Joint Pathology Center
Veterinary Pathology Services

WEDNESDAY SLIDE CONFERENCE 2023-2024

Conference #11 29 November 2023

CASE I:

Signalment:
20-month-old female spayed Domestic Shorthair cat, feline (*Felis catus*)

History:
Sudden death with no previously identified clinical signs. The submitting veterinarian suggested congestive heart failure, feline infectious peritonitis, or toxin exposure as possible differentials.

Gross Pathology:
The subcutaneous and internal fat stores were mildly icteric. The liver was small and diffusely orange to brown with multifocal, slightly raised yellow nodules on the hepatic surface and throughout the parenchyma on cut section. An impression smear of the liver showed frequent spindle cells suggestive of fibroblasts or oval cells, and cells with multiple small, clear cytoplasmic vacuoles suggestive of hepatic lipidosis.

Laboratory Results:
A sample of fresh liver was submitted for mass spectrometry. A copper level of 611.2 ppm on a wet matter basis was found (normal <45ppm), which is equivalent to 2139.2 ppm on a dry matter basis.

Microscopic Description:
Liver: There are large coalescing areas of degeneration and necrosis of hepatocytes

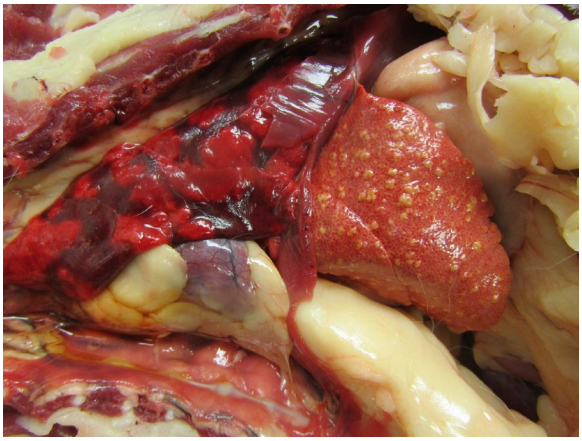

Figure 1-1. Liver, cat. The liver was small and diffusely orange to brown with multifocal, slightly raised yellow nodules on the hepatic surface. (*Photo courtesy of:* Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Dr, Saskatoon, SK, Canada, S7N 5B4, https://wcvm.usask.ca/departments/vet-pathology.php)

affecting all zones (massive pattern) interspersed with randomly distributed, well-delimited nodules of viable hepatocytes. Portal areas and periacinar regions are close to each other (parenchymal collapse, loss of hepatocytes). Hepatocytes show marked distension of cytoplasm by small to large vacuoles (microvesicular to macrovesicular steatosis, positive Oil-red-O staining), ill-defined borders, granular eosinophilic cytoplasm, a prominent nucleus characterized by pyknosis, karyorrhexis or karyolysis. Endothelial cells are often plump (reactive) and there are reactive spindle cells (perisinusoidal cells), free pyknotic debris, and ill-

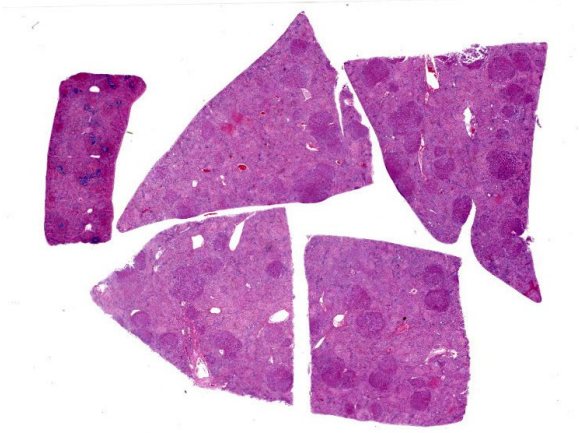

Figure 1-2. Liver, cat. Four sections of liver and one section of spleen are submitted. At subgross magnification, areas of pallor and few regenerative nodules are evident. (HE, 5X)

defined ducts (oval cell hyperplasia). Large numbers of Kupffer cells with pale brown granules and rare red blood cells are present and similar pigment is seen in few hepatocytes.

The nodules consist of thick, disorganized hepatic plates, large hepatocytes with fine cytoplasmic vacuolation (fat, positive Oil-red-O staining), and one to two prominent nuclei. Bile canaliculi are distended by bile plugs and Kupffer cells contain large amounts of pale brown pigment (nodular regeneration). Portal areas are infiltrated by small numbers of plasma cells and lymphocytes, small amounts of collagen, and there is bile duct and oval cell hyperplasia with extension and destruction of the limiting plate. There is multifocal bridging portal-to-portal fibrosis. Terminal hepatic venules are difficult to discern. Sublobular veins are often infiltrated by small numbers of plasma cells and fat-laden macrophages. There are few foci of extramedullary hematopoiesis.

