Joint Pathology Center
Veterinary Pathology Services

WEDNESDAY SLIDE CONFERENCE 2018-2019

C o n f e r e n c e 8

24 October 2018

CASE I: Case 1 6981 R23 (JPC 41078788-00).

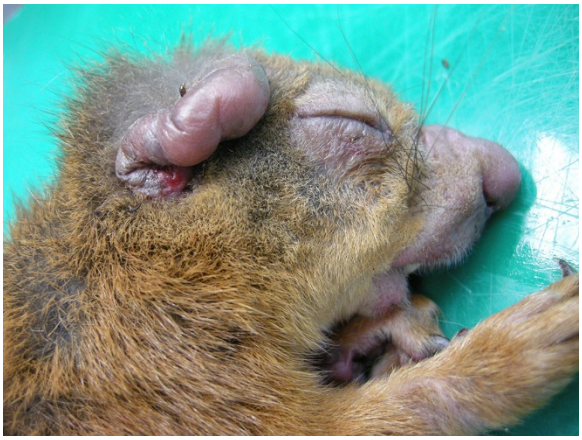
Signalment: Adult, female, N/A, *Sciurus vulgaris*, red Eurasian squirrel

History: A frozen, adult red squirrel was presented for postmortem examination as part of a surveillance scheme aiming to understand the causes behind the decline in red squirrel populations in the UK.[4]

Gross Pathology: This squirrel had bilateral areas of alopecia and cutaneous swelling at the snout, lips, eyelids, pinna and the ventral and distal aspect of all limbs (figure 1). Internally, the thoracic cavity was filled by a moderate amount of pale pink, opaque fluid (chylothorax).

Laboratory results: Hsp65 PCR followed by sequencing[6], and ulterior whole genome sequence work[1] were positive for *Mycobacterium lepromatosis*.

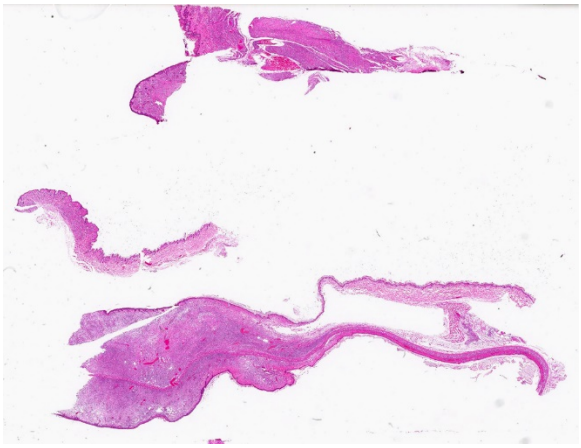
Microscopic Description: Description of the pinna only: At the tip of the pinna, extending from the aural cartilage to the superficial dermis, there is severe, focally extensive expansion by intercellular spacing (oedema)



Pinna, squirrel. The squired had bilateral areas of alopecia and swelling on the snout, lips, eyelids, and pinnae. (Photo courtesy of: Easter Bush Pathology, College of Medicine and Veterinary Medicine, University of Edinburgh, UK (http://www.ed.ac.uk/vet/services/easter-bush-pathology).

and severe infiltration by inflammatory cells. The latter are mostly foamy macrophages, with fewer lymphocytes and plasma cells and rare multinucleated giant cells. Scattered throughout this area, there are spindle shaped cells (fibroblasts – fibrosis, suspected) and accumulation of loose eosinophilic fibrillary material (collagen). Within this there are clear spaces containing 50-150µm wide, loose aggregates of pale basophilic cotton-like material. In the superficial dermis, all the hair follicles are small to non-existent (follicular atrophy, suspected). The overlaying epidermis is approximately 5 cells thick (mild, focally extensive, epidermal hyperplasia), and has a thick stratum corneum composed of several, parallel layers of keratin (lamellar hyperkeratosis).

In areas of skin adjacent to the above described lesion (at the base of the pinna), the superficial dermis is diffusely infiltrated by small numbers of lymphocytes and fewer eosinophils. In the panniculus and subcutaneous muscle associated, there is mostly perivascular infiltration by lymphocytes, with very rare mast cells.



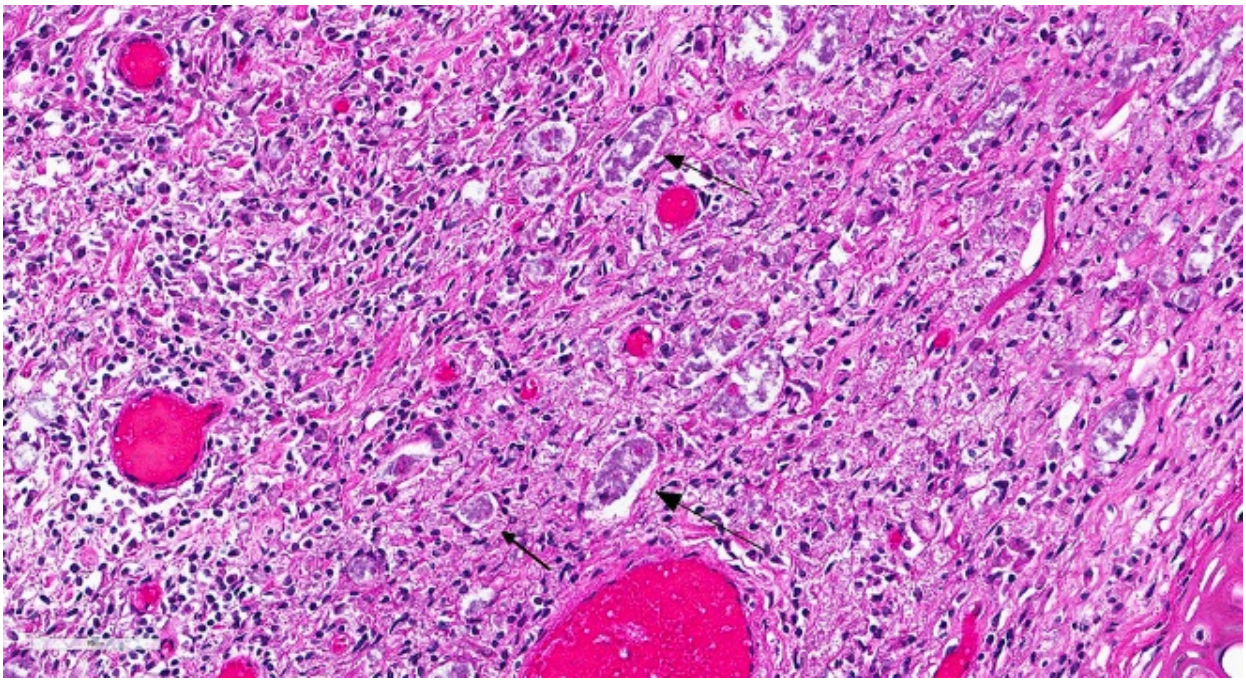
Pinna, squirrel. A section of haired skin and a cross-section of the pinna is submitted for examination. The dermis of both is markedly expanded by a dermal infiltrate that causes marked thickening of the pinna. (HE, 5X)

Contributor's Morphologic Diagnoses: Severe, focally extensive, granulomatous dermatitis with fibrosis, superficial oedema, epidermal hyperkeratosis and basophilic bacteria – haired skin, pinna

Contributor's Comment: This is the first red squirrel that was diagnosed with squirrel leprosy a novel disease of red squirrels that has only been detected in the UK so far.[7].

In this case, the diagnosis of mycobacterial disease was first confirmed by Ziehl-Neelsen staining, which revealed intrahistiocytic and intraneural acid-fast bacteria. The role of the gram-positive filamentous bacteria noted within the pinnal lesions was interpreted at first either as a co-infection, or as a secondary infection. Since this first diagnosis, we have recorded the presence of filamentous bacteria in some -but not all- further cases of squirrel leprosy, frequently associated with ulceration of the lepromatous lesions. This suggests that the primary etiological agent are the acid-fast bacteria. Work on the characterization of this sporadic association, as well as the taxonomical identity of these filamentous bacteria is pending.

