Joint Pathology Center
Veterinary Pathology Services

WEDNESDAY SLIDE CONFERENCE 2021-2022

C o n f e r e n c e 20

23 March 2022

CASE I: D21-018285 (JPC 4167865)

Signalment:
10-month-old, male castrated European ferret (*Mustela putorius furo*)

History:
The ferret initially presented to the Zoological Medicine Service for an acute onset of inappetence, sneezing, coughing, and lethargy. The animal was laterally recumbent unless propped up and was febrile (106.4 °F). Clear, serous discharge came from both nostrils, the animal was sensitive to abdominal palpation, and a firm semi-mobile 1.5 cm mass was felt in the skin of the dorsum between the scapulae. No wheezes or crackles were heard on auscultation. The animal was treated empirically with Clavamox and enrofloxacin for a suspected abscess.

The ferret re-presented the same day to the Emergency Service. At re-presentation, his temperature was 105.7 °F. Full body radiographs were performed and mild splenomegaly and trace peritoneal effusion were seen. CBC showed leukocytosis (WBC 18,000/ µL). A splenic fine needle aspirate was performed and extramedullary hematopoiesis, primarily myeloid in origin, was seen. The animal was treated with cyclophosphamide, chloramphenicol, and prednisolone and improved within the first week, but acutely decompensated approx.-imately three weeks after presentation and died.

Gross Pathology:
All skeletal muscles were mildly to moderately atrophic; the temporal muscles and muscles of the proximal hind limbs appeared most severely affected. The spleen was moderately enlarged, diffusely light

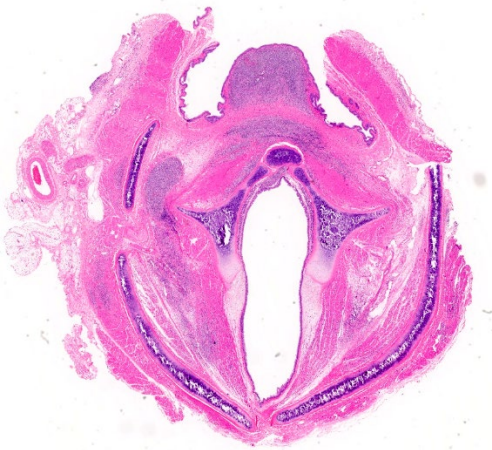

Figure 1-1. Larynx, ferret. Multifocal inflammatory infiltrates replace skeletal muscle of the larynx and overlying esophagus. (H&E, 5X) (Kansas State Veterinary Diagnostic Laboratory, Department of Diagnostic Medicine/Patho-biology, www.ksvdl.org)

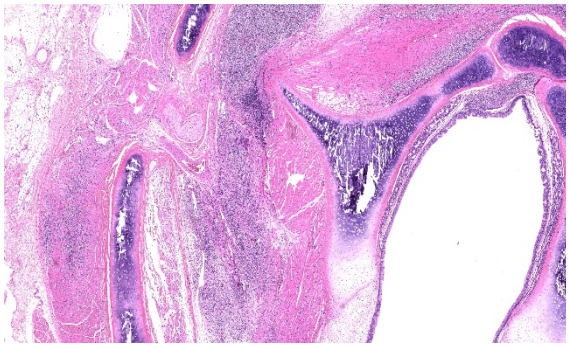

Figure 1-2. Larynx, ferret: Skeletal muscle of the larynx is multifocally infiltrated and effaced by a profound cellular infiltrate. (HE, 26X)

brown, and firm. Numerous pinpoint hemorrhages were present along the adventitia of the esophagus throughout its entire length.

Laboratory Results:
None submitted.

Microscopic Description:
Skeletal muscle; esophagus (muscularis propria), larynx: Multifocally throughout the section, skeletal muscle fibers are separated, expanded, and replaced by moderate to large numbers of degenerate and non-degenerate neutrophils and macrophages, which dissect through and expand the fascia and endomysium. Large infiltrates of these inflammatory cells obliterate the architecture of the muscle fibers. Remaining entrapped islands of myocytes are frequently degenerate with cell swelling or shrinking, hypereosinophilia, fragmentation of sarcoplasm, and loss of cross striations.

Contributor's Morphologic Diagnoses:
Skeletal muscle: Myositis and fasciitis, pyogranulomatous to suppurative, multifocal to coalescing, severe with abundant myocyte degeneration

Spleen (not submitted): Extramedullary hematopoiesis, diffuse, marked

Contributor's Comment:
The histologic changes in the skeletal muscle of this animal are consistent with disseminated idiopathic myofasciitis (DIM), an inflammatory myopathy in ferrets.

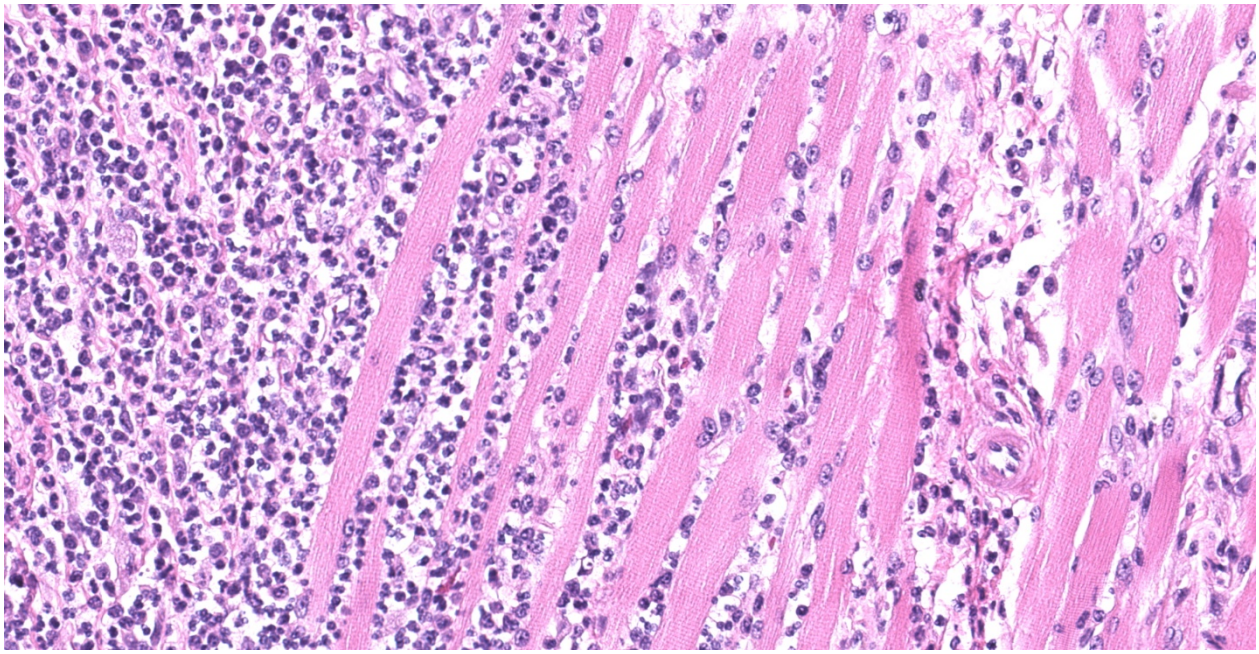

Figure 1-3. Thyroarytenoideus muscle, ferret: The cellular infiltrate is primarily composed of viable neutrophils, with fewer scattered macrophages. (HE, 336X)

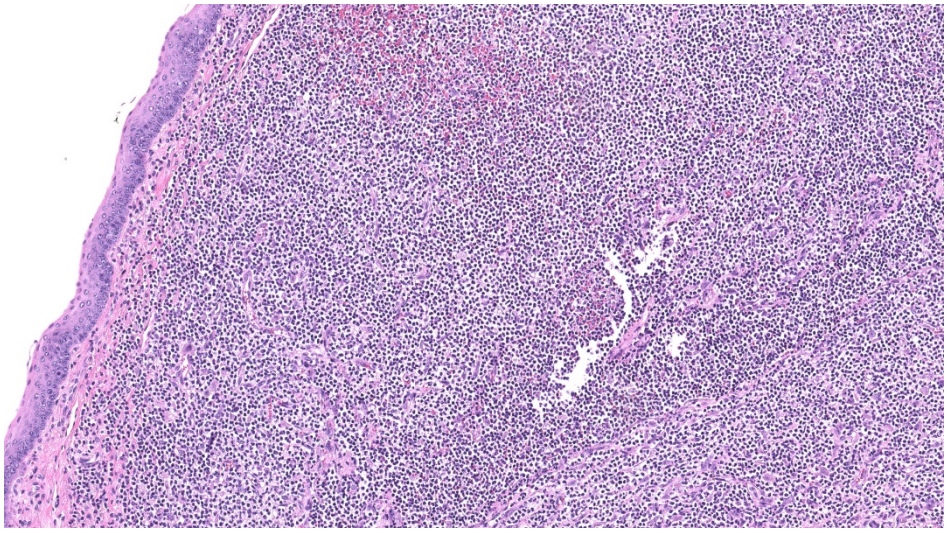

Figure 1-4. Esophagus, ferret: The cellular infiltrate effaces the skeletal muscle of the ventral esophagus and extends into the overlying submucosa. (HE, 128X)

First described by Garner et al in 2007, gross findings of DIM include whole body muscle mass loss, esophageal mottling, white streaks in muscles, lymphadenomegaly, and splenomegaly. Histologic lesions consist of suppurative to pyogranulomatous inflammation in muscle, fascia, and adipose throughout the body with associated atrophy, as well as myeloid-predominant extramedullary hematopoiesis in the spleen.[3,5] Inflammation in the muscularis of the esophagus with sparing of the mucosa is considered an important histologic lesion in DIM.[2] Affected ferrets are generally young (<18 months), however DIM has been reported in ferrets as young as 4 months and as old as 24 months[5], with no sex predilection.