Special stains: There are large numbers of copper granules with rhodanine stain in macrophages and to a lesser degree in hepatocytes in areas of necrosis. There is a very small amount of copper in areas of regeneration. This is accompanied by large number of positive granules on Schmorl's stain (lipofuscin) and there is a moderate amount of hemosiderin in macrophages on Perl's stain. There is mild collapse and mild to moderate thickening of reticulin fibers in periacinar regions. These areas correspond to deposition of collagen with Masson's trichrome stain. There is a small amount of collagen in periportal parenchyma.

Contributor's Morphologic Diagnoses:
Liver: Hepatic fatty degeneration and necrosis, acute to subacute, with bile duct hyperplasia, fibrosis, nodular regeneration.

Contributor's Comment:
Primary copper toxicity has been well-described in dogs and humans; however, it is poorly described in cats.[3] The most well-known primary copper toxicity of animals occurs in Bedlington terriers, which has an autosomal recessive inheritance pattern that may be linked to a deletion in the *COMMD1* genes.[1] Similarly, Wilson's disease in humans is an autosomal recessive disorder that affects the *ATP7B* gene, which affects secretion of copper.[1] Both genes are used in copper secretion into the blood and bile.[2] Without proper copper secretion, copper accumulates in hepatocytes, leading to increased

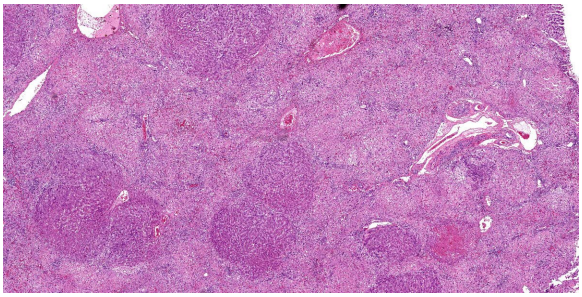

Figure 1-3. Liver, cat. Regenerative nodules are evident in areas of hepatocellular degeneration and necrosis. (HE, 30X)

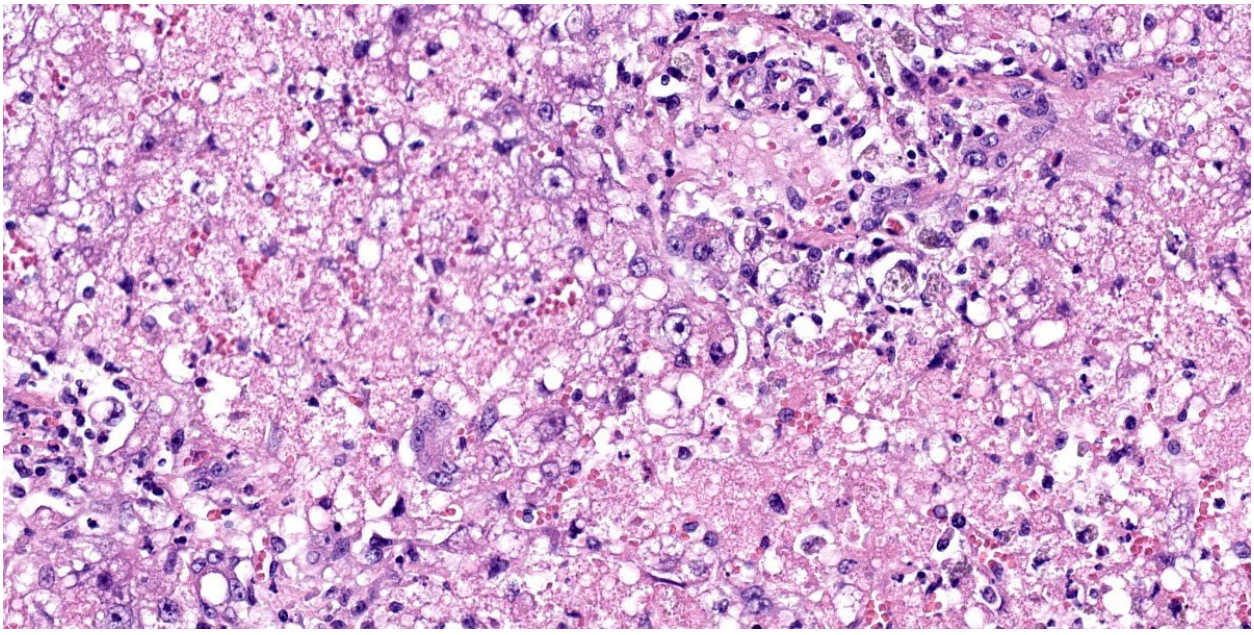

Figure 1-4. Liver, cat. There are large areas of hepatocellular degeneration and necrosis with infiltration of small numbers of neutrophils. (HE, 340X)

cellular oxidative stress that quickly uses up intracellular glutathione.[2] Ultimately, oxidative stress leads to hepatocyte necrosis.[2]