Following this diagnosis, further sporadic red squirrel cases with similar gross lesions were submitted for examination from diverse areas of Scotland, and we were able to collect six cases from 2006 to 2013. Three of these cases were lost to follow up due to freezer break down, and the gross and histological examination was followed by PCR analyses for the three remaining squirrels (including this case). Briefly, fresh and fixed samples from these were analyzed by PCR for the detection of IS900 and F57 (specific for *Mycobacterium avium* subspecies *paratuberculosis* [MAP]), IS901 (specific for MAP), and the gene encoding heat shock protein 65 (hsp65=vgg – present in all *Mycobacterium* spp). Only hsp65 yielded



Pinna, squirrel. The infiltrate is composed of numerous epithelial macrophages with vacuolated cytoplasm, admixed with fewer lymphocytes and plasma cells. Some macrophages are up to 60um in diameter with stacks of greyish filamentous bacilli and cellular debris in their cytoplasm (lepra cells) (arrows). (HE, 320X)

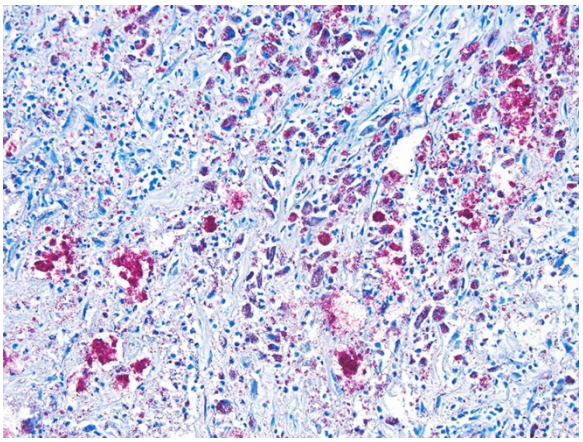
positive results, and of the amplicons revealed 99% sequence homology with *Mycobacterium lepromatosis* (FJ924). This was an unexpected finding, as *M. lepromatosis* had only been reported in human leprosy patients in Mexico from 2008,[3] and since then in other human cases from additional locations (e.g. Costa Rica and Singapore).[2] There are no human cases of *M. lepromatosis* reported in the United Kingdom, or any report of *M. lepromatosis* in other non-human mammal worldwide.

The first report of human leprosy by *M. lepromatosis* in 2008 has expanded the range of etiological agents of human leprosy to two bacterial species (previously only *Mycobacterium leprae* was known to cause human leprosy). Human infection with *M. lepromatosis* is associated with diffuse leprosy, a severe form of leprosy with spreading lesions containing foamy macrophages laden with large numbers of intracytoplasmic acid fast bacteria. The histological presentation we saw in these squirrels is consistent with lepromatous leprosy. The spectrum of human leprosy lesions as described for *Mycobacterium leprae* is summarized in figure 6. At the extremes of this spectrum *M. leprae* causes two distinct histological patterns[5]:

- *Tuberculoid leprosy*, with dry, insensitive, scaly cutaneous lesions, which histologically feature asymmetric involvement of large peripheral nerves, which are enclosed within granulomas containing low numbers of intracellular acid fast bacilli (paucibacillary). This is associated with a Th1 response, with production of IL-2 and IFN-γ.
- *Lepromatous leprosy*, with symmetric skin thickening and nodules in distal areas (earlobes, feet). This presentation involves widespread nervous invasion of nerves by Mycobacteria, which are found within Schwann cells, endoneural and perineural macrophages with minimal

inflammation. The lesions are composed of large, dermal aggregates of lipid-laden macrophages containing large numbers of acid-fast bacteria (multibacillary). Other organs that may be involved are regional lymph nodes, spleen, liver and testicles. This presentation is also known as anergic leprosy, because of immune unresponsiveness, and features a weak Th1 response and relative increase in Th2 response.

The spectrum contain intermediate categories between the two poles, and is described by the Ridley-Joplin classification scheme.[7] This scheme also includes early indeterminate leprosy (IL – usually early lesions that may evolve to one or another pole). The entire scale includes polar tuberculoid leprosy (TT), borderline tuberculoid leprosy (BT), mid-borderline leprosy (BB), borderline lepromatous leprosy (BL), and polar lepromatous leprosy (LL). A further layer of information is provided by the determination of the number of acid-fast bacilli present in the lesions, which is expressed on a 1 to 6 score based on a logarithmic scale called the bacteriologic index (BI). At the extremes are score 1= 1-10bacilli in 100 oil immersion fields (paucibacillary) to score 6= ≥1000 bacilli in 1 oil immersion field (multibacillary).



Pinna, squirrel. Epithelioid macrophages and lepra cells contain numerous intracytoplasmic acid-fast bacilli. (Fite-Furaco, 200X)

An important diagnostic feature of leprosy, is the presence of neural lesions, as noted in the red squirrel cases. Intraneural acid fast bacteria are a diagnostic feature, and these may be associated with neuritis or perineuritis.[5] Neural lesions are present throughout the spectrum of leprosy lesions, and lead to skin anaesthesia and muscular atrophy that render affected areas susceptible to trauma-associated lesions. It is possible to hypothesize that the filamentous bacteria noted in red squirrels may have entered the skin lesion after trauma, as their presence is associated with ulceration.

Other patterns may be associated with human leprosy, which have not been reported in red squirrels. The Type I reaction, or reversal reaction, involves an increase in cell-mediated immunity in lesions at the borderline part of the spectrum. This leads to progression towards the tuberculoid pole, but also to reactivation of the lesions. This reactivation in results in pain, the appearance of new lesions, erythema/oedema of old lesions, and nerve tenderness and swelling. The type II reaction, or erythema nodosum, is a type III hypersensitivity that occurs on patients with lepromatoid leprosy. These patients develop neutrophilic infiltration of the lesions, oedema, vasculitis/panniculitis, and visceral manifestations.

Raised awareness as a result of the work on Scottish squirrels infected with *M. lepromatosis* led to the detection of a cluster of similar cases in Brownsea Island, a small island off the south coast of England. Strikingly, sampling and PCR analysis of these cases revealed infection of 25 red squirrels with *M. leprae*[1]. This expands the range of possible *M. leprae* hosts previously, which were humans and nine-banded

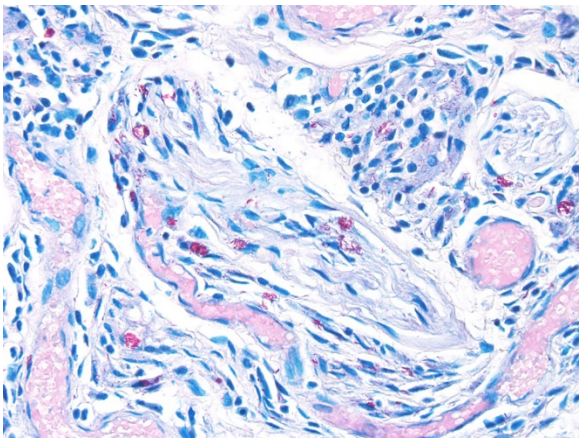
armadillos.[9] Another isolated case of *M. lepromatosis* was detected in Ireland as part of this further work. The gross and microscopic lesions are very similar in red squirrels infected with *M. leprae* or *M. lepromatosis* (indistinguishable in the sample set available).

Further mycobacterial whole genome analysis was possible using enriched techniques (e.g. host DNA depletion tools) on tissues from all the squirrel samples.[1] This enabled whole genome sequencing of these bacteria, and phylogenetic comparisons using Bayesian statistics. Comparison of British and Irish *M. lepromatosis* with two Mexican strains from humans show that they diverged from a common ancestor around 27,000 years ago, whereas the *M. leprae* strain is closest to one that circulated in Medieval England. Furthermore, comparison between the English and Irish *M. lepromatosis* isolates revealed divergence approximately 300 years ago, coinciding with the reintroduction of red squirrels in Ireland from British stocks.

Contributing Institution:
Easter Bush Pathology, College of Medicine and Veterinary Medicine, University of Edinburgh, UK (http://www.ed.ac.uk/vet/services/easter-bush-pathology).

JPC Diagnosis:

1. Ear, pinna: Dermatitis and neuritis, granulomatous, multifocal to coalescing, severe, with numerous intracytoplasmic bacilli.
2. Ear, pinna: Dermatitis, superficial, hyperkeratotic, multifocal to coalescing, moderate, with rare arthropods (acariasis).



Haired skin, squirrel. Macrophages containing acid-fast bacilli are present within rootlets of peripheral nerves. (HE, 320X)

JPC Comment: The contributor has provided an outstanding write up on this emerging disease a unique species as well as a fine review on the various types of leprosy, allowing us to examine the history of one of the world's oldest scourges.

In 2005, genetic analysis was performed on the incredibly stable genome of MA in order to map its origins.[6] Incredibly, a cases of leprosy throughout history are derived from a single clone throughout history, and very rare polymorphisms in single nucleotides allow for insight into the disease's roots. The disease originated in Eastern Africa or the Near East, and spread into Asia and Europe with progressive human migrations, often accompanying patterns of colonization and the slave trade. Leprosy was introduced into the Americas with the advent of slavery, either by Europeans or Western Africans.

Sadly, no disease has resulted in more transiently or social stigmata than leprosy. The etymology of the word "leprosy" is derived from Greek or from old English, meaning (scaly skin). While it is difficult to make retrospective diagnoses from symptoms of diseases in ancient writings, lesions strongly suggestive of leprosy known

as “tzaraath” (and reputed to be healed by Jesus in the New Testament) are found in the Bible and the Talmud, and similar lesions are described in the Feng Zhen Shi (266-246 B.C., China). In 2009, a 4000-year old skeleton was uncovered in India demonstrating patterned lesions of leprosy.