Clinical signs include acute onset of severe pyrexia, lethargy, pain, paresis, and recumbency. Additional reported clinical signs include inappetence, dehydration, lymphadenomegaly, serous nasal or ocular discharge, and diarrhea.[3,5] Clinical pathology may show leukophilia due to mature neutrophilia, increases in ALT and glucose, and mild hypoalbuminemia. Creatinine kinase, generally associated with muscle damage, is not elevated. Animals generally decline until death or euthanasia and the disease is resistant to attempts at treatment, which has included antibiotics, steroids, NSAIDs, and immune modulators.[5]

The inciting cause of DIM is not known. Histologic, immunohistologic, and electron microscopy testing for infectious etiologies, including fungi, bacteria, and protozoa such as *Sarcocystis neurona*, *Toxoplasma gondii*, and *Neospora caninum* have been unrewarding.[3,5] An association with the Fervac-D canine distemper vaccine was considered; however, this vaccine is no longer produced, and more recently affected ferrets have been vaccinated with several different distemper vaccines, including Distox-Plus, Distem-R TC, Purevax, and Galaxy-D.[3,5] A heritable component is being considered, as some forms of inflammatory myositis in humans and dogs are inheritable[5], and immune mediated myositis (IMM) in horses is suspected to be inheritable as well due to its prevalence in reining and cow bred Quarter Horses and Quarter Horse outcross breeds such as the American Paint Horse and Appaloosa.[1,4]

Immune-mediated inflammatory myopathies exist in several other species; however, the inflammation in these diseases is generally mononuclear, particularly lymphocytes, in contrast to the suppurative to pyogranulomatous inflammation seen in ferrets with DIM. Horses with IMM are generally between 8 and 17 years old, and

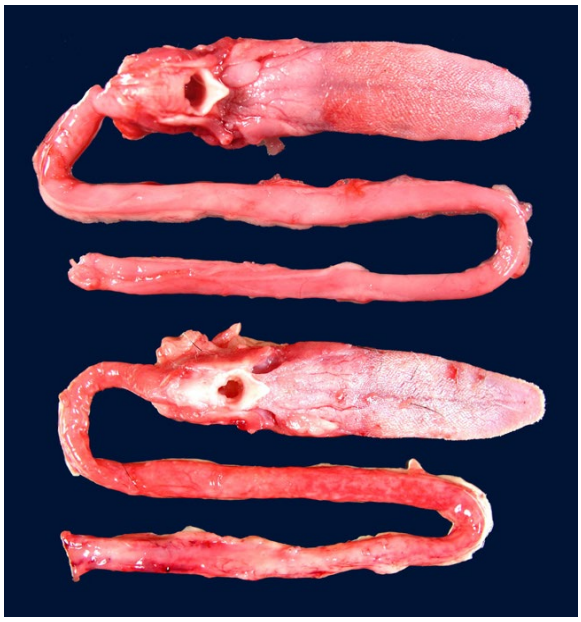

Figure 1-5. Esophagus, ferret. Esophagus from a ferret with DIM versus normal control (top). There is multifocal pallor and thinning of the skeletal muscle, most prominently in the distal esophagus. (*Photo courtesy of Dr. Michael Garner, Northwest Zoopath*.)

39% have been recently exposed to a suspected immune trigger such as *Streptococcus equi* or other respiratory viral infection, or vaccination.[1] Horses with IMM have lymphocytic infiltration of myocytes and frequently lymphocytic vascular cuffing, with the epaxial and gluteal muscles most commonly affected and other muscles generally being spared.[1] In dogs, immune-mediated myositis may be confined to the masticatory muscles or may be widespread as a polymyositis (PM). Masticatory myositis results from autoantibodies to type 2M myosin, a myosin isoform that is only found in the masticatory muscles, and inflammation generally consists of eosinophils and lymphocytes.[6] PM in dogs is most common in large breed dogs, particularly German Shepherds. A breed associated PM also occurs in Newfoundlands with primarily lymphocytic inflammation.[6] In humans, immune mediated polymyositis results from development of autoantibodies to muscle components. Human PM consists primarily of non-suppurative inflammation, predominantly T lymphocytes and macrophages.[3]

Contributing Institution:
Kansas State Veterinary Diagnostic Laboratory
Department of Diagnostic Medicine/Pathobiology
Ksvdl.org

JPC Diagnosis:
1. Larynx and esophagus: Rhabdomyositis, neutrophilic, chronic-active, diffuse, severe, with myofiber atrophy.

2. Perilaryngeal soft tissues: Cellulitis, neutronphilic, chronic-active, multifocal, moderate.

JPC Comment:
The contributor provides an excellent review of disseminated idiopathic myofasciitis (DIM) in ferrets, an inflammatory myopathy with a high mortality rate, no sexual predisposition, and predominantly affects ferrets less than 18 months of age.[5]

Although astute diagnosticians may suspect DIM based on the previously noted clinical signs and clinical pathology abnormalities, definitive antemortem diagnosis requires the biopsy of skeletal muscle, preferably from 2-3 locations given DIM's multifocal distribution. Recommended sites include the lumbar, pelvic limb, shoulder, or temporal regions. Essential tissues collected from necropsies include esophagus, skeletal muscle, and heart, although lesions may be observed other tissues, such as the larynx in this case; therefore a comprehensive set of tissues should ideally be collected. Finally, given the pathogenesis is unknown, it is recommended to collect paired tissue samples that may be frozen and used in future studies.[5]

Two diseases with similar clinical and pathologic features that are also commonly fatal in this species include ferret systemic coronavirus infection and mycobacteriosis.[2]

Ferret systemic coronavirus (FSCV) infections were first recognized around 2003, during the same time period as initial cases now known as DIM were reported. FSCV causes gross and histologic lesions identical to those of feline infectious peritonitis, which arise as the result of systemic pyogranulomatous inflammation, vasculitis, and necrosis that predominantly targets visceral organs and lymph nodes. Young adult ferrets are most commonly affected and clinical signs may include intra-abdominal masses, lymphadenomegaly, vomiting, diarrhea, inappetance, dyspnea, CNS signs, and mild ascites (uncommon). Gross findings include white to tan nodules on the serosa of thoracic and abdominal organs, as well as within the parenchyma and lymph nodes. Biopsy of affected tissue, typically lymph node or mesentery, is necessary for antemortem diagnosis. Immunohisto-chemical reagents developed for FIP may be utilized for the diagnosis of FSCV due to cross-reactivity. Interestingly, analysis FSCV's spike protein indicates the virus is more closely related to ferret enteric coronavirus (etiologic agent of epizootic catarrhal enteritis) than feline coronaviruses, despite the nearly identical gross and histologic lesions shared with FIP.[2]

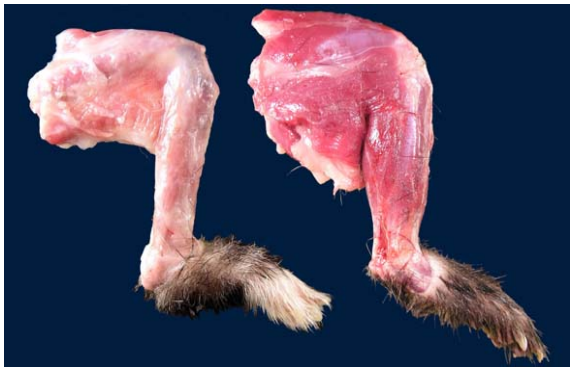

Figure 1-6. Skeletal muscle, hindlimb, ferret. There is marked skeletal muscle wasting and pallor in the affected leg (left; normal control at right) (*Photo courtesy of Dr. Michael Garner, Northwest Zoopath*)

In regard to mycobacteriosis, ferrets appear to be particularly susceptible to human, bovid, and avian strains of mycobacteria and serve as reservoir hosts, most notably in New Zealand where transmission of *Mycobacterium bovis* from feral ferrets to humans and sheep is of concern. *Mycobacterium avium* is the most common etiology identified in the United States and is typically associated with granulomatous gastroenteritis, lymphadenitis, otitis, and meningoencephalitis with large numbers (i.e. multibacillary) of acid-fast bacteria within macrophages. Atypical mycobacteriosis may also occur, with very few bacteria (i.e. paucibacillary), most commonly being found extracellularly within areas of necrosis or surrounded by inflammatory cells. Depending on the tissue affected, clinical signs may include nasal discharge, head tilt, circling, vomiting, diarrhea, and enlarged visceral and peripheral lymph nodes. As with DIM and FSCV, antemortem diagnosis requires biopsy of affected tissue, with subsequent diagnostics including acid-fast stains, PCR, and culture.[2]

Given mycobacteriosis shares some similar clinical and pathologic features with DIM and FECV, one should consider risk of potential zoonotic infection during tissue collection and handing in cases suspected of the two latter entities.[2] Additional sections submitted with this case were subjected to a battery of stains including acid-fast, Gram, Grocott's methenamine silver, and periodic acid-Schiff. No infectious agents were identified, further supporting the diagnosis of DIM.