Previous reports in cats have been infrequent, but are often diagnosed based on being young, having histological findings consistent with copper toxicity, and no history of excessive copper intake.[1,4,5] Reported histological findings in these reports include regenerative nodules, hepatic fibrosis, and hepatocytes containing brown granules that stain positively on rhodanine stain.[1,5] These hepatocytes were most frequently found in the centrilobular areas.[1,5] Similar rhodanine-positive granules were identified in the kidney and lung.[4] These findings are similar to copper toxicity in dogs.[1,5] In a study examining 104 feline liver biopsies, only 5 cases were identified with centrilobular copper.[7] A similar study examining liver samples from 100 cases of hepatic disease in cats found 11 presumed cases of primary copper toxicity.[5] Both of these studies suggest that primary copper toxicity is very rare.[5,7]

To date, the causative gene and potential inheritance pattern of primary copper toxicity in cats is unknown. One reported case of two sibling cats with primary copper toxicity showed two single-nucleotide variations in the *ATP7B* gene, the same gene affected in humans.[1] A subsequent study identified single-nucleotide variations in *ATP7B* in three of four cats examined, suggesting this is the most likely cause of primary copper toxicity in cats.[2]

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JPC Diagnosis:
1. Liver: Hepatocellular degeneration and necrosis, massive, with marked hepatocellular lipidosis.
2. Liver: Nodular hepatocellular regeneration, multifocal, moderate with mild biliary hyperplasia.

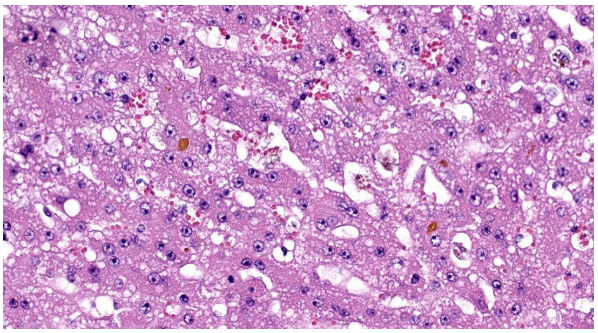

Figure 1-5. Liver, cat. Remaining hepatocytes contain abundant lipid vacuoles and there is cholestasis. (HE, 340X)

JPC Comment:

Copper is an essential heavy metal that is required for the proper function of a wide variety of enzymes, including the cytochrome oxidases (mitochondrial respiration), lysyl oxidase (collagen synthesis), tyrosinase (melanin synthesis), ceruloplasmin (iron synthesis), and antioxidant defense (superoxide dismutase).[3] In excess, the multiple redox states of copper can lead to free radical production and oxidative damage to cellular components, and animals have responded to this threat by developing regulatory mechanisms to ensure excess copper is rendered inert and excreted.

Copper metabolism begins in the gut, where dietary copper is actively transported across the mucosal surface of small intestinal enterocytes. This function is performed by a specific copper transporter, Ctr1, and by the non-specific transporter, divalent metal transporter (DMT1), which also transports several trace elements (e.g., iron and zinc) with which copper competes for transport. Once in the enterocyte, copper is incorporated into enzymes or, if in excess, bound to metallothionein and stored in lysosomes to protect the cell from free copper.[6] Once metallothionein is saturated, copper is excreted into the blood, where it is bound to the carrier proteins ceruloplasmin and transcuprein, or to albumin, and transported to the liver via the portal blood.[6]

Copper enters the hepatocyte via the same Ctr1 transporter found in the enterocytes. Once in the hepatocyte cytoplasm, chaperone proteins send copper to mitochondria and to the Golgi body, where copper is transported into the Golgi by the transporter ATP7B. ATP7B directs the incorporation of copper into ceruloplasmin for return to the systemic circulation and distribution to other tissues.[6] If the ceruloplasmin/copper complex returns to the liver, it is taken up into hepatocytes and secreted into bile for excretion. If copper accumulates in excess of metabolic requirements within the hepatocytes, it is complexed with metallothionein or glutathione and stored within lysosomes as in enterocytes.[6]

Perturbations in any of the above processes, such as a primary metabolic defects in hepatic copper metabolism, altered hepatic biliary excretion of copper, or excess dietary copper intake, can result in hepatic copper toxicosis.[3] Species susceptibility to copper toxicosis varies widely, with sheep being most prone to copper poisoning due to a lack of sufficient capacity to excrete copper into the bile and to a very small copper metallothionein binding capacity.[3] When faced with a sudden dietary excess, biliary excretion can't keep up with the influx of copper, leading to very high levels of copper deposition within the liver. When the binding capacity of metallothionein is exceeded, a sudden, generally fatal intravascular hemolytic crises can occur.[3,6]