The stigmata associated with leprosy began in the Middle Ages – afflicted individuals were often required to wear bells on their persons to announce their passage in the streets and literally thousands of leprosariums sheltered the infected (as well as many others with other diseases whose only affront was being a family member of an infected individual or simply indigent. Mandatory segregation of inflicted individuals was practice in a number of countries (with Japan in 1996 being the final country to rescind this stigmatizing law) and many countries (including the UK) made it a practice to segregate patients by gender in leprosaria, in the unfounded belief that children arising from two infected individuals would be born with the disease. Some of the most restrictive practices were the norm in India which in the 1900s regionally prohibited infected individuals from traveling by train, diving, running in local elections and even made it a legal justification for divorce. Some of these laws, written prior to the development of successful multidrug therapy and the Indian healthcare’s systems very successful campaign against the diseases, still remain on the books.

In 1873, Dr. G.H Armauer Hansen discovered the causative agent, making leprosy the first disease to be recognized as being caused by a bacterium. The disease today is often referred to as “Hansen’s disease”, a term preferred by patients over the term “leper”. In the 1940’s the sulfone drug promin became the first effecting therapy.

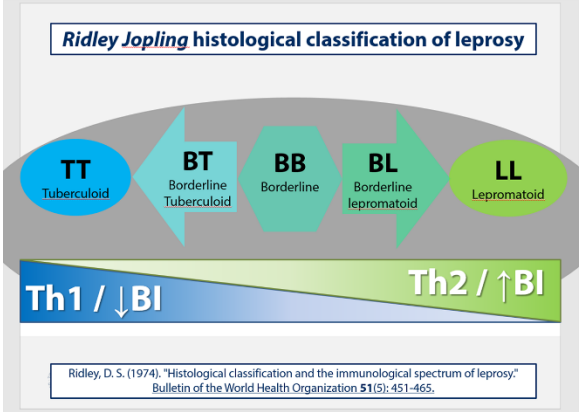


Diagram of the Ridley-Jopling histological classification of leprosy. (Image courtesy of: Easter Bush Pathology, College of Medicine and Veterinary Medicine, University of Edinburgh, UK (http://www.ed.ac.uk/vet/services/easter-bush-pathology).

Today, multidrug therapy of rifampicin, clofazimine, and dapsone (a derivative of promin) are highly affected in treating leprosy.

Due to effectiveness of multidrug therapies, the sole remaining leprosarium in the US in Carville, Louisiana was closed in 1999 in favor of outpatient treatment of those afflicted with Hansen’s disease. Approximately 200 cases are diagnosed yearly in the U.S, and treated effectively if the disease is recognized early. Interestingly, zoonotic leprosy, transmitted from armadillos to humans has been confirmed in parts of the southern U.S.[9]

In this case, the amount of hyperkeratosis and superficial inflammation seemed excessive for mycobacteriosis. In the electronic disc provided with this series, there are rare cross sections of a chitinous exoskeleton, suggestive of an arthropod, overlying the epidermis. On serial sections of the slide provided for special stains, multiple intact arthropods were identified within regions of hyperkeratosis. This is consistent with acariasis in addition to leprosy in this animal.

A recent publication on "atypical histiocytosis" in red squirrels described animals with similar gross and histologic lesions to those in this case, but IHC, special stains, and PCR were negative for *Mycobacterium* spp.[8]

Following the review of the case, the moderator discussed the widespread distribution of mycobacteria in a number of other species including fish, reptiles and amphibians. Other species of note for mycobacterial infections include elephants, which are often infected by *M. tuberculosis*, noting that it may is advantageous to wear personal protective gear when performing necropsies on elephants. She also noted the prevalence and severity of mycobacterial in sygnathid fish, including sea horses, sea dragons, and pipefish.

References:

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5 McAdam AJ, Sharpe AH. Leprosy. In: Kumar V, Abbas AK, Fausto N, Aster JC, ed. *Robbins & Cotran pathologic basis of disease*. 8th ed. Philadelphia, US: Saunders Elsevier; 2010:372-373

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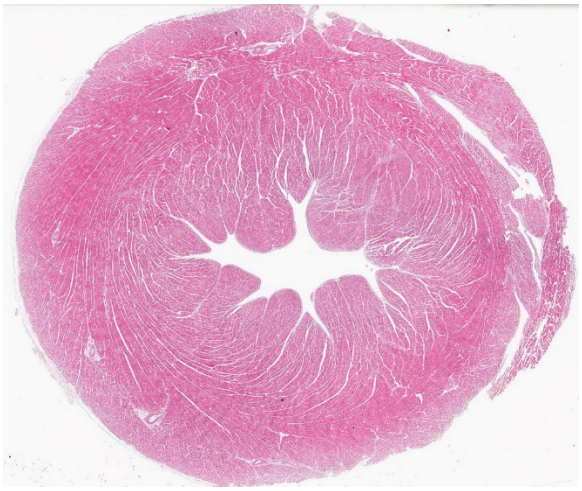
CASE II: D15-060481 (JPC 4082891-00).

Signalment: Hatch-year female bald eagle (*Haliaeetus leukocephalus*)

History: This animal was admitted to The Raptor Center (TRC) of the University of Minnesota on December 13, 2015. It was recumbent and had head tremors. The animal was severely dyspneic. Small radio-dense fragments were detected in the ventriculus by x-ray. The animal had a PCV of 27% (considered to be anemic). It was euthanatized the same day after blood analysis revealed a markedly elevated lead level (3.3ppm).

Gross Pathology: The bird was in a good nutritional state. It was anemic. The pericardial sac contained 45ml of clear



Heart, bald eagle: A cross section of the myocardium is submitted. Subgross examination does not reveal any significant lesions. (HE, 6X)

yellowish watery fluid with a small amount of flocculent material (heart weight: 53g). The myocardium of the left and right ventricle was multifocally beige discolored. Approximately 30% of the myocardium, particularly subjacent to the endocardium of the left ventricle, were affected. The proventriculus and ventriculus contained multiple small metallic fragments.

Laboratory results: None performed.

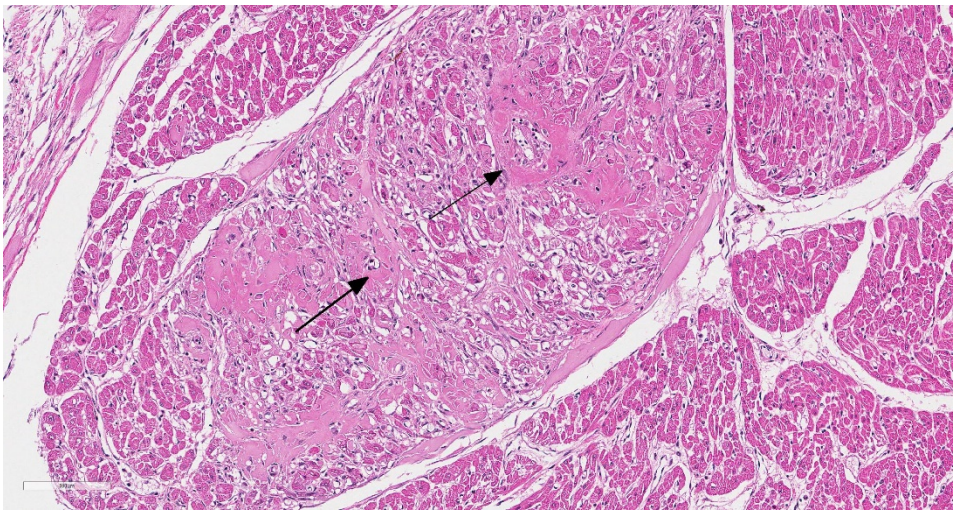
Microscopic Description: Heart (left ventricle) – The lesions are most pronounced in one papillary muscle of the left ventriculus but to a lesser degree are present at other sites of the myocardium (eg subepicardial myocardium). Cardiomyocytes in numerous fascicles of the aforementioned papillary muscle are shrunken and slightly hyper-eosinophilic containing homogenous ("hyalinized") material. Occasional pyknotic nuclei of cardiomyocytes are present. The number of cardiomyocytes in some fascicles appears to be reduced and the endomysium appears more prominent and slightly vacuolated. The number of fibrocytes in the endomysium is slightly increased in few locations with subtle deposition of collagenous matrix. In more affected fascicles, groups of cardiomyocytes are replaced by pools of homogenous eosinophilic acellular ("hyalinized") material. The smooth muscle cells in the media of occasional small to medium caliber myocardial arteries are mildly vacuolated. The endothelial cells of these arteries are slightly hypertrophied. In few arteries, homogenous eosinophilic ("hyalinized") material replaced the media or portions of the media and adventitia.

Contributor's Morphologic Diagnoses: Heart:

a. fibrinoid necrosis of small and medium caliber myocardial arteries, multifocal, acute
b. myocardial necrosis, multifocal, moderate, acute.
c. myocardial fibrosis, multifocal, mild.

Contributor's Comment: The cardiac lesions in this case are most consistent with a degenerative or toxic cardiomyopathy. The result of the blood lead analysis is diagnostic of lead poisoning. The blood lead level of 3.3ppm is high considering that bald eagles with levels above 1ppm have a poor prognosis for survival even when treated by chelation and are frequently euthanized at admission.[12] The good nutritional state of the bird at the time of death suggests a fairly acute course of the disease. Accordingly, the myocardial lesions were dominated by rather acute changes with only subtle early myocardial fibrosis.