Conference participants discussed the temporal characterization of inflammation in this case given neutrophilic inflammation is

generally associated with an acute process. It is unknown why neutrophils are the predominant inflammatory cell associated with DIM despite being a chronic process. However, histologic features such as skeletal muscle atrophy and fibrosis are also common features of DIM and consistent with a chronic inflammatory process. Therefore, conference participants favored the temporal modifier of "chronic-active".

References:

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CASE II: SP-18-1795 (JPC 4117534)

Signalment:
4-year-old female marine toad (*Rhinella marina*)

History:
The toad had a coelomic mass of unknown duration.

Gross Pathology:
This toad had an approximately 3 cm encapsulated mass present on the left ovary, as well as a 1.5 cm subcutaneous mass.

Laboratory Results:
Numerous *Brucella* spp. were isolated from the ovarian mass. *Brucella* isolates were identified to the genus level using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Partial 16s rDNA sequencing was performed and confirmed a *Brucella inopinata*- like spp.

Microscopic Description:
Effacing the normal ovarian architecture and compressing normal ova is a large mass primarily composed of sheets of histiocytes mixed with scattered lymphocytes and heterophils. There are multifocal to coalescing areas of necrosis characterized by degenerate cells with pyknotic nuclei and eosinophilic and karyorrhectic cellular debris. Contained within the cytoplasm of histiocytes and often freely within necrotic foci are numerous gram-negative 1-2 um diameter small bacilli. Surrounding necrotic areas are large numbers of reactive fibroblasts and epithelial macrophages interspersed by bands of collagen. The ovary contains abundant multifocal melanin pigment. Organisms are acid fast-negative.

Contributor's Morphologic Diagnoses:
Ovary: Granulomatous oophoritis with numerous intracytoplasmic gram-negative bacilli.

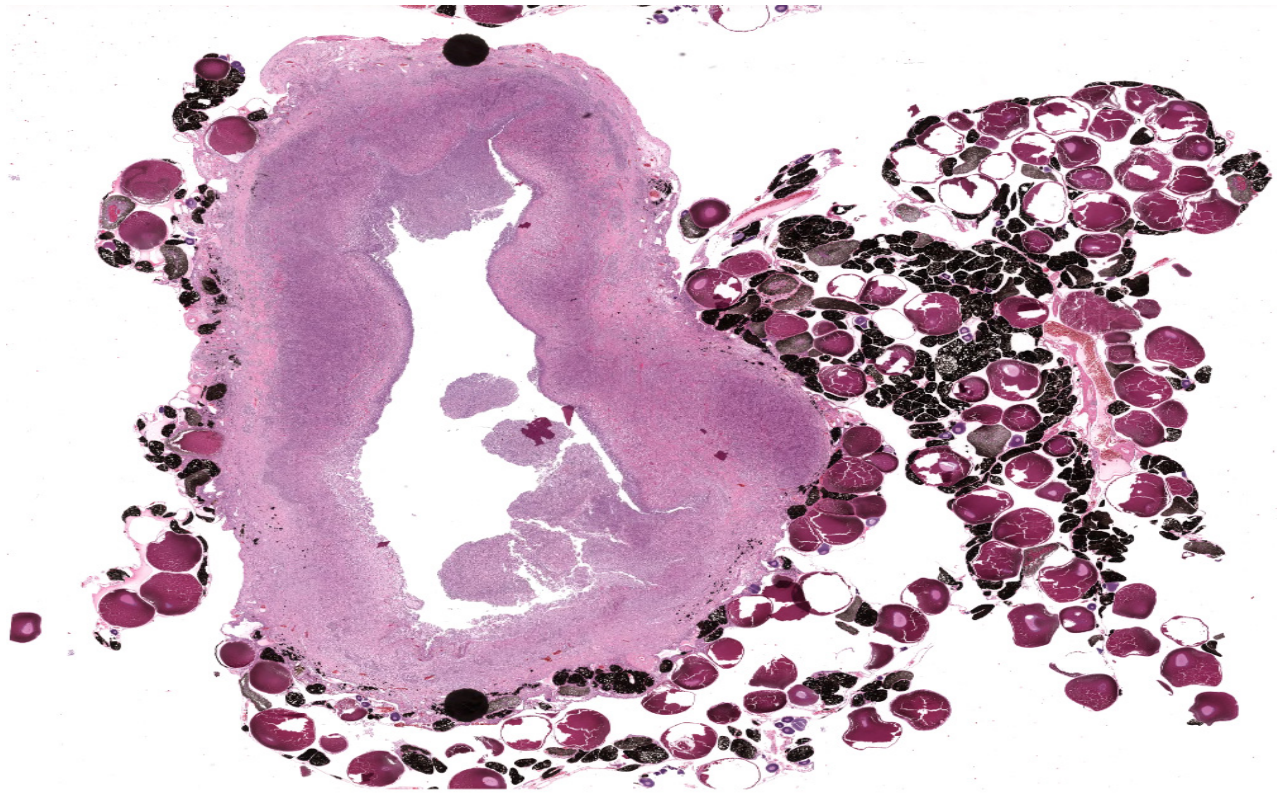

Figure 2-1. Ovary, marine toad: A large inflammatory nodule with a central area of necrosis expands the ovary. (HE, 5X)

Contributor's Comment:

Brucella spp. are an important worldwide zoonotic pathogens of both human and veterinary medical significance. This genus was previous classified into six "classical" species based on preferential mammalian hosts including *Brucella melitensis* (goats), *Brucella suis* (suidae), *Brucella abortus* (cows), *Brucella ovis* (sheep), *Brucella canis* (dogs), and *Brucella neotomae* (desert rats).[5] Newly identified marine mammal species include *Brucella ceti* (cetaceae) and *Brucella pinnipedialis* (pinnipeds).[3] More recently, 'atypical' *Brucella* strains have been isolated from non-mammalian hosts including amphibians; a big-eyed tree frog, several African bullfrogs, a White's tree frog and a Pac-Man frog,[1,2,8] however to the best of our knowledge, have not been previously described in toads.

Brucella spp. are facultative intracellular bacteria which exhibit a variety of lesions in amphibians, ranging from localized manifestations (e.g. subcutaneous abscess, skin lesions, swollen paravertebral ganglia, panopthalmitis) to systemic infection with high mortality in zoological exhibitions.[6] In some species, however, the lack of pathogenic lesions suggests that *Brucella* spp. may be a commensal microorganism or at least a facultative pathogen. The precise epidemiology and pathogenesis of brucellosis in amphibians remains largely unknown, and limited data is available for the zoonotic potential of novel or 'atypical' members of the *Brucella* genus.

The marine toad, also known as the cane toad, is considered to be the most widely introduced species in the world (Global Invasive Species Database. http://www.iucngisd.org/gisd). Originally

used to control insect pests of sugarcane and other crops, it is now considered to be a pest species itself as it preys on and outcompetes native amphibians. Its toxic secretions (bufotoxin) are known to cause illness and death in dogs, cats, wildlife, and humans. This particular female toad and her mate were used in a zoo outreach and education program. Due to the unknown zoonotic disease risk of *Brucella inopinata*- like spp. in humans, all exposed personnel were administered prophylactic antibiotics. This animal and two others from the same shipment were culled. One of the other animals was also infected with *Brucella inopinata*- like spp.

Contributing Institution:
Michigan State University Veterinary Diagnostic Laboratory
College of Veterinary Medicine
4125 Beaumont Road, Lansing, MI 48910
Phone: (517) 353-1683
animalhealth.msu.edu

JPC Diagnosis:
Ovary: Granuloma, focal.

JPC Comment:
As noted by the contributor, this case represents one of multiple recent discoveries of *Brucella* spp. in non-domestic species, particularly amphibians. *Brucella sp.* are small (0.6 µm x 0.6-1.5 µm) gram-negative bacilli or coccobacilli that are typically intracellular, associated with chronic infections and abortion in both domestic and wildlife species.[6]

Host adapted species of *Brucella* have been emerging for millennia since the domestication of canines and ungulates. However, the disease now known as brucellosis was not identified until British Royal Army Medical Corps officers serving on the island of Malta during the 1850s noted British servicemen were developing fevers of unknown origin. In 1861, Dr. Jeffrey Alan