The contributor provides an excellent survey of the few cases of feline copper toxicity

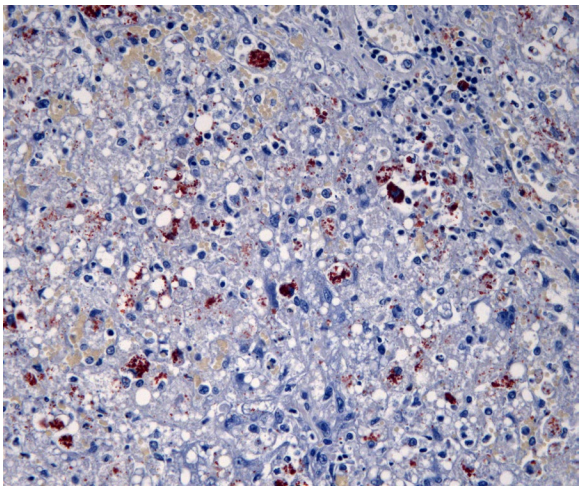

Figure 1-6. Liver, cat. There are small to moderate amounts of copper, but only in macrophages. (Rhodanine, 200X)

reported in the veterinary literature which, for all their rarity, present with familiar histologic features: severe fibrosis, regenerative nodules, and excessive intracytoplasmic copper as evidenced by rhodanine staining. Copper toxicity does not, however, typically present with abundant hepatic necrosis, and the significant necrosis in the examined section provoked robust discussion before, during, and after this week's conference.

The moderator of today's conference was Dr. Anna Travis, Chief of Education Operations at the Joint Pathology Center, who began discussion of this case with a review of copper metabolism, as summarized above, and a review of the typical histologic changes associated with copper toxicity. Conference participants noted the small amount of fibrosis evident on Masson trichrome stain, which was surprising given the significant number of regenerative nodules.

Conference participants also noted the abundant, multifocal to coalescing hepatic necrosis, which for many was the predominate histologic feature of this case. The abundant necrosis also hampered qualitative evaluation of the rhodanine staining, which, in the remaining intact hepatocytes, appeared sparse in relation to the incredibly high copper levels measured in this patient. While participants agreed that the amount of rhodanine staining was abnormal, many felt that the amount of staining was not consistent with the dry weight of copper value obtained in the lab results. Finally, the participants felt that a second, acute insult resulted in widespread necrosis which may have obscured a more traditional pattern of feline copper toxicity.

This case was sent for post-conference consult with Dr. John Cullen, Distinguished Professor at the North Carolina State University College of Veterinary Medicine. Dr. Cullen agreed that the histologic picture in this case is not a perfect match for copper toxicity as a sole insult. Copper accumulation in reported feline cases is typically centrilobular and has a substantial inflammatory component, neither of which is apparent in this case.

Though a tidy resolution for this case remains elusive based on a complex interplay of acute and chronic pathogeneses, conference participants continue to believe that the primary lesion in this case is an acute, necrotizing insult, the cause of which is not apparent. The excessively high copper dry weight almost certainly contributed to the observed pathology in this case; however, the substantial acute necrosis is confounding, and participants preferred to separate the morphologic diagnoses into acute and chronic effects to emphasize the presumed dual pathologic processes in this fascinating case.

Figure 2-1. Presentation, Pacific pond turtle: Multifocal coalescing ulcers and depressions are present along the midline of the carapace, as well as on the plastron, where there is occasional bone exposure. (*Photo courtesy of:* Disease Investigations, Institute for Conservation Research, San Diego Zoo Wildlife Alliance, http://institute.sandiegozoo.org/disease-investigations)

Getting short shrift this week was the forlorn section of spleen, located literally and figuratively on the periphery and characterized by mild to moderate chronic diffuse congestion with hemosiderin-laden macrophages.

References:

1. Asada H, Kojima M, Nagahara T, et al. Hepatic copper accumulation in a young cat with familial variations in the *ATP7B* gene. *J Vet Intern Med*. 2019;33:874–878.
2. Asada H, Chambers JK, Kojima M, et al. Variations in ATP7B in cats with primary copper-associated hepatopathy. *J Feline Med Surg*. 2020;22:753-759.
3. Cullen JM, Stalker MJ. Liver and Biliary System. In: Maxie GM, ed. Vol. 2, *Jubb, Kennedy, and Palmer's Pathology of Domestic Animals.* Elsevier, Inc;2016: 302, 342-343.
4. Haynes JS, Wade PR. Hepatopathy associated with excessive hepatic copper in a Siamese cat. *Vet Pathol*. 1995; 32:427–429.
5. Hurwitz BM, Center SA, Randolph JF, et al. Presumed primary and secondary hepatic copper accumulation in cats. *J Am Vet Med Assoc*. 2014;244:68–77.
6. Lopez-Alonso M, Miranda M. Copper supplementation in cattle: a challenge. *Animals (Basel)*. 2020;10(10):1890.
7. Whittemore JC, Newkirk KM, Reel DM, et al. Hepatic copper and iron accumulation and histologic findings in 104 feline liver biopsies. *J Vet Diagn Invest*. 2012; 24:656–661.