Lead toxicity is a common cause of death in scavenging raptors such as eagles and vultures as well as waterfowl worldwide. Cardiac lesions including hydropericardium, myocardial necrosis, fibrinoid necrosis of myocardial vessels and myocardial fibrosis



Heart, bald eagle: Multifocally, the walls of arterioles near the endocardial surface are effaced by a dense hyaline material with admixed cellular debris (fibrinoid necrosis).

have been described in bald eagles.[2,6,7,10] Approximately 36% of lead intoxicated eagles submitted to the National Wildlife Health Center (Madison, Wisconsin, USA) had microscopic cardiac lesions. Myocardial infarction with angiopathy is also common in waterfowl with fatal lead intoxication.[4]. Besides the cardiac lesions, tubulonephrosis and brain lesions, including hemorrhages and parenchymal necrosis, have been reported in eagles with plumbism.[8,10] Anemia, bile stasis and bile staining of gastrointestinal mucosa are considered to be common gross findings in lead intoxicated eagles but are unspecific as to the cause and highly subjective findings.[7] Lead is known to have a wide range of pathophysiologic effects. Lead interferes in the avian host with sulfhydryl-dependent enzyme function (eg, delta aminolevulinic acid dehydrase); mimics calcium hereby interfering with neurologic function and mitochondrial respiration; and adversely affects DNA and RNA synthesis.[3] However, the exact pathogenesis of the lead-associated fibrinoid vascular necrosis, cardiomyocyte degeneration, and myocardial fibrosis is uncertain.

While waterfowl pick up lead pellets (accumulated in lakes after hundreds of years of hunting with lead-based ammunition and ongoing illegal use of lead-based ammunition), lead sinkers, etc. while dabbling, lead exposure in eagles and scavenging birds occurs almost exclusively by ingestion of carcasses and offal of animals (upland birds and ungulates) killed with lead-based ammunition.[1] Despite the ban of lead-based ammunition for waterfowl hunting in the USA and Canada in the seventies, the flow of cases of lead-intoxicated scavenging birds to wildlife rehabilitation centers and diagnostic labs has not slowed.[5] Extending the ban of lead-based ammunition to hunting of upland birds, wild turkey and ungulates would prevent lead poisoning in scavenging birds.[1]

Contributing Institution:
University of Minnesota Veterinary Diagnostic Laboratory
http://www.vdl.umn.edu

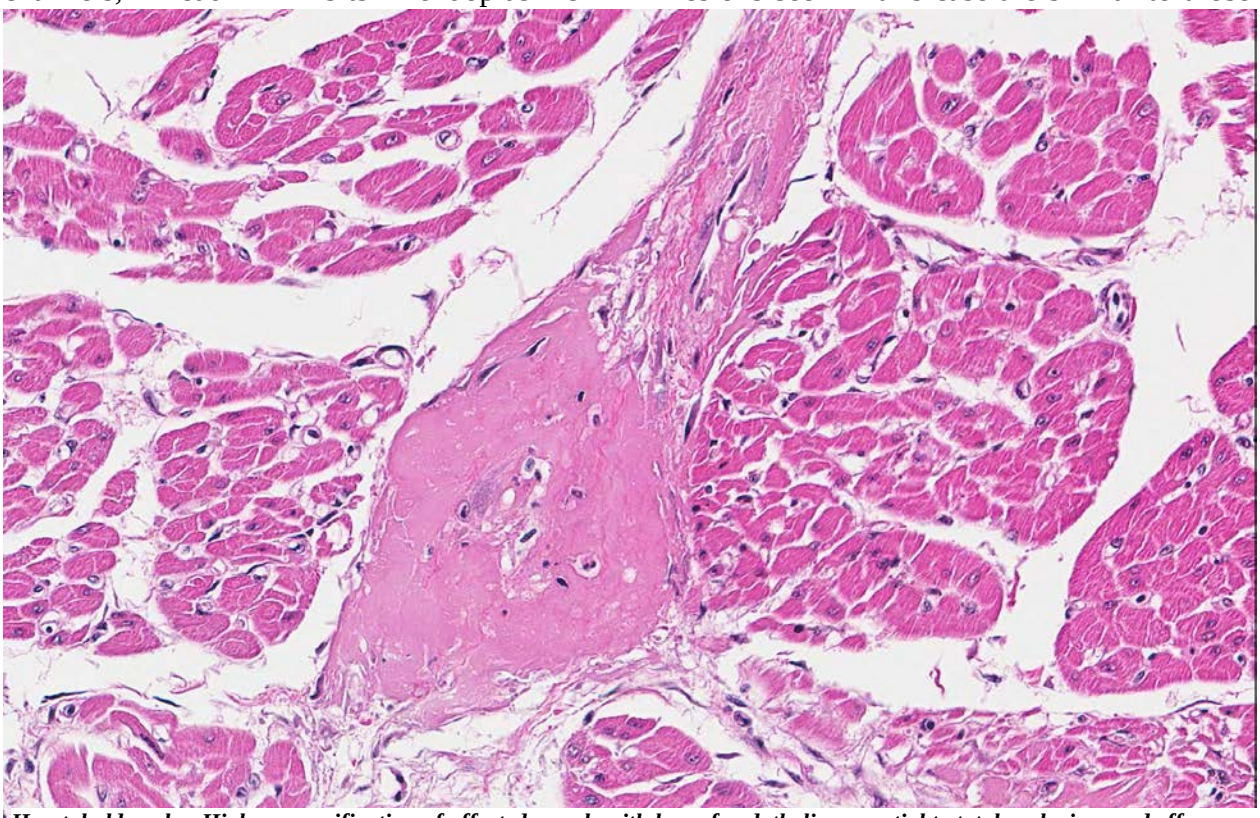
JPC Diagnosis: Heart, small- and medium-sized arteries: Fibrinoid necrosis, multifocal, severe, with myofiber degeneration, necrosis and atrophy and marked myocardial fibrosis.

JPC Comment: Lead is a well-known and potent environmental and industrial contaminant. It is widely distributed in the body and stored in the kidney, liver, and bone (where it may reside for up to 35 years!). Its effects in the hematopoietic, urinary, musculoskeletal, and nervous systems are

well known, even if many gaps yet persist in our knowledge of its pathophysiology in these systems.

One of the more recent areas of investigation in lead toxicosis are its effects upon the cardiovascular system. Damage to the vascular system as a result of lead intoxication is likely multifactorial, and lead exposure in humans has been identified in an increased incidence of cardiovascular disorders such as hypertension, organic heart disease, and peripheral arterial diseases, including atherosclerosis.[9] Research in animal models and human populations has also shown a direct and causal relationship between low-level lead exposure and hypertension. [11]

At the cellular level, lead is a major driver in free radical damage affecting both endothelial cells and smooth muscle cells. Entering the cells through normal calcium channels, lead inhibits endoplasmic reticulum (ER) Ca^{2+}-ATPase, resulting a release of calcium into the cytoplasm, and triggering ER stress as a result of release of calcium-dependent signaling and chaperone proteins contained within the ER.[11] In addition, it can bind directly to the calcium-binding protein, glucose-regulated protein 78 (GRP78), another ER-based chaperone protein involved in ER stress. [11] Activation of these proteins results in elevated levels of reactive nitrogen species in damaged cells and measurable increases in the cytotoxic effects of lead. *In vitro* studies of cardiofibroblasts has also shown that administration of lead induces autophagy (a protective response) through inhibiting the mammalian target of rapamycin complex 1 (mTORC1) pathway.[13]While speculative, the vascular changes seen in this case suggest a correlation between hypertension, endothelial damage and the toxic principles of lead at the cellular level. The arterial lesions seen in this case are similar to those

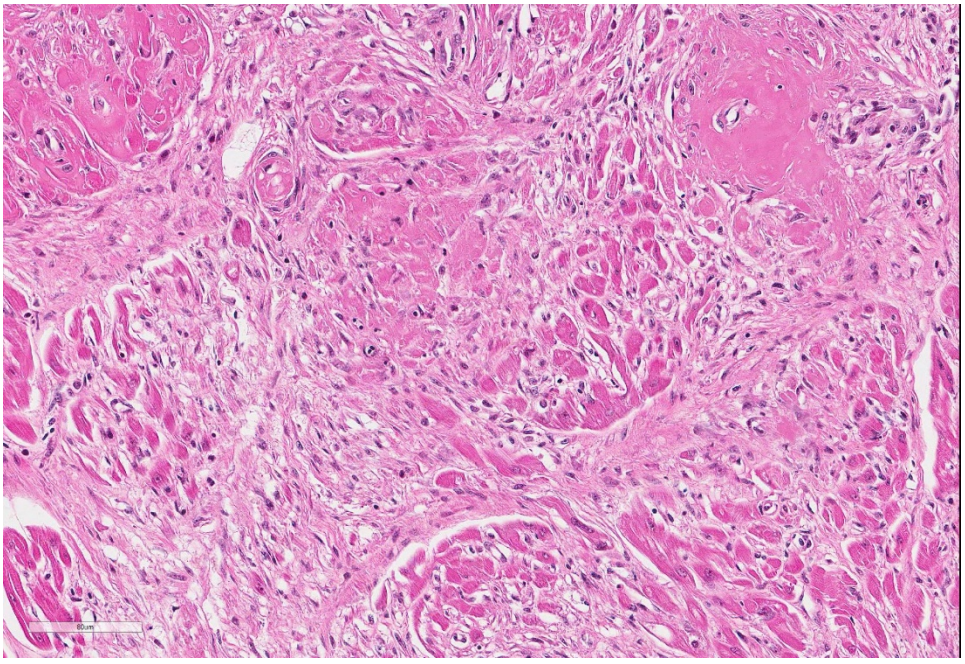


Heart, bald eagle: Higher magnification of affected vessels with loss of endothelium, partial to total occlusion, and effacement of the media and adventitia. (HE, 354X)

of polyarteritis nodosa (PN), a change often seen in hypertensive rat models. A vicious cycle of endothelial damage and hypertension may result in similar lesions. (One difference between this lesion and PN, however, is the lack of cellular proliferation in the adventitia associated with many cases of PN).