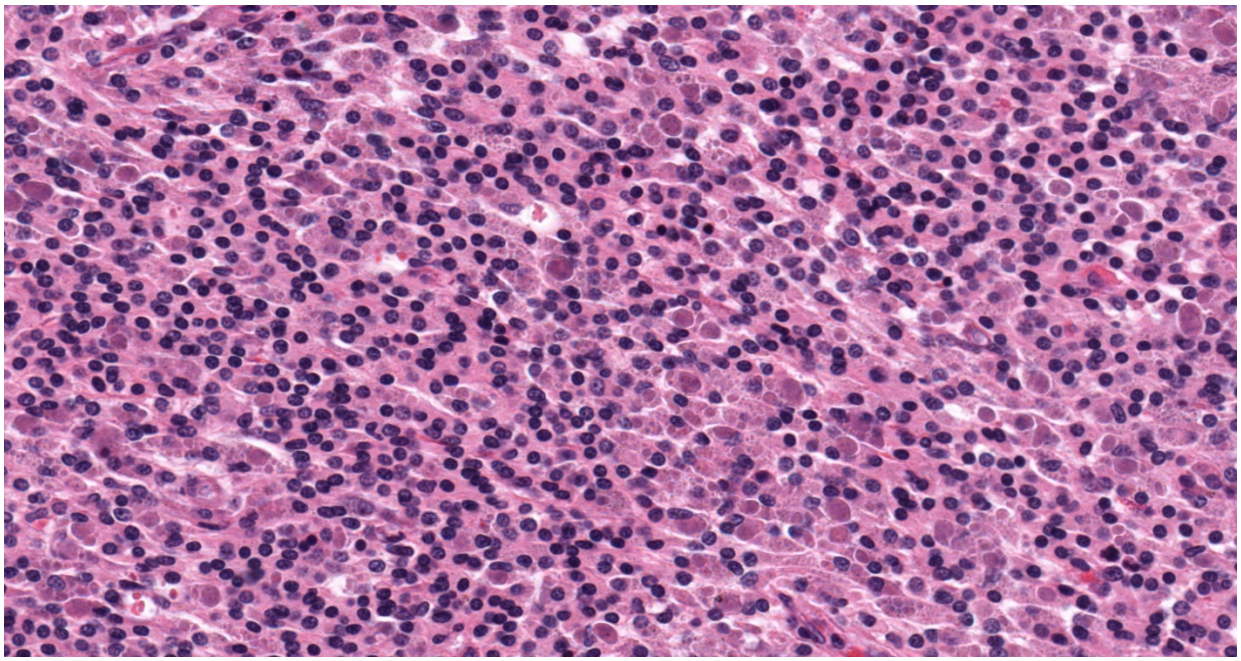

Figure 2-2. Ovary, marine toad: The inflammatory nodules is composed of innumerable macrophages, admixed with fewer lymphocytes, rare granulocytes, and abundant cellular debris. (HE, 333X)

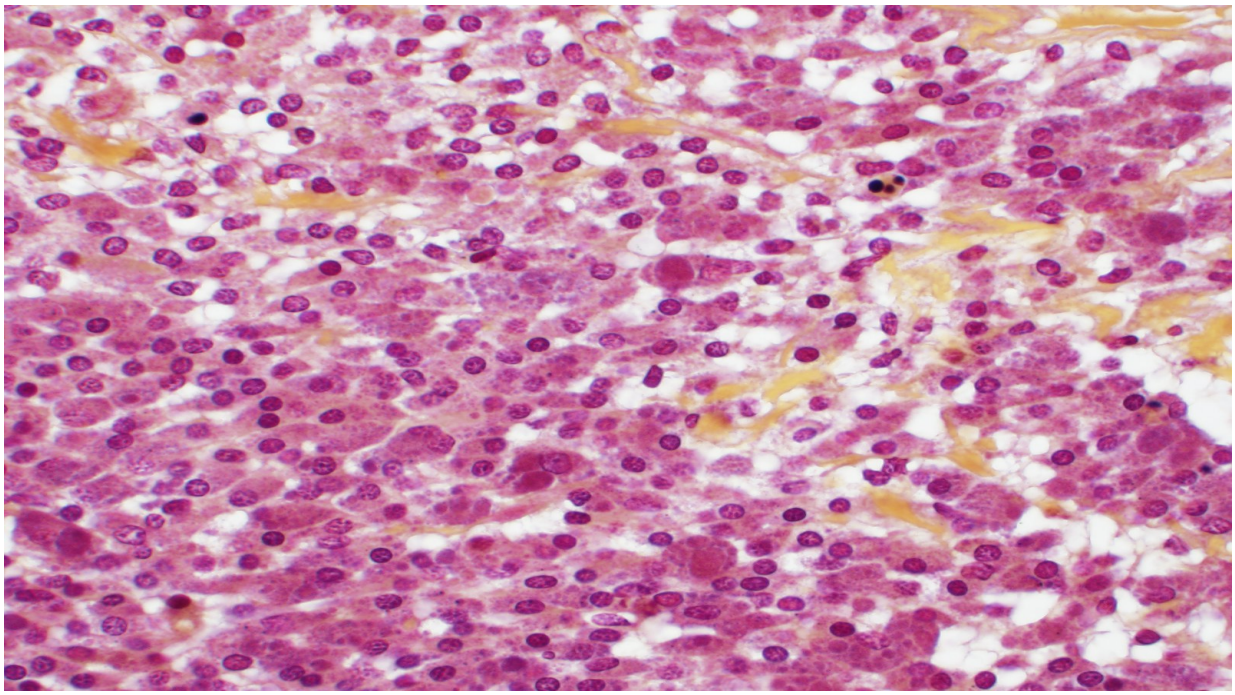

Figure 2-3. Ovary, marine toad. Macrophages are filled with gram-negative bacilli (Brown Hopps, 400X)

Marston, a British Army surgeon, described the features of typhoid fever, differentiating it from the 'Undulant Fever' he observed on Malta during the previous decade. Over two decades later, David Bruce and Lady Bruce, with the assistance of Guiseppe Caruna Scicluna, first cultured and discovered the genus now known as *Brucella.* However, the source and route of infection remained unknown until Dr. Themistocles Zammit, a Maltese microbiologist, discovered the bacteria in the urine, blood, and milk of goats on the island in 1904. Further investigation found approximately 50% of Maltese goats to be seropositive, with approximately 10% secreting the pathogen into the milk. This was significant given the British military procured goat milk for consumption by its service members. The British military subsequently banned procurement of goat milk in 1906, resulting in a drop from 3631 cases between 1900 and 1906 to only 21 in 1907 and zero by 1909. Despite this early success, brucellosis persisted on Malta until the island was finally declared brucellosis free in 2005, nearly a century after Zammit's discoveries. This issue was largely due to approximately two-thirds of the goats on the island belonging to back-yard breeders with only one or two goats and exempt from or failed to cooperate with government driven eradication efforts.[9]

As noted by the contributor, *Brucella* sp. may be classified as 'typical' or 'atypical', with the former representing 'classical' or 'core' species such as *B. melitensis* as well as additional species such as *B. pinnipedialis, B. ceti,* and *B. papionis* that share >99% of the genome with identical or nearly identical 16S rRNA and *recA* gene sequences. In contrast, atypical species remain within the proposed species boundary of 95-96% nucleotide similarity but resemble members of genus *Ochrobactrum*, the nearest genetic neighbor within family Brucellaceae. Atypical species are often motile due to the presence of flagella and also demonstrate an increased ability to survive in low pH environments due to the presence of a glutamate decarboxylase-dependent system, a useful marker for

distinguishing between typical and atypical genera.[6]

The similarity between atypical *Brucella* sp. and *Ochrobactrum* sp. has resulted in the former being misidentified as the latter when rapid identification techniques (e.g. API® 20NE test strips) are utilized. *Ochrabactrum anthropi* is an opportunistic pathogen associated with severe *Brucella*-like disease in immunocompromised patients and is closely related to *Brucella* sp. Therefore, real-time or conventional PCR targeting the IS711 insertion sequence common to all *Brucella* species or 16S rRNA sequencing should be considered following the diagnosis of *Ochrobacterum* sp. by conventional methods to rule out atypical *Brucella.*[4]

It is currently unknown if amphibian brucellosis occurs as the result of a commensal or facultative pathogen or as the result of direct pathogenicity. Furthermore, host species, geographic range, and range of affected species are currently unknown. Including this case, atypical *Brucella* isolates have been identified in at least ten amphibian species native to North and South America, Australia, and Africa. These isolates were obtained from zoologic collections (as in this case), wild caught specimens, private breeders, and pet stores. Given the emergence of brucellosis within amphibians, personnel responsible for care and treatment of these animals should not only be aware of the risk of brucellosis as a cause of morbidity and mortality in these species, but also the potential risk of zoonotic infection, though no reports of transmission have been reported.[4]

Based on the moderator's experience, anuran brucellosis frequently manifests as a musculoskeletal abscess associated with spherical foci of discoloration of the overlying skin, although coelomitis, and/or visceral abscesses may also be seen, such as in this case.

Brucella sp. in this case are readily identified using Gram stains (i.e. Brown-Hopps), however, this gram-negative organism is often poorly discernable in tissue sections stained with both standard histochemical stains such as hematoxylin and eosin (H&E) as well as Gram stains. The moderator suggested performing acid-fast stains in such cases to exclude mycobacteria (which can cause similar lesions), followed by PCR as previously described.

Although intrahistocytic eosinophilic material is present within macrophages, participants agreed that coccobacilli could not be readily identified on H&E stained sections and therefore could not rule out other phagocytized material. In addition, participants preferred the morphologic diagnosis of "granuloma" rather than "oophoritis" due to the focally extensive distribution of the lesion, which was centered on regions of dropout interpreted to be necrosis, as well as features of granulomas, such as epithelioid macrophages surrounded by peripheral fibrosis and lymphocytes.

References:

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CASE III: 12-143 (JPC 4065944).