CASE II:

Signalment:

17-year-old, male, intact, Pacific pond turtle (*Actinemys marmorata pallida*)

History:

A wild-hatched turtle housed at the zoological institution had a history of severe chronic pitting and ulcerated lesions on the shell for over a year. Recently, it had a rapid clinical decline with anorexia and obtunded mentation. Given the poor prognosis, humane euthanasia was elected.

Laboratory Results:

Panfungal (ITS3-ITS-4) PCR assay on frozen shell yielded a 203 base pair fragment with 100% identity and 100% query cover

Figure 2-2. Dorsal aspect of the head, Pacific pond turtle. The skin has a focal brown crusty depression on the dorsal aspect (arrowhead). (*Photo courtesy of:* Disease Investigations, Institute for Conservation Research, San Diego Zoo Wildlife Alliance)

age to MG780506, *Emydomyces* sp. isolate 13-1796.

Microscopic Description:
Head, dorsal: Extending from a focal ulcer on the skin surface and through the dermis to efface 60% of the dorsal calvarium, expand the meninges, and invade into the brain, as well as extending ventrally along the lateral aspect of the skull, are numerous coalescing epithelial inclusion cysts containing fungal hyphae. The epithelial inclusion cysts are lined by keratinized squamous epithelium and contain lamellated keratin, necrotic debris, entrapped necrotic bony fragments, fungal hyphae, and mixed bacteria. The fungal hyphae are 1 to 4 µm in diameter, regularly septated, and have acute to right-angle branching. The effaced bone has empty lacunae with loss of osteocytes (osteonecrosis), with moth-eaten margins and osteoclasts in Howship's lacunae (osteolysis).

The affected meninges are also expanded by increased fibrous tissue. In this area, the epithelial inclusion cysts penetrate into the brain parenchyma and are associated with moderate heterophilic and histiocytic inflammation, necrotic debris, and rarefaction of the neuropil. In other sections, fungal invasion disrupts the ependymal lining of the ventricle. Variable numbers of heterophils, histiocytes, and lymphocytes are associated with the cysts and fungi throughout the section, but inflammation is most prominent in the skeletal muscle along the lateral aspect of the skull, where there are numerous, coalescing fungal heterophilic granulomas.

Contributor's Morphologic Diagnosis:
Multiple tissues, dorsal head: Severe, regionally extensive, chronic-active, necroulcerative heterophilic and granulomatous dermatitis, myositis, osteomyelitis, and meningoencephalitis with epithelial inclusion cysts, osteonecrosis and osteolysis, and intralesional fungal hyphae (consistent with *Emydomyces* sp., presumably *E. testavorans*).

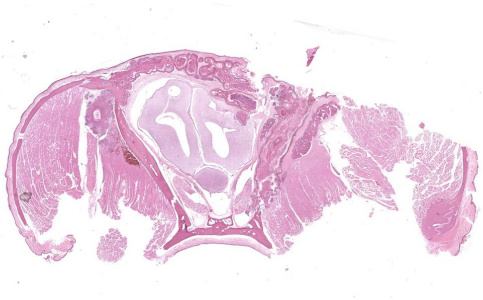

Figure 2-3. Head, Pacific pond turtle. A cross section of the head through the olfactory lobes of the brain is submitted for examination. There are numerous epithelial inclusion cysts within the dermis that extend downward to replace the bone of the calvarium and compress the underlying cerebrum. (HE, 5X)