In this section, myocardial damage appears most severe in the areas of vascular necrosis, suggesting a cause-and-effect scenario, bolstered by the concurrent presence of myofiber degeneration, necrosis, and fibrosis, denoting a polyphasic timeline consistent with a toxic vascular injury. It is certainly possible that a similar direct effect of lead on cardiocytes, in addition to that seen in the vessels may have contributed to the widespread myocardial damage in this section.

The most diagnostic tissue to submit for lead toxicosis post mortem is the liver. Although lead accumulates in bone, bone levels of lead represent a lifetime accumulation of this metal and therefore are difficult to correlate to point in time toxicosis if acute toxicity is suspected. As stated by the contributor, exposure to lead in these animals is via ingestion. Although embedded lead shot is common in wildlife, intramuscular lead does not readily dissolve, and poses little risk for toxicosis. It is unusual that presumptive lead fragments were identified in the proventriculus and ventriculus in this case as birds of prey with plumbism frequently cast out metallic fragments prior to death. An additional histologic finding of lead toxicosis, though rare, is intranuclear, acid-fast inclusions in renal tubular epithelial cells. These lead inclusions have not been reported in eagles or California condors, but have been seen in turkey vultures and Andean condors.



Heart, bald eagle: Scattered throughout the myocardium, and often within papillary muscles, there are areas of myocardial necrosis with myofiber swelling, loss of cross striations, and rare pyknotic nuclei. (HE, 400X)

Attendees discussed a number of differential diagnoses for this case including West Nile virus, capture myopathy, nutritional imbalance of Vitamin E and selenium, and even electrocution (which may result in hemopericardium at necropsy). The absence of significant inflammation argues against West Nile Virus infection (although interestingly, the heart, brain, and eye are preferentially affected - the same triad of organs that exhibit vasculitis in

lead–intoxicated birds.) The polyphasic nature of the lesion and concomitant vasculitis argues against the possibility of capture myopathy, and vasculitis is uncommon in most species with Vitamin E/Se imbalance (with the possible exception of swine).

It is not uncommon for particulate matter containing lead to be absent from poisoned birds at autopsy. When submitting avian tissues for lead measurement, most reference ranges are based on liver accumulation, which would best represent acute lead storage (as opposed to bone levels, which may reflect a lifetime accumulation of lead). When searching histologically for evidence of lead toxicosis, kidneys are a good choice for the identification of intranuclear inclusions.

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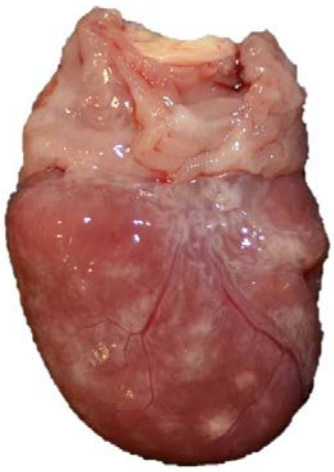
CASE III: L16-618 (JPC 4083860-00).

Signalment: One juvenile female alligator (*Alligator mississippiensis*)

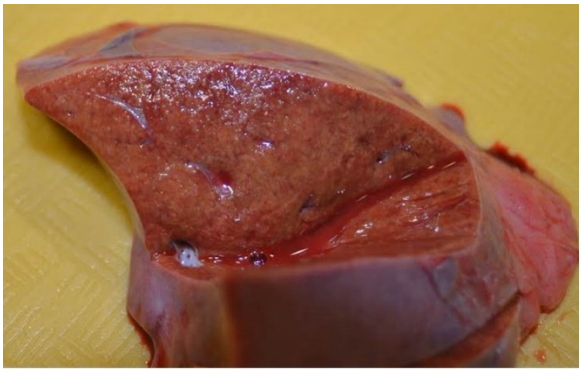
History: Out of a cohort of 5,000 alligators, five had decreased appetite but no associated mortalities had been recorded at the time of submission. Alligators of this farm had previously been diagnosed with chlamydia infection. The client also requested testing for West Nile Virus.

Gross Pathology: The alligator was in good body condition, weighing 2.8 kg. A scant amount of clear free fluid was in the coelomic cavity. The liver was pale tan to green on cut surface . The spleen measured 4 x 1.5 x 1 cm (approximately twice the expected size), was mottled red to dark red, and bulged on cut surface. On the epicardial surface were multiple 1 mm, pale tan foci that did not extend into the myocardium . Minimal red-tinged fluid was in the lower trachea.



Heart, alligator. Multiple pale tan foci are on the epicardial surface. (Image courtesy of: Louisiana Animal Disease Diagnostic Laboratory, http://www1.vetmed.lsu.edu/laddl/index.html).

Laboratory results:
Molecular (PCR):
Liver, heart, conjunctiva (pooled) and conjunctival swab: Positive for Chlamydiaceae
Liver, kidney, heart, brain, spleen (pooled): Negative for West Nile Virus
Special stains:
Liver: Gimenez-positive microorganisms
Immunohistochemistry
Liver: Microorganisms are immunopositive using a monoclonal anti-chlamydia lipopolysaccharide antibody



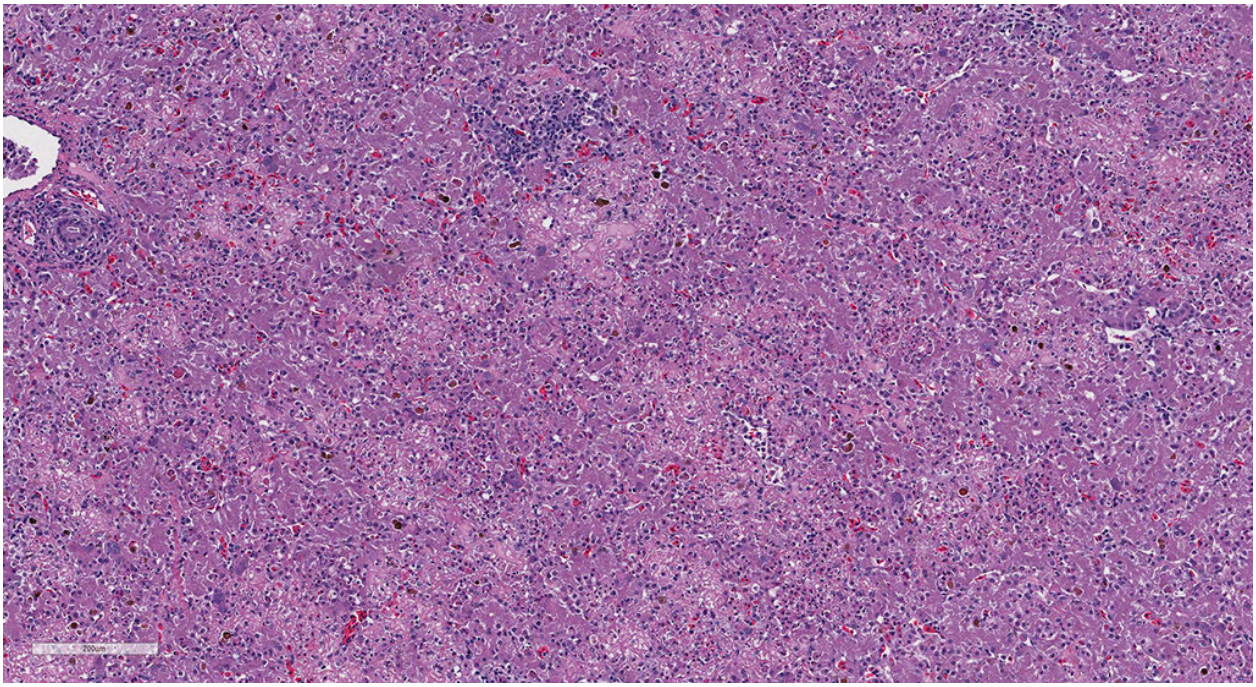
Liver, alligator. On cut surface the hepatic parenchyma is slightly orange to brown with multiple pinpoint pale tan-to-yellow foci. (Image courtesy of: Louisiana Animal Disease Diagnostic Laboratory, http://www1.vetmed.lsu.edu/laddl/index.html).