Signalment:
2 month old female snowy owl (*Bubo scandiacus (*formerly *Nyctea scandiaca))*

History:
This captive bird was one of a clutch of two eggs hatched in the late spring. Owl was vaccinated for WNV 3 weeks prior to death. Found dead in enclosure with no premonitory signs.

Gross Pathology:
At necropsy, there was mildly reduced breast muscle mass with a prominent keel. There was mild hepatosplenomegaly.

Laboratory Results:
A blood sample obtained from the clutch mate was grossly watery, with a hematocrit of 5% (normal 38-50%). On a smear, there were visible hemoparasites (including trophozoites, round gametocytes and rare schizonts) with hemozoin pigment, as well as polychromasia and schistocytes.

Histopathologic Description:
Submitted tissues are liver and spleen. In the liver centrilobular hepatocytes are frequently pale and vacuolated, with dissociation of hepatic cords, rounded swollen hyalinized cells and pyknotic, karyolytic or karyorr-hectic nuclei (centrilobular necrosis). Large numbers of Kupffer cells are present in sinusoids. There is abundant refractile finely globular brown-black pigment (hemozoin) with yellow-orange birefringence within Kupffer cells and erythrocytes. Within erythrocytes are frequent protozoal

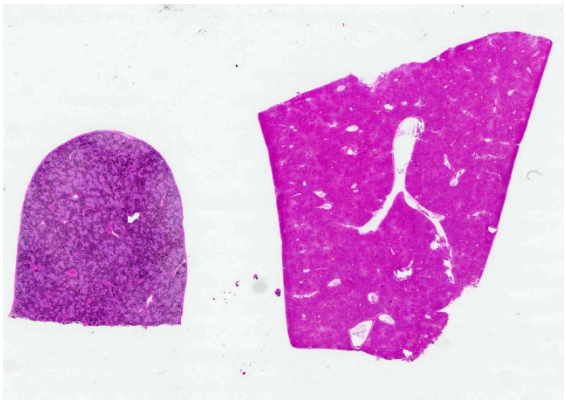

Figure 3-1. Spleen, liver, snowy owl. A section of spleen and liver from a 2-month-old snowy owl are presented for examination. There are no significant lesions at this magnification. (HE, 5X)

hemoparasites including trophozoites and round gametocytes. Bile casts are visible in some large intrahepatic bile ducts. The splenic red pulp is markedly expanded by large numbers of macrophages, the majority of which are hemozoin-laden. In both organs, erythrophagocytosis is evident. Autolysis is present at the margin of the spleen in some sections.

Additional findings included perivascular (ring) hemorrhages in the brain with accumulations of macrophages in the Virchow-Robbins spaces (not submitted).

Contributor's Morphologic Diagnoses:
Syndrome, hemoparasitism, malaria

Liver and spleen, histiocytosis, diffuse, moderate, with intrahistiocytic hemozoin pigment

Liver, centrilobular hepatocellular degeneration and necrosis, multifocal, acute, mild to moderate

Contributor's Comment:
The three main erythroparasites of birds are *Plasmodium, Leucocytozoon* and *Haemoproteus*.[1,2] Evaluation of Giemsa- stained blood smears is the standard for diagnosis. Unlike *Leucocytozoon* and *Haemoproteus,*

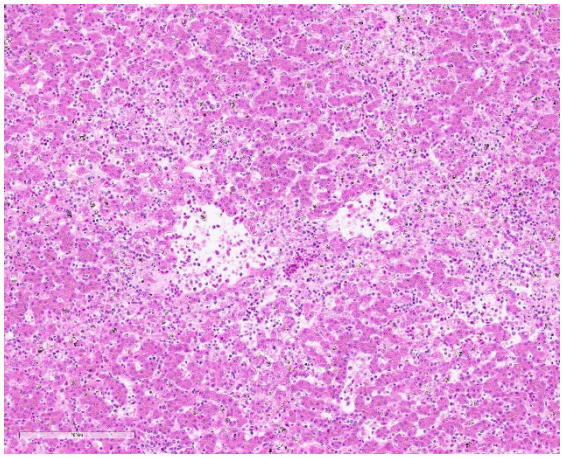

Figure 3-2. Liver, snowy owl. There is bridging necrosis of centrilobular hepatocytes. (HE, 120X)

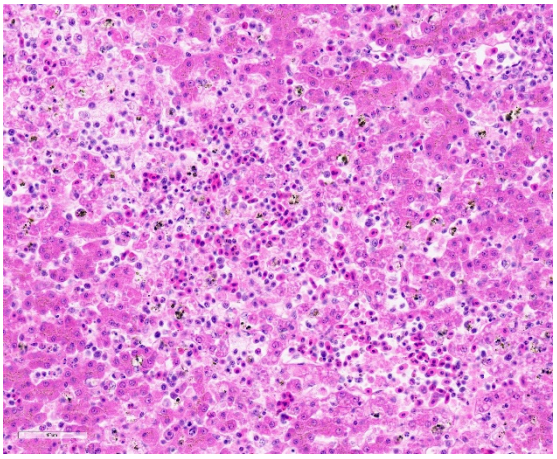

Figure 3-3. Liver, snowy owl. Within centrilobular areas of the hepatic lobule, hepatocytes are individualize, rounded up, and pyknotic, with small amounts of hemorrhage and fibrin in areas of hepatocellular loss. Diffusely, Kupffer cells contain a brown black granular pigment (hemozoin). (HE, 400X)

Plasmodium undergoes asexual reproduction (merogony) in circulating erythrocytes. Both *Plasmodium* and *Haemoproteus* produce hemozoin pigment from the digestion of host cell hemoglobin; *Leucocytozoon* does not. *Haemoproteus* forms large sausage shaped gametocytes that partially or completely envelop the erythrocyte nucleus, generally without displacing it; hemozoin pigment granules only appear late within development.

Plasmodium are generally host adapted, causing subclinical infections. Epizootics occur when the parasite and/or mosquito vector are introduced to new areas (such as the Hawaiian Islands), or when hosts native to areas that lack the parasite or requisite mosquito vectors are moved (classically penguins displayed in temperate zoos). After infective sporozoites of *Plasmodium* are injected by the mosquito, the sporozoites invade local fibroblasts and macrophages and undergo asexual reproduction (merogony), releasing merozoites that invade macrophages for second generation merogony and dissemination.[2] Second-generation mero-

zoites invade capillary endothelial cells or hepatocytes in the liver (exoerythrocytic merogony). Released merozoites may re-invade endothelium or may invade erythrocytes for asexual merogony or sexual gametogenesis.

Leucocytozoon form large megalomeronts (megaloschizonts) in infected tissues, while *Haemoproteus* forms sinuous schizonts in lung capillary endothelium (both absent in this case). All three parasites have been reported in captive snowy owls.[4,5] The young age in this bird was likely a contributing factor. All three blood parasites have also been identified in other species of owls.[6,9] Hepatosplenomegaly with pigmentation is the classic gross appearance of malaria.

Centrilobular hepatocellular degeneration and necrosis in the liver is consistent with ischemia secondary to severe anemia. The centrilobular hepatocytes are located the furthest from the arterial blood supply, and consequently have the lowest oxygen delivery within the lobule.[3] Paradoxically, these hepatocytes also have the largest concentration of phase I cytochrome p450s.

Contributing Institution:
Department of Comparative Medicine
Penn State College of Medicine
Penn State Hershey Medical Center
http://www.hmc.psu.edu/comparativemedicine/

JPC Diagnosis:
1. Liver, spleen, macrophages and erythrocytes: Hemozoin pigment.

2. Liver: Necrosis, multifocal to coalescing, with hemorrhage and extramedullary hematopoiesis.

3. Spleen: Extramedullary hematopoiesis, multifocal, moderate, with erythrophagocytosis.

JPC Comment:
The contributor provides an excellent summary of three main erythroparasites of birds: *Plasmodium, Leucocytozoon* and *Haemoproteus*.

Avian malaria was discovered in 1885, shortly following Alfonse Laveran's discovery of protozoans as the causative agent of human malaria. As a result, avians were subsequently utilized as the primary animal model for malaria research until being overtaken by rodents following the discovery of *Plasmodium* parasites in Central African rats in 1948. By 1940, the genera responsible for avian malaria were separated into those utilized today: *Plasmodium, Haemoproteus,* and *Leukocytozoon.*[8]

In regard to *Plasmodium*, the parasite has been identified in over 800 species of birds and on every continent, with the exception of Antarctica where the mosquito vectors responsible for its transmission do not occur.

