in eastern bongo.

Chronic interstitial nephritis (CIN) was the most common inflammatory condition identified, present in 38% of individuals. Interstitial nephritis is a non-specific inflammatory disease and can be the result of multiple aetiological agents including metabolic disease, drug hypersensitivity, autoimmune disorders, toxins, and infection.[23] As the most frequently diagnosed inflammatory condition in eastern bongo, identifying the cause of CIN is imperative. Leptospirosis has been identified as a major cause of interstitial nephritis in cattle.[29,35] A seroprevalence of 15% has been identified within European cattle but the prevalence of leptospirosis in captive exotic ungulates is currently unknown.[4] No

leptospirosis diagnostics were performed in this study, however, investigating leptospirosis as a potential aetiological agent is of high importance. The ability to prevent and manage this condition may also result in a decline in amyloidosis cases and improve the health of the captive population.

Based on the significantly lower mean kinship values found in this study, a genetic component to amyloidosis is unlikely. These low mean kinship values indicate a low degree of relatedness to the current population. Poor reproduction can also result in lower values, however, this was ruled out as amyloidosis positive animals had a significantly larger number of descendants. Although no genetic component could be identified, further genetic studies would be required to confirm these findings.

Where organs were examined with Congo red, the liver, kidneys, and small intestine were most commonly affected. This organ predilection is consistent with findings in the American eastern bongo population,[3] and also in domestic cattle.[34] The mechanisms that govern the target tissue sites for the deposition of amyloid in different species are currently unknown.[21,26] This precludes the development of measures to prevent amyloid deposition in particular tissues. However, understanding predilection sites in this species will allow further investigation into the clinical implications of deposition at these sites and the development of potential supportive measures.

The distribution of amyloid within the liver and small intestine was consistent with the distribution sites within these organs identified in domestic cattle.[22] In affected kidneys, amyloid deposition was localized in the renal medulla. This pattern of deposition has previously been reported in felid species[5,25,28] and Dorcas gazelle[27] leading to compression atrophy to the surrounding collecting ducts and blood vessels, and ultimately progressing to renal failure. However, in domestic cattle, medullary amyloidosis is less common and is

typically associated with subclinical renal disease.[14] The clinical implications of renal medullary amyloidosis were not investigated in this study but some of the cases were classified as severe, therefore, clinicians should be aware of the potential for medullary amyloidosis to cause clinical renal disease.

A number of cases of amyloidosis in this study were only detected upon Congo red staining. Based on these findings, performing Congo red as well as routine H&E stains during post-mortem histopathological assessment of eastern bongo is advisable. As the liver and kidneys were amyloid positive on Congo red examination in 94% of individuals and were the most severely affected organs in 80% of systemic amyloidosis cases, where resources are limited, focussing Congo red examination on the liver and kidneys may be a practical alternative. However, it is important to be aware that some cases may be missed by limiting assessment to just these two organs.

A single measurement of SAA concentrations up to 6 months prior to death did not correlate with the presence of amyloidosis in the animals in this study. This unfortunately precludes the use of SAA as an ante-mortem test for amyloidosis. As an acute phase protein, SAA levels can increase up to 100-fold in the presence of acute and chronic inflammation, infection, and tissue damage[30] as well as in the presence of physical stress.[1] Many of the blood samples analyzed in this study will have been taken from medically unwell animals following general anesthesia with the concomitant stresses this produces, also potentially affecting SAA levels. In domestic cattle affected by amyloidosis, SAA levels were higher than in control animals, but not different to individuals with inflammatory disease.[9] It is likely that the lack of correlation of SAA with the presence of amyloidosis in this study was due to the confounding interaction of concurrent inflammatory conditions and physical stress.

Little is known about the SAA response in eastern bongo and notable species variation in acute phase protein response to inflammatory disease has been reported.[7,8] Further research looking at serial SAA values or the effect of acute physical stress from samples obtained under GA versus samples obtained via behavioral restraint is needed to increase understanding of the SAA response in eastern bongo and whether it could be a valid predictor for amyloidosis. However, based on these results, at present single sample SAA assessment is not a practical or useful diagnostic tool for amyloidosis in this captive population.

As with any retrospective study, there were variable levels of participation and data availability from institutions. Inconsistent necropsy protocols and sample collection were the main limiting factors in this study. Incomplete reports, including the variable use of special stains, resulted in inherent bias, which may have resulted in a degree of over or underestimation of the prevalence of disease in the population. Not all organs were available for histopathological examination due to inconsistent sampling and variable tissue preservation which affected the ability to identify the exact organs affected and may have resulted in bias in organ prevalence assessment. The use of a full and consistent necropsy protocol would have precluded these deficiencies and allowed for a more complete disease evaluation.

This study demonstrated a high prevalence of amyloidosis within the captive European eastern bongo population. An association between amyloidosis and chronic inflammatory conditions was also identified. It is anticipated that the clinical effects of amyloidosis superimposed over pre-existing inflammatory conditions would worsen the prognosis for treatment and long-term survival. Further genetic research is required along with research into the underlying primary causes of chronic inflammation in the eastern bongo population so measures can be taken to prevent and manage this condition. Ante-

mortem diagnosis of amyloidosis remains challenging, and our results indicate that SAA levels are not a reliable indicator for amyloidosis.

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Table 1: Prevalence of amyloidosis within organs of eastern bongo (*Tragelaphus eurycerus isaaci*) identified using Congo red analysis.

Organ	Number available for analysis	Amyloidosis present
Liver	22	14 (63.6%)
Kidney	24	14 (58.3%)
Rumen	13	6 (46.2%)
Omasum	4	0
Abomasum	15	2 (13.3%)
Small intestine	23	12 (52.2%)
Large intestine	9	1 (11.1%)
Pancreas	5	0
Spleen	17	1 (5.9%)
Heart	19	1 (5.3%)
Lung	23	2 (8.7%)
Lymph node	11	2 (18.2%)
Reproductive tract	7	0
Adrenal gland	7	3 (42.9%)
Thyroid gland	8	0
Brain	4	0
Spinal cord	1	0
Skeletal muscle	3	0
Peripheral muscle	1	0

Figure 1: Congo red stained histological sections of renal tissue from eastern bongo (*Tragelaphus eurycerus isaaci*) demonstrating an amyloidosis severity scoring system depicting **A.** mild **B.** mild to moderate **C.** moderate **D.** moderate to severe and **E.** severe cases (x200)

Figure 2: Immunohistochemistry stained histological section of renal tissue from an eastern bongo (*Tragelaphus eurycerus isaaci*) with brown staining (*) indicating the presence of AA amyloid (x1000)