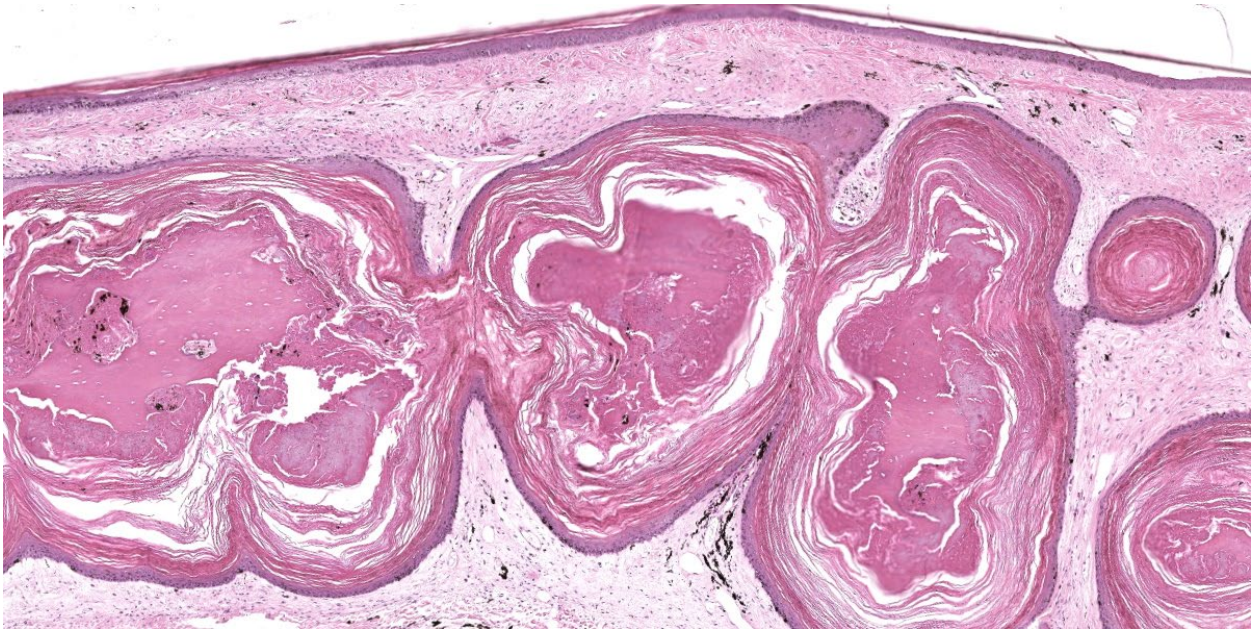

Figure 2-4. Head, Pacific pond turtle. Epithelial inclusion cysts measure 2mm in diameter, often coalesce, and are lined by 4-5 layers of basally pigmented epithelium which undergoes gradual keratinization. The cyst lumina contain abundant lamellated keratin, fragments of necrotic bone, and brightly eosinophilic cellular debris. (HE, 69X)

Contributor's Comment:

Emydomyces testavorans, a newly described fungus, is strongly associated with 'ulcerative shell disease' or 'pond turtle shell disease' in free-ranging and captive aquatic turtles in North America.[2,4,5] The classical shell lesions associated with emydomycosis are ulcerations in the carapace and/or plastron with increased pliability. In some cases, the turtles have expansile nodular masses within the shell, displacing the coelomic membrane, distorting the shell contour, and compressing internal viscera.[4] The present case has an unusual presentation, in which the fungal infection affected the tissues of the head, in addition to the shell, and invades deeply into the brain causing meningoencephalitis with antemortem neurological signs.

Histologic features associated with *E. testavorans* infection include multilocular intradermal and intraosseous epithelial inclusion cysts, which are lined by keratinized stratified squamous epithelium and contain keratin debris and necrotic bone.[2,4] The cysts are also associated with squamous metaplasia, hyperkeratosis, osteonecrosis, and inflammation, all of which are consistent with the present case of the Pacific pond turtle in our collection.[4] Although *E. testavorans* is strongly associated with ulcerative shell disease in freshwater aquatic chelonians, causation has not been proven through experimental infections.

Very little is known about the pathogenesis and lesion progression of emydomycosis, and it is unclear if it is a primary pathogen or if a combination of factors is required to elicit disease. Reported histologic features do not appear to be consistently pathognomonic for *E. testavorans* infection. Instead, they could be non-specific and indicative of a chronic healing process with cysts representing walling off of the damaged tissue or

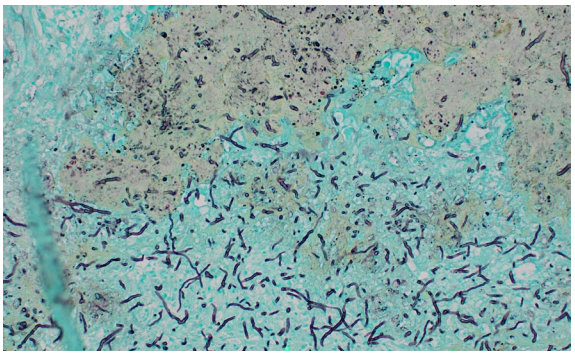

Figure 2-5. Head, Pacific pond turtle. A GMS stain on the inclusion cysts demonstrates numerous 2-4µm diameter, parallel-walled, dichotomously branching, septate fungal hyphae. (GMS, 200X)

dyskeratosis.[4] Other differential diagnoses for ulcerative shell lesions in aquatic turtles include septicemic cutaneous ulcerative disease (SCUD), which is caused by a combination of trauma, bacterial infection, and poor water quality. Epithelial inclusion cysts have also been reported in one case of mycobacteriosis, with no evidence of concurrent fungal infection.[4]