Microscopic Description: Liver: There are are multifocal to coalescing areas of hepatocellular vacuolar degeneration, necrosis, and loss and replacement by aggregates of fibrin, cellular debris, and heterophils. Clusters of basophilic, Gimenez-positive microorganisms are present in multiple remaining hepatocytes. Aggregates

of lymphocytes and plasma cells infiltrate portal areas. Biliary epithelial cells, Kupffer cells and, to a lesser extent, hepatocytes contain intracytoplasmic golden yellow-to-dark brown, iron stain-positive and bile stain-negative pigment granules.

Contributor's Morphologic Diagnoses: Liver: Hepatitis, necrotizing, multifocal-to-coalescing, marked, acute, with intra-hepatocellular coccobacilli

Contributor's Comment: Chlamydiae represent a phylum of obligate, intracellular bacteria that parasitize an array of hosts including vertebrates, a few arthropod species, and several free-living amebae.[2] Ubiquitous in the environment, chlamydiae are further classified into nine different families, all of which share similar developmental cycles and reproductive requirements but differ in their morphology, host specificity and capacity for disease.[4] Chlamydiae exist in either one of three stages: an extracellular infectious elementary body (or the dispersal form, analogous to a spore), an intracellular vegetative reticulate body, or, rarely, as described for the Parachlamydiaceae family, a crescent body (also an infective stage).[2] The family Chlamydiaceae is comprised of the genera *Chlamydia* and *Chlamydophila*. The most significant human pathogens include *Chlamydia trachomatis* (responsible for urogenital infections and the agent of trachoma) and *Chlamydophila pneumoniae* (associated with respiratory infections and neurodegenerative syndromes). The majority of the other related species are veterinary pathogens with few anthropozoonotic exceptions, notably *Chlamydophila psittaci* (psittacosis) and *Chlamydophila abortus* (spontaneous fetal loss).[6] Besides mammals, *Chlamydophila* also targets birds, amphibians, and reptiles, and chlamydiosis has been described in both free-ranging and captive cold-blooded animals including a variety of snakes, frogs, crocodiles, chameleons, and tortoises.[6] Associated lesions in these species are commonly observed in the spleen, heart, lung and liver, characterized by granulomatous



Liver, alligator. A retiform pattern of pallor resulting from coalescing areas of lytic necrosis is present across the section. (HE, 100X)

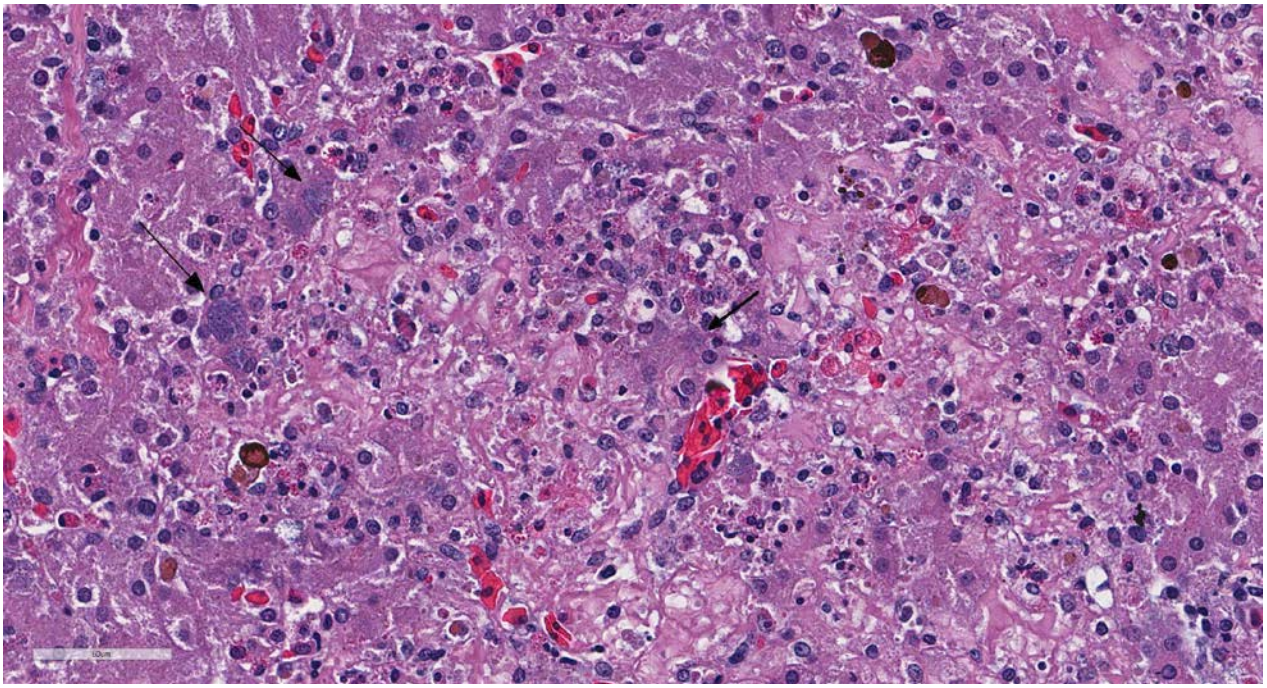
inflammation within whichever organ is infected. There are also rare reported incidences of wasting disease; gastrointestinal involvement; and necrotizing myocarditis, enteritis and splenitis.[6]

The Louisiana Animal Disease Diagnostic Laboratory has recently diagnosed an outbreak of chlamydiosis with high mortalities in a group of farmed American alligators, the first documented report of chlamydiosis in the species.[6] A novel reptilian *Chlamydophila* isolate was identified via PCR with 86% homology to *C. psittaci* using OmpA gene sequencing. The main pathological findings included necrotizing, heterophilic and granulomatous hepatitis with intracellular bacteria, necrotizing myocarditis, and lymphoplasmacytic, mixed histiocytic and heterophilic conjunctivitis. The alligator of the present submission had similar lesions in both the liver and heart (namely a heterophilic and lymphoplasmacytic necrotizing hepatitis, epicarditis and pericarditis). Additional molecular characterization of this novel *Chlamydophila* species is warranted to understand its host specificity and infective potential.

In conference, the moderator reviewed some anatomic peculiarities of the reptile liver. The reptile liver is not organized into distinct lobules as in mammals, and hepatic cores are not always seen radially around central veins. Hepatic veins and bile ductules mark the edge of a lobule, and hepatic arteries may or may not be present. She also commented on the difficulty of precisely identifying pigments within the liver of reptiles, which in this case, the attendees variously identified as bile, hemosiderin, or melanin. In reality, many of the aggregates of pigment may stain positively for none, one, or more of these particular pigments on a variety of histochemical stains.

Contributing Institution:

Louisiana Animal Disease Diagnostic Laboratory



Liver, alligator. At the periphery of areas of necrosis, hepatocytes contain an intracytoplasmic bacterial inclusion characteristic of chlamydiae. (HE, 400X)

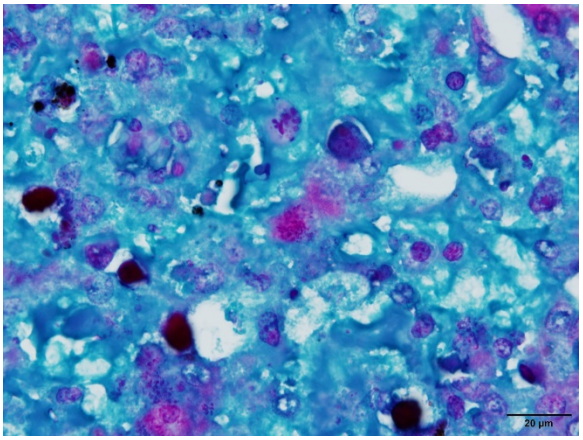
http://www1.vetmed.lsu.edu/laddl/index.html

JPC Diagnosis: Liver: Hepatitis, necrotizing, multifocal to coalescing, severe, with rare intrahepatocellular intracytoplasmic bacterial inclusions.