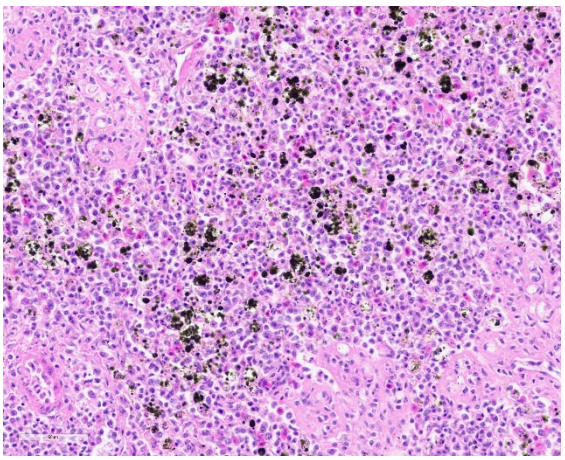

Figure 3-4. Spleen, snowy owl. Macrophages within the red pulp contain abundant granular black brown pigment (hemozoin). (HE, 400X)

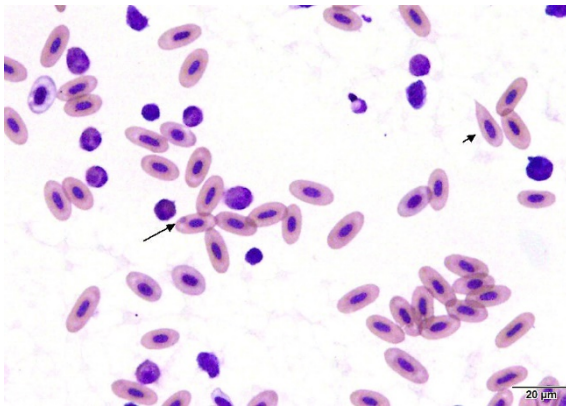

Figure 3-5. Blood smear, snowy owl. Erythrocytes contain malarial trophozoites (arrow), and there are occasional schistocytes (arrowhead.) (*Photo courtesy of*: Department of Comparative Medicine, Penn State College of Medicine, Penn State Hershey Medical Center, http://www.hmc.psu.edu/comparativemedicine/) (Wright-Giemsa, 1000X)

As noted by the contributor, species native to Antarctica relocated to temperate regions (such as penguins) are also susceptible.[8]

As with avian species, malaria continues to be a scourge of the human population with over 40% of the world's population at risk. In 2019, there were 229 million cases and 409,000 deaths attributed to the disease, with children most commonly affected. Dozens of species within genus *Plasmodium* have been identified within avian species while five are known to be pathogenic in humans: *P. falciparum, vivax, ovale, malariae,* and occasionally *knowlesi.* Amongst these five species, *P. falciparum* is the most prevalent and is often fatal.[7]

Ronald Ross, an army surgeon working in India in 1898, utilized avian models to conclusively prove malaria's transmission by mosquito vectors. Frustrated by a lack of willing human participants, Ross turned to avian models and later stated, "I should have been wise to have begun my researches in birds in 1895." His research demonstrated mosquitoes were required to obtain blood meals from separate hosts to complete the cycle of transmission. Following an initial blood meal from an infected host, male and female gametocytes ingested by the mosquito differentiate into gametes within the midgut and fuse, forming a zygote. Zygotes subsequently develop into motile ookinetes and migrate to the stomach wall where they form oocytes. After several days, each oocyte produces thousands of sporozoites that eventually localize to the mosquito's salivary glands, ready to infect the next susceptible vertebrate host from which the mosquito takes a second blood meal. Within the vertebrate host, *Plasmodium* sp. undergo the lifecycle previously described by the contributor, which facilitates subsequent transmission to additional mosquitoes, completing the cycle of transmission. Prior to this discovery, it was believed humans became infected by drinking water contaminated by dead infected mosquitoes.[8]

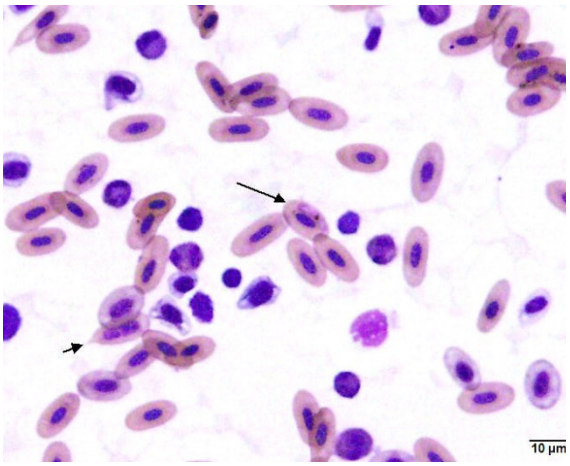

Figure 3-6. Blood smear, snowy owl. Erythrocytes contain hemozoin pigment (arrow), and there are occasional schistocytes (arrowhead). (*Photo courtesy of*: Department of Comparative Medicine, Penn State College of Medicine, Penn State Hershey Medical Center, http://www.hmc.psu.edu/comparativemedicine/) (Wright-Giemsa, 1000X)

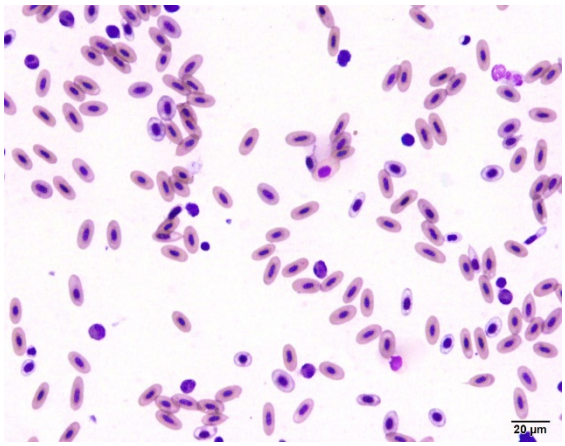

Figure 3-7. Blood smear, snowy owl. There is marked polychromasia. (*Photo courtesy of*: Department of Comparative Medicine, Penn State College of Medicine, Penn State Hershey Medical Center, http://www.hmc.psu.edu/comparativemedicine/) (Wright-Giemsa, 1000X)

Due to the disruption of quinine supplies from Indonesia during World War I, avians were utilized as the first animal models for the development of synthetic antimalarial medications such as plasmoquin and atabrin. In addition, early research in regard to development of antimalarial resistance was conducted during the 1920s and 1930s by administering gradually increasing doses of the medications to infected canaries.[8]

A hallmark feature of malaria is the presence of black pigment (hemozoin) in tissue sections, which was first noted by Meckel in 1847 in post-mortem blood and splenic samples. Virchow attributed this pigment to malaria two years later. The pigment was believed to be hematin (ferriprotoporphyrin IX hydroxide) until Carbone identified spectroscopic differences between hematin and hemozoin in 1891. Ronald Ross, whose aforementioned efforts proved the transmission cycle between mosquitoes and vertebrates, identified the pigment within the mosquitoes in 1897, further supporting the insect's role as a vector.[7]

Hemozoin plays a significant role in the survival of *Plasmodium* sp. The organism digests up to 75% of the hemoglobin within erythrocytes, resulting in the release of free heme (Fe^{II}-protoporphyrin IX) which left unsequestered induces the formation of free radicals as the result of free iron and the Fenton reaction. However, the parasite neutralizes this threat by dimerizing heme to form Fe ^{III}-protoporphyrin, which is then stored in insoluble sub-micron sized hemozoin crystals in parasitic digestive vacuoles. Interestingly, quinolines and other antimalarials inhibit the formation of hemozoin within the digestive vacuole, which eventually perforates with spillage of toxic substances such as iron into the parasite's cytoplasm.[7]

During the conference, participants discussed the significance of hemozoin pigment in tissue sections such as in this case. Although the presence of hemozoin significantly increases the index of suspicion of avian malaria, the pigment is not a pathognomonic lesion. As noted by the moderator, the parasite is often difficult to identify in tissue sections and advanced diagnostics, such as PCR, are often required for definitive diagnosis.

There was spirited discussion amongst participants in regard to the pattern of distribution of hepatic necrosis in this case, with multifocal to coalescing being preferred over centrilobular by a majority participants. However, the pathogenesis associated with this pattern of distribution is unclear given the most likely underlying cause of hepatic necrosis in this case is hypoxia secondary to anemia, which is typically associated with centrilobular necrosis. Although this pattern may have manifested as the result of a comorbidity, a battery of histochemical stains including Gram, acid-fast, Giemsa, periodic acid-Schiff, and rhodanine did not reveal

additional significant findings in examined sections.

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CASE IV: N2017-0533 (JPC 4117674).

Signalment:
Juvenile, undetermined sex, tentacled snake *(Erpeton tentaculatum)*

History:
Multifocal skin bumps were noticed on the skin of a juvenile tentacled snake, and two days later the snake was found dead.

Gross Pathology:
Along the skin, predominantly along the caudal half of the body, are dozens of multifocal, 0.1 cm to 0.2 cm, white to tan, soft to gritty, variably raised up to 0.1 cm, exophytic foci. On cut section these foci are limited to the scaled skin and do not involve subjacent tissue.