Emydomyces testavorans is in the order Onygenales, along with other emerging pathogenic reptile keratinophilic fungi, such as *Ophidiomyces ophiodicola* and *Nannizziopsis guarroi*.[5] Presumably, like many fungi in this order, *E. testavorans* is also an environmental saprophyte.[5] *Emydomyces testavorans* was first reported in free-living turtles in California in 2020, but has been isolated from turtles in Illinois and Washington in samples from as early as 2016, suggesting a potentially broad geographic distribution.[2,5] As Pacific pond turtles are listed as a vulnerable species, there is some concern that this recently described and likely underdiagnosed disease could pose a significant threat to already at-risk populations.[1]

Contributing Institution:
Disease Investigations
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JPC Diagnosis:
Head: Epithelial inclusion cysts with osteonecrosis, granulomatous dermatitis, cellulitis, myositis, and meningitis, and fungal hyphae.

JPC Comment:
As the contributor notes, the key histologic features of emydomycosis are ulcerative dermatitis, necrotizing osteomyelitis, and inclusion cysts lined by keratinized stratified squamous epithelium containing keratin debris. Fungal hyphae that are thin-walled, regularly septate, and occasionally branched can be demonstrated with GMS or PAS staining, making for a relatively straightforward histologic diagnosis.[5] Grossly, however, the lesions of emydomycosis may be difficult to identify as they can be highly local-

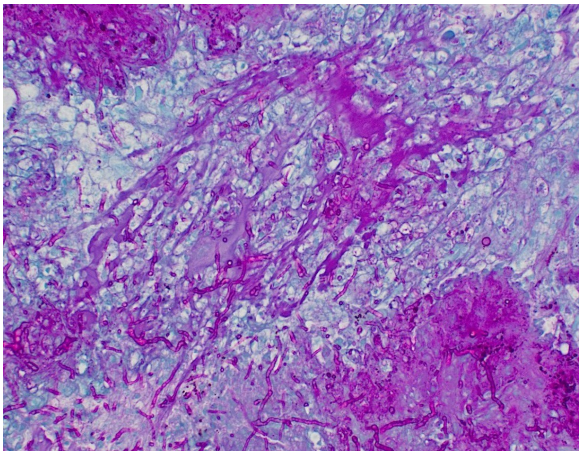

Figure 2-6. Head, Pacific pond turtle. Morphology of the fungal hyphae are better observed with a PAS stain (with malachite green)

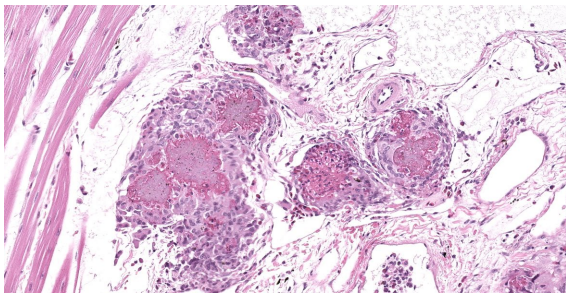

Figure 2-7. Head, Pacific pond turtle. In some areas, such as infiltrated skeletal muscle, there are fungal granulomas without inclusion cyst formation. (HE, 200X)

ized lesions and may lurk under the shell surface after the initial shell defect has healed over.[2] The contributor describes typical gross lesions in the excellent summary above, but gross lesions may also be rather subtle, and consist only of carapace flaking or bleaching.[2] In a recent study of affected pond turtles in Washington state, emydomycosis was diagnosed based on external exam in 25-50% of wild caught animals, but in greater than 80% of animals based on CT scans, which revealed the inclusion cysts and osteolytic lesions that characterize the disease.[2]

Emydomycosis presents similarly to its main differential diagnosis, septicemic cutaneous ulcerative disease (SCUD), which can cause ulcerated lesions on the plastron and carapace, petechial hemorrhage, emaciation, lethargy, and death.[3] SCUD is currently considered a multifactorial disease caused by poor water conditions and environmental stressors which predispose the animal to bacterial infection. SCUD was originally associated with *Citrobacter freundii* infection, but it is now understood that a variety of gram-negative organisms can cause the syndrome.

Conference discussion focused on reptilian dermatomycoses generally and on specific dermatologic disease of chelonids. In addition to SCUD and mycotic shell disease caused by *E. testavorans*, participants also discussed fibropapillomatosis in sea turtles, caused by chelonid herpesvirus-5, which are characteristic tumors of the skin around the eyes, mouth, limbs, shell, and cornea. These tumors can vary in appearance from flat, plaque-like lesions to exophytic or verrucous masses. They may also form on internal organs and grow large enough to impair buoyancy, leading to death.

Discussion of the morphologic diagnosis centered on whether the rather exuberant epithelial inclusion cysts were invading or merely compressing the brain. Participants felt the brain was only being compressed and thus preferred a diagnosis of meningitis rather than meningoencephalitis.