JPC Comment: Prior to any further discussion on this case, it appears (to the delight of pathologists everywhere), all members of the genus *Chlamydophila* have been replaced in the genus *Chlamydia*, and the controversial creation of the genus *Chlamydophila* has been reversed.[1]

While largely known as an intracellular "energy parasite" of mammals and birds, chlamydiae are also well-established as parasites of reptiles, fish, insects, crustaceans, and bivalves. Chlamydia (or *Chlamydia*-related bacteria) are the causative agent of "epitheliocystis", a poorly-named but common parasitic gill disease of fresh and saltwater fish species.[1]

One of the most interesting parts of the life cycle of chlamydiae is their dependence on



Liver, alligator. A Gimenez stain highlights the clusters of microorganisms within the hepatocytes (Gimenez, 400X)(Image courtesy of: Louisiana Animal Disease Diagnostic Laboratory, http://www1.vetmed.lsu.edu/laddl/index.html).

host cells for energy. Traditionally, chlamydiae have been considered "energy parasites", entirely dependent on host cells to supply ATP requirements. Much of this early research was apparently performed on elementary body life stages (a rather metabolically inert form) revealing a lack of flavoproteins and cytochromes associated with traditional mitochondrial function, as well as the presence of two ATP-ADP translocases which allow for energy uptake from the host cell. Recent genomic investigations of other life stages have revealed genetic encoding for many energy pathways, including glycolysis, the Krebs cycle, and the pentose phosphate pathway. Enyzmes in these pathways have been identified in reticulate bodies suggesting a potential for inherent energy creation in more metabolically active life stages than previously thought.[5]

Another interesting feature of many species of chlamydiae is their symbiotic relationship in the absence of a suitable host with free-living amoeba, to include *Acanthamoeba* and *Hartmanella*. While present within the cytoplasm of amoeba, the bacteria are not digested or harmed, and some species may even continue to excrete elementary bodies (the environmentally resistant life stage) into the surrounding extracellular environment. The presence of intracellular chlamydiae have differential effects on the growth rate of various host amebae, and have been documented to increase the cytopathic effects of certain ameba species. In human medicine, combined free-living amebic and chlamydial infections have been documented, likely as a result of infection of susceptible individuals by ameba/chlamydial symbionts.[2]

Since the submission of this case, a second report of fatal systemic *Chlamydia* infection in juvenile Siamese crocodiles, with lesions in the heart, liver and spleen similar to those reported in this outbreak, has been published.

PCR also suggested a novel *Chlamydia* sp. The lesions seen in this case, as well as the subsequent report by Thongkamkoon *et al.*, are similar to the lesions seen in estuarine and Nile crocodiles, in which hepatitis is the predominant lesion of systemic chlamydiosis[8] and differs from other types of chlamydia-associated disease previously identified in crocodiles, which include infection of the conjunctiva and upper respiratory tract, and death as a result of fibrinous suppurative upper airway and tracheal disease.[3]

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CASE IV: 13-46415 (JPC 4048668-00).

Signalment: Adult, male, eastern massasauga rattlesnake (*Sistrurus catenatus catenatus*)

History: Free-ranging eastern massasauga rattlesnake from Michigan.

Gross Pathology: The mandible is markedly enlarged and distorted by firm swelling of the subcutis, up to 0.9 cm in thickness. Along the rostral aspect of mandible, a 1.5 cm x 0.4 cm region of the oral mucosa is red-brown and ulcerated. The spectacles are bilaterally light blue and opaque. Adhered along the entire length of the body, there are focally extensive regions of dull, retained scales (dysecdysis).

Mandible, rattlesnake. A 1.5x04cm ulcerated nodule involves the oral mucosa. The spectacle is moderately opaque. (Photo courtesy of: University of Illinois College of Veterinary Medicine Department of Pathobiology and Veterinary Diagnostic Laboratory. http://vetmed.illinois.edu/path/)

Laboratory results:
Real time PCR positive for *Ophidiomyces ophiodiicola*

Microscopic Description: Effacing and expanding the dermis and subcutis of the right caudoventral mandible, compressing the adjacent skeletal muscle and dorsally elevating the overlying mucosa of the oral cavity are multiple coalescing granulomas centered on eosinophilic necrotic debris and small numbers of fungal hyphae. The hyphae are 3-5 um in diameter, parallel walled, septate, and occasionally branching. Areas of necrosis are surrounded by numerous epithelioid macrophages and few multinucleated giant cells containing up to 6 nuclei. The granulomas are further circumscribed by plump fibroblasts and dense bands of fibrous connective tissue that are infiltrated by many heterophils, fewer lymphocytes and plasma cells. There is also a free crust composed of numerous degenerate and viable heterophils, necrotic cellular debris, myriad small coccobacilli and a few fungal hyphae. The fungal hyphae stain black with Grocott's Methenamine Silver (GMS).

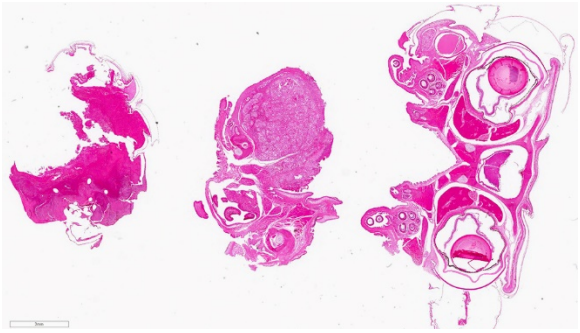
Contributor's Morphologic Diagnoses:

Head, mandible: Dermatitis and cellulitis, heterophilic and granulomatous, focally extensive, severe with fungi

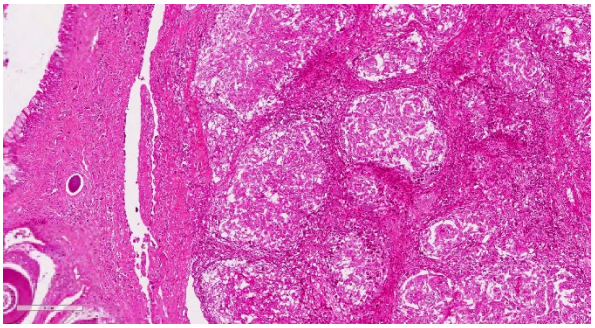
Contributor's Comment: Over the last decade, Snake Fungal disease (SFD) has become an emerging skin disease in certain populations of wild snakes in the Eastern and Midwestern United States. The keratinophilic fungus *Ophidiomyces* (formerly *Chrysosporium*) *ophiodiicola* has been consistently associated with SFD. Unlike other fungal members of the family Onygenaceae (order Onygenales), *O. ophiodiicola* has been recovered only from snakes. Published reports of fungal isolates confirmed via DNA sequencing or PCR assays have been reported in at least 12 different snake species.[2,12]

While clinical signs and disease severity may vary by species, fungal infection often leads to a fatal outcome, especially in eastern massasauga rattlesnakes.[2] The most common clinical signs of *O. ophidiicola* are severe facial swelling and disfiguration. Other cutaneous lesions include scabs or crusty scales, subcutaneous nodules, skin ulcers, dysecdysis, and hyperkeratosis.[2,11-13] Occasionally, fungal invasion may further progress to disseminated or systemic mycosis. Histologic lesions typically consist of cutaneous ulcers with thick adherent serocellular crusts and multiple granulomas within deeper tissues that are centered on variable numbers of fungal hyphae.[2,11] Although not seen histologically in this snake, notable morphological characteristics for *O. ophiodiicola* may include formation of short, undulate, sparsely septate lateral branches and chains of cylindrical arthroconidia usually along the epidermal surface.[8]

The origin, transmission, and predisposing factors of infection with *O. ophiodiicola* remain poorly understood. Occurrence of infection across different locations over a span of years suggests fungal presence within the environment. Histopathologic evidence of primary skin involvement is also consistent with environmental acquisition of infection.[2] Recent culture and molecular based surveys of healthy captive and wild snakes have shown that the fungus is not a common constituent of the normal snake skin microflora.[1,3]



Head, rattlesnake. Three sections of decalcified head are submitted. The middle section contains a large inflammatory nodule. The section on the right is primarily composed of a serocellular crust. (HE, 5X)



Mandible, rattlesnake. The inflammatory nodule is composed on well-defined coalescing granulomas ("a granuloma composed of smaller granulomas".) (HE, 122X)

Aside from presence of the fungal elements in disease-associated lesions, specific pathological criteria for *O. ophiodiicola* associated SFD have not yet been established. Differentials for dermatomycosis of snakes should include other members of the genera *Nannizziopsis*, *Paranannizziopsis*, which have been well-detailed by Sigler et al.[12] Proper diagnosis of *O. ophiodiicola* typically requires a combination of physical examination, histopathology, culture and/or molecular analysis such as PCR or DNA sequencing.[11-13]

Contributing Institution:
University of Illinois College of Veterinary Medicine Department of Pathobiology and Veterinary Diagnostic Laboratory. http://vetmed.illinois.edu/path/

JPC Diagnosis: Mandible: Osteomyelitis, rhabdomyositis, cellulitis, and stomatitis, granulomatous, focally extensive, severe,