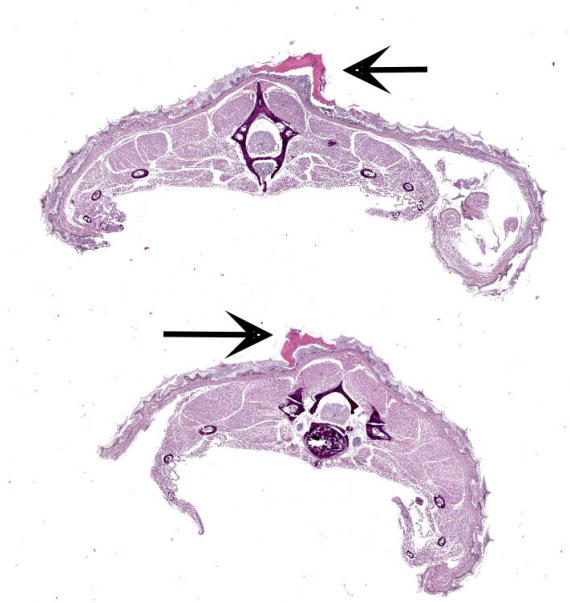

Figure 4-1. Scaled skin, tentacled snake. Multifocally, along the dorsal midline, there is partial to full thickness epidermal necrosis with replacement by an eosinophilic coagulum (arrows). (HE, 5X)

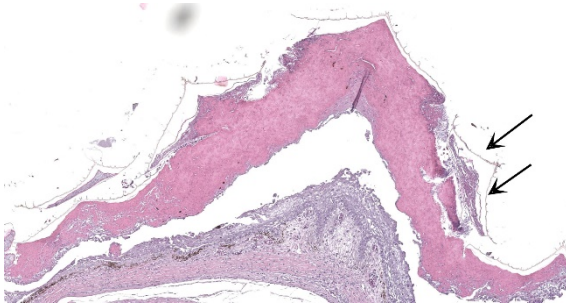

Figure 4-2. Scaled skin, tentacled snake. Higher magnification of epidermal necrosis extending into the deeper germinative layers (HE, 89X)

Laboratory Results:

Cytology: A touch impression of a skin lesion stained with Diff-Quik shows small aggregates of amorphous purple-staining keratinaceous debris mixed with numerous oval to pyriform conidia, rare elongating hyphal forms, and moderate mixed, extracellular bacteria (predominately bacilli with few cocci).

A fresh-frozen sample of the skin lesions was PCR positive for fungus at the Zoological Medicine and Wildlife Disease Laboratory at the University of Florida, and direct sequencing identified *Paranannizziopsis australasiensis.*

Microscopic Description:

Scaled skin: Multifocally, there is mild to marked expansion and replacement of the epidermis by dense, brightly eosinophilic, degenerate heterophils, necrotic debris, degenerate keratin and variably discernable shrunken or vacuolated keratinocytes with pyknotic nuclei. Within the necroinflammatory crusts are moderate numbers of non-pigmented fungal hyphae, approximately 3-5 µm wide, with parallel walls, regular septa, and frequent acute to right angle branching. Along the surface and often elevating the beta-keratin, are small to moderate numbers of oval-shaped conidia, approximately 2 x 4 µm. Small to moderate numbers of mixed bacilli and cocci bacteria, and occasional foreign debris are also present along the epidermal surface. The adjacent epidermis is moderately hyperplastic with mild to moderate intracellular edema and low numbers of infiltrating heterophils. Frequently, there is segmental retention of

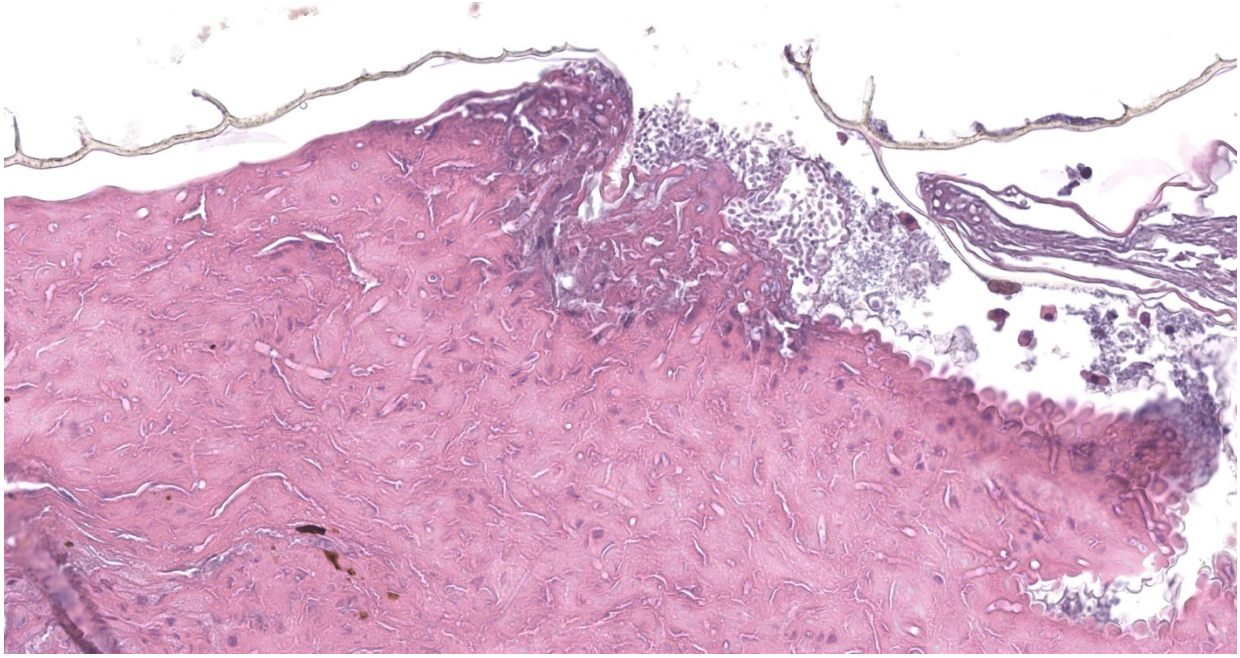

Figure 4-3. Scaled skin, tentacled snake. High magnification of the necrotic coagulum demonstrating large numbers of fungal hyphae, which form a mat at the surface of the necrotic material (arrow). (HE 381X)

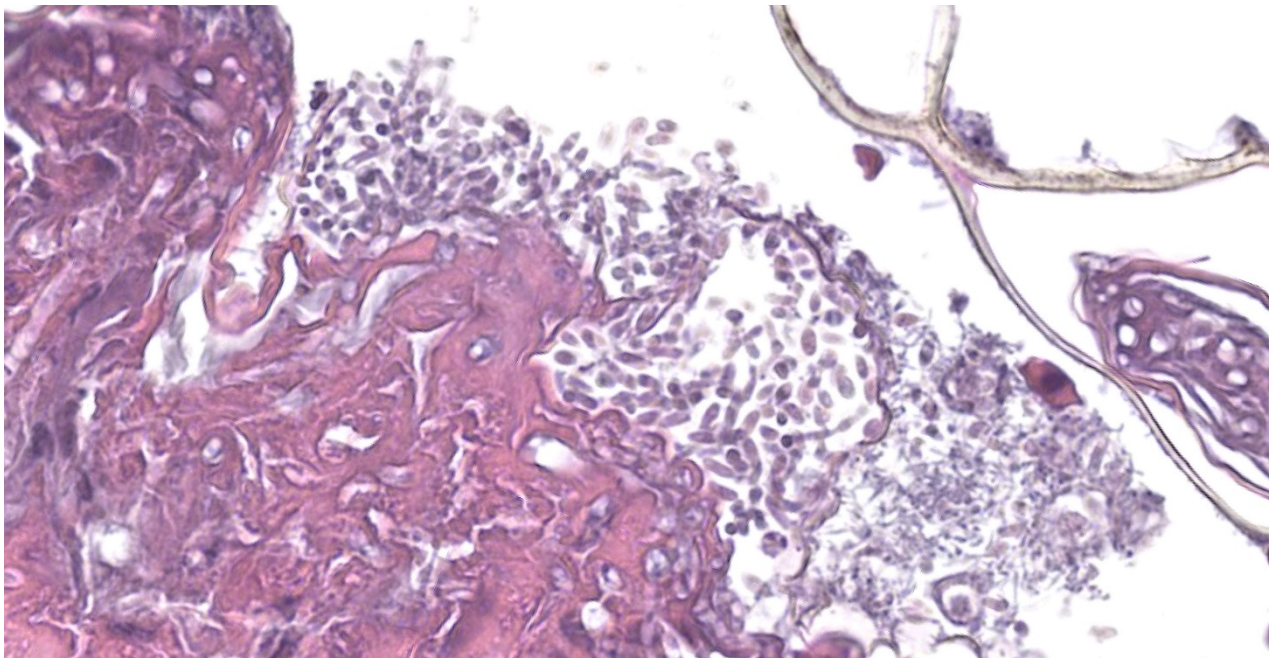

Figure 4-4. High magnification of numerous arthroconidia and septate fungal hyphae of *paranannizziopsis australensis.* (HE, 1000X)

the outer epithelial generation with a vacuolated lacunar tissue of outer generation, and separation from the beta-layer of inner generation (dysecdysis). Within the inner epidermis there is mild vacuolation of basal keratinocytes and occasional, mild, intercellular edema. The dermis is infiltrated by small to moderate numbers of heterophils, and small numbers of perivascular to interstitial lymphocytes and histiocytes.

Contributor's Morphologic Diagnoses:
Scaled skin: Epidermitis, heterophilic and necrotizing, multifocal, chronic, moderate with moderate numbers of intralesional fungal hyphae and conidia, moderate mixed superficial bacteria, moderate epidermal hyperplasia, mild lymphohistiocytic dermatitis, and mild to moderate dysecdysis.