References:

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2. Lambert MR, Hernandez-Gomez O, Krohn AR, et al. Turtle shell disease fungus (*Emydomyces testavorans*): first documented occurrence in California and prevaelence in free-living turtles. *Ichthyology & Herpetology*. 2021;109 (4):958-962.
3. Mitchell MA, Tully TN. *Current therapy in exotic pet practice*. Elsevier;2016.
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5. Woodburn DB, Miller AN, Allender MC, Maddox CW, Terio KA. *Emydomyces testavorans*, a new genus and species of Onygenalean fungus isolated from shell lesions of freshwater aquatic turtles. *J Clin Microbiol*. 2019;57(2).

CASE III:

Signalment:
Fetus aborted at approximately 120 days of gestation, ovine, (*Ovis aries*)

History:
In July 2015, two of approximately 200 pastured sheep in a family farm in Colonia, Uruguay, aborted three fetuses at approximately 4 months of gestation.

Gross Pathology:
The fetus was a female, with a crown-to-rump length of 32 cm (estimated gestational age: 120 days) with a complete wool/hair coat, in a moderate state of post-mortem decomposition (moderate autolysis). The fetus was fully formed, with a complete wool/hair coat and no external abnormalities. The liver was moderately enlarged, with rounded edges, and there were numerous, discrete, white to yellowish, pinpoint to ≤2 mm foci throughout the hepatic parenchyma with a multifocal widespread (disseminated) distribution (necrotizing hepatitis). There were fibrin strands in the abdominal and pericardial cavities (moderate fibrinous peritonitis and pericarditis). No other gross lesions were noted. The lungs were unventilated/unexpanded, and there were no colostrum/milk curds in the lumen of the abomasum.

Laboratory Results:
IHC using a monoclonal primary antibody against *Francisella tularensis* lipopolysaccharide (LPS) revealed abundant and strong immunoreactivity colocalizing extracellularly with the hepatic lesions and intracellularly in infiltrating inflammatory cells.

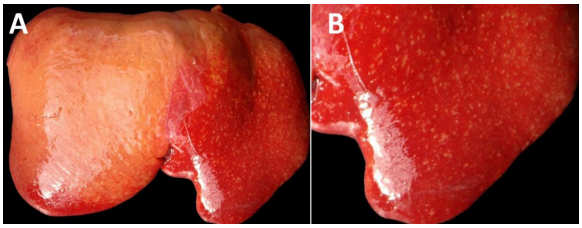

Figure 3-1. Liver, ovine abortus. The liver contains numerous miliary foci of necrosis. (Photo courtesy of: The University of Liverpool (Reprinted from: Giannitti F, Dorsch MA, Schild CO, Caffarena RD, Sverlow K, Armién AG, Riet-Correa F. Pathologic and immunohistochemical evidence of possible Francisellaceae among aborted ovine fetuses, Uruguay. Emerg Infect Dis. 2023 Jan;29(1):141-144.)

Microscopic Description:
Liver: Randomly throughout the parenchyma, there are multiple foci of necrosis and inflammation. These foci are characterized by disruption and effacement of the hepatic cord architecture and replacement by necrotic cell debris; the hepatocytes have angular cell borders, hypereosinophilic cytoplasm, and either nuclear pyknosis or karyorrhexis (necrosis). Within these foci, there is an accumulation of eosinophilic fibrillar material (fibrin), and inflammatory cell infiltrates, mostly neutrophils and macrophages. There is an increased number of neutrophils in the sinusoids (circulating neutrophilia).

Contributor's Morphologic Diagnosis:
Liver: Hepatitis, necrotizing and fibrinosuppurative, multifocal widespread, random, severe.

Figure 3-2. Liver, ovine abortus. One section of liver is submitted for examination. (HE, 5X)

Contributor's Comment:
The hepatic lesions in this fetus were suggestive of an infectious etiology. In addition, the microscopic examination of other fetal tissues revealed multifocal neutrophilic myocarditis, multifocal neutrophilic bronchiolitis and alveolitis, and fibrinous splenic capsulitis (peritonitis). Altogether, these lesions were suggestive of a bacterial cause.

Ancillary testing to assess for possible abortifacients of sheep, in this case, included immunohistochemistry (IHC) for *Chlamydia* spp., *Listeria monocytogenes*, *Coxiella burnetii*, *Salmonella enterica*, and *Toxoplasma gondii*, all of which yielded negative results. Additionally, no curved bacilli (i.e., *Campylobacter*), spirochetes (i.e., *Leptospira*, *Flexispira*) or fungi were identified in sections of liver stained with Steiner silver stain and Gomori´s methenamine silver stain, and no intralesional bacteria were visualized with tissue Gram stain. However, IHC using a monoclonal primary antibody against *Francisella tularensis* lipopolysaccharide (LPS) revealed abundant and strong immunoreactivity colocalizing extracellularly with