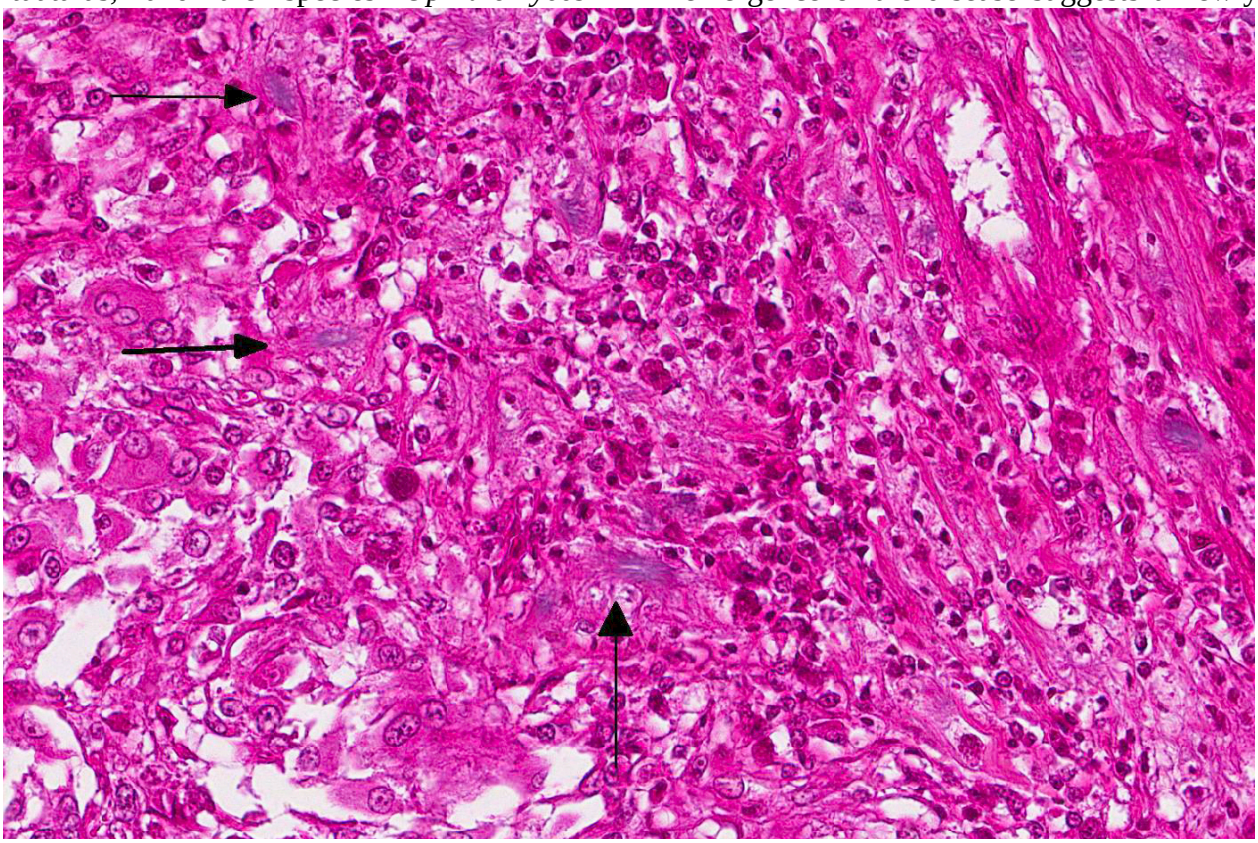
with myofiber atrophy, ulcerative stomatitis, and fungal hyphae.

JPC Comment:

Within the last generation, cutaneous and systemic fungal infections have resulted in dramatic declines of varies animal species on a global scale – *Batrachochytridium dendrobatidis* in amphibians (and a related species, *B salamandrivorans* in European salamanders), *Pseudogymnoascus destructans* in bats, and now, related species of *Chrysosporium (Nannizziopsis)* in various species of reptiles.[3,12]

Three lineages of closely related genera cause dermal or systemic infections in reptiles: the genus *Nannizziopsis* (*N. vriesis, N. guarroi*, and six additional species) infect various lizard species. *Paranannizziopsis* has four species which affect snakes and tuataras, and the species *Ophidiomyces ophiodiicola* affects snakes.[10,13]

While considered an emerging threat, opinions differ as to whether *O. ophiodiicola* is a recently introduced pathogen, or a pathogen which has emerged and spread as a result of recent environmental changes.[4] The first report of *O. ophiodiicola* was considered to be associated with a captive black rat snake with facial granulomas[11], however, the agent was identified retrospectively in samples of imported brown tree snake suffering from rapidly progressive systemic mycosis.[6] then has been identified in over 30 snake species, 6 families, and outbreaks in wild snakes in almost every state in eastern half of the United States.[4] A number of cases of dermatomycosis in various species of snakes were subsequently re-evaluated and *O. ophiodiicola* was determined to be the culprit. While this rapid emergence of the disease suggests a newly

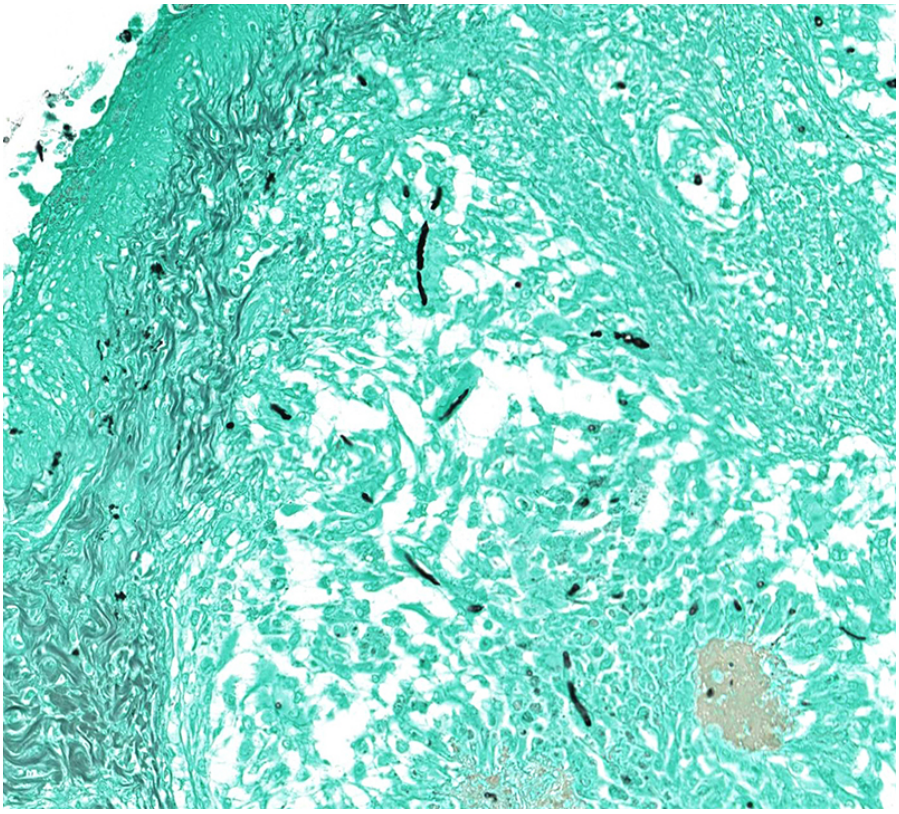


Mandible, rattlesnake. Scattered randomly throughout the granulomas are cross sections of negatively stained, 3-5um diameter fungal hyphae (arrows) (HE, 400X)

introduced pathogen, subsequent cases may be identified 500-1000 miles distant from the closest report, activity not consistent with the "new pathogen" theory. Moreover, investigation of historical outbreaks of skin disease in wild snakes, couple with new molecular techniques has identified *O. ophiodiicola* in previous cases. One recent study documented a 74% incidence of *O. ophidocola* in cases of "hibernation sores" – a condition well known to herpetologists for decades.[4]

On a comparative note, invasive, occasionally fatal infections caused by *Nannizziopsis* sp. have been rarely identified in humans, often in immunosuppressed patients. *N. obscura*, *N. infrequens* (both good names for rare pathogens) have been identified in patients sharing risk factors of HIV infection and/or recent travel to Africa. Abscesses in the brain, bone, viscera or soft tissues predominate; treatment with antifungals were successful in several cases. There is currently no evidence of zoonotic infections associated with *Nannizziopsis* sp.[5]

Reptiles have a diverse array of normal mycobiota of the skin. As such, PCR or culture should never be performed to diagnose fungal disease without paired histologic samples. Many fungi colonize the shed of snakes, and ecdysis may be a mechanism for snakes to prevent pathogenic fungal infections. Fungi that are primary pathogens in snakes are uncommon.[7] Invasiveness, granulomatous dermatitis, and arthroconidia may increase the index of suspicion for SFD (and warrant culture) and help the pathologist to diagnose this condition over other, common, secondary fungal infections. Molecular testing is now not only considered the diagnostic test of choice, but has also resulted in the correct assignment of these morphologically similar fungi to appropriate genera (most importantly, *Ophidi-omyces ophidiicola*.).[10-13] Secondary, opportunistic, fungal infections are very common in snakes, and are frequently caused by hyalo-hyphomycotic fungi; this underscores the importance of evaluating fungal morphology and using ancillary diagnostics to screen for primary fungal diseases, such as SFD.[7]



Mandible, rattlesnake. A silver stain highlights the presence of non-branching fungal hyphae with non-parallel walls (GMS, 200X)

The moderator discussed the diverse mycobiota of the skin of snakes, which is often composed of a number of secondary

pathogens; however, subcutaneous infection and granuloma formation is very characteristic of this particular pathogen. Acid-fast staining to rule out mycobacteriosis is advisable in these cases from a practical aspect.

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