Contributor's Comment:
This case demonstrates a mycotic dermatitis (dermatomyosis) caused by *Paranannizziopsis australasiensis.* While previously known under the name *Chrysosporium* anamorph of *Nannizziopsis vriesii* (CANV), more recent phylogenetic studies have separated CANV into the genera *Nannizziopsis*, *Ophidiomyces* and *Paranannizziopsis*.[6,8] Multiple different species of these genera are important causes of dermatitis in reptiles. Two of the more widely reported members of these genera include *Nannizziopsis guarroi,* the primary agent in "yellow fungus disease", a deep granulomatous dermatomycosis affecting bearded dragons, and *Ophidiomyces ophiodiicola* the causative agent of snake fungal disease (SFD). SFD is an important cause of morbidity and mortality in both captive and wild snakes, and is recognized as a major threat to wild snakes in the north central and northeastern United States.[3,6]

The fungus *Paranannizziopsis australasiensis,* has been recognized as a cause of dermatitis in reptiles (snakes, lizards and tuataras) in New Zealand and Australia; however, it has not been previously reported in North America.[1,2,5] Other *Paranannizziopsis* spp. (*P. californiensis, P. crustacea,* and *P. longispora)* are reported to

cause dermatitis in captive tentacled snakes in North America and are a significant infectious disease.[6]

All species in the Nannizziopsiaceae family produce single-celled aleurioconidia, and most species additionally produce arthroconidia. The production of conidia differs between fungal species and between in vitro versus in vivo environments. Aleurioconidia can take multiple shapes (e.g., oval, pyriform, clavate), whereas arthroconidia are classically cylindrical and appear rectangular on side view.[6,8] In culture, *P. australasiensis* is reported to produce pyriform to clavate aleurioconidia and occasional arthroconidia.[8]

Dermatomycosis in snakes can result in extensive disruption of the skin with subsequent secondary bacterial infections, dysecdysis, and osmotic imbalances. Aquatic snakes, such as tentacle snakes, may be particularly susceptible to osmotic imbalances associated with cutaneous lesions.[1] Additionally, lesions on the head and around the mouth, which have been reported to be particularly severe with *Ophidiomyces ophiodiicola,* can result in facial deformity and emaciation.[3,6]

Contributing Institution:
https://www.wcs.org/

JPC Diagnosis:
Scaled skin: Epidermitis, necrotizing, multifocal, marked with numerous fungal hyphae and arthroconidia.

JPC Comment:
The contributor provides an excellent overview of three significant causes of mycotic dermatitis in reptiles formerly classified as *Chrysosporium* anamorph of *Nannizziopsis vriesii* (CANV). In addition to snakes, these etiologies have been reported in multiple species including crocodilians, lizards, and tuataras.[9]

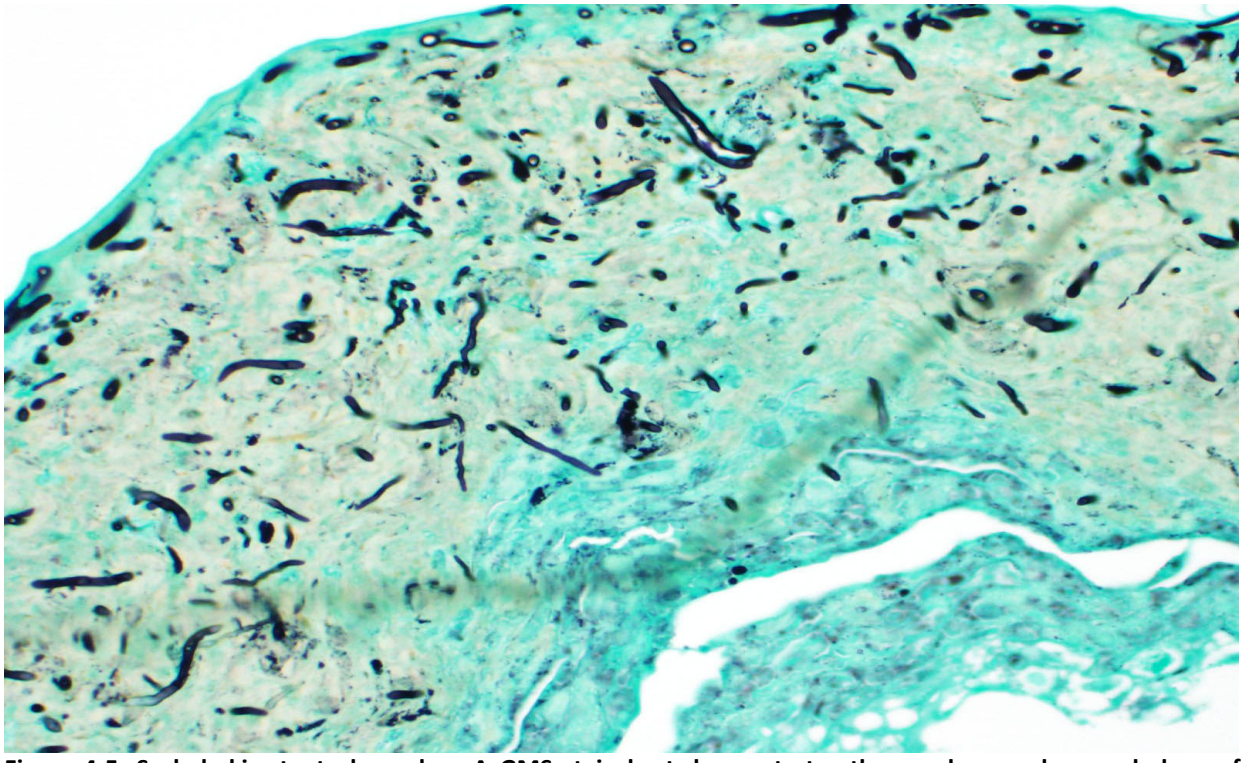

Figure 4-5. Scaled skin, tentacle snake. A GMS stain best demonstrates the numbers and morophology of arthroconidia and septate fungal hyphae of *Paranannizziopsis australensis.* (HE, 1000X)

Tentacled snakes (*Erpeton tentaculatum*) are an aquatic species commonly sought by zoological collections for exhibits due to their unique appearance and feeding behavior. A 2021 review[4] of 125 pathology reports of tentacled snakes at multiple New York City zoos between 1966 and 2017 found over half the deaths in this species were attributed to infectious disease. Over half (n=40) of these deaths were due to fungal dermatitis, with the majority of cases of fungal dermatitis demonstrating histo-morphology consistent with *Paranannizziopsis* spp. Fungal dermatitis was followed by bacterial infection as a common cause of death (n=21), with 7 of these cases attributed to *Mycobacterium* spp.[4]

In addition to the species previously listed by the contributor, an additional species (*P. tardicrescens*) has been identified following the submission of this case to the WSC in 2018. Before this discovery, North American cases were restricted to only captive tentacle snakes with identified species included *P. crustacea, P. californiensis, P. longispora*, and *P. australasiensis*. Although the newly identified *P. tardicrescens* was identified in two tentacle snakes, the pathogen was also identified in two additional snake species, a Wagler's viper (*Tropidolamemus wagleri*) and a rhinoceros snake (*Rhynchophis boulengeri*) that had been housed in the same room prior to developing lesions. Although the source and route of transmission with these cases is unknown, a misting system servicing terrestrial aquaria adjacent to tentacled snake enclosure may have inadvertently resulted in the pathogen's distribution via water droplets. Given this finding, it is suspected *P. tardicrescens* is capable of spreading via fomites, highlighting the need for basic but strict biosecurity measures such as cleaning and disinfecting tools, workspaces, scales, as well as wearing and changing gloves between enclosures and hand washing. In addition, the water in both tentacled snake enclosures was found to have a pH of >7, which may have predisposed these snakes to infection. Wild tentacle snakes are typically found in slightly acidic water which is ideally mimicked in captivity by maintaining a pH range of of 6.0-6.5.[7]

P. tardicrescens is unique from other *Paranannizziopsis* species in that it demonstrates a variable growth pattern when cultured at 35ºC depending on the medium while other species now no growth or strong growth restriction. Additional distinguishing features of *P. tardicrescens* include the production of urease and lack of undulate hyphal branches. In contrast, both *P. australasiensis* and *P. californiensis* are weak urease producers and *P. crustacea* has undulant hyphal branches.[7]

Although *Nannizziopsis*, *Ophidiomyces*, and *Paranannizziopsis* have been found to infect multiple reptilian species, they have not been found to infect aquatic turtles (order Testudines). However, another notable species within fungal order Onygenales is *Emydomyces testavorans.* This species was recently identified following its isolation from ulcerative shell lesions of freshwater aquatic turtles, including a variety of zoological collections as well as wild western pond turtles (*Actinemys marmorata*) in Washington State. *E. testavorans* forms narrow, septate hyphae similar to *Nannizziopsis*, *Ophidiomyces*,and *Parananni zziopsis*, however, arthroco-nidia are not observed in sections of shell or tissue lesions. In addition, conidia formed in culture are smaller than those of the previously described fungi.[9]

The moderator noted tentacle snakes infected with *Paranannizziopsis* spp. most commonly develop facial lesions whereas the previously

described Wagler's viper and rhinocerous snakes developed lesions on the lateral aspect of the body. In addition, the most common cause of death of tentacle snakes infected with this entity is drowning.

The subject of dysecdysis (abnormal or impaired shedding of skin) was a topic of discussion amongst participants in while discussing this case. The prevailing opinion amongst the majority was dysecdysis could not be differentiated from ecdysis (the normal periodic process of sloughing and renewal of skin) due to lack of more definitive features such as inflammation associated with retained outer generational layers of skin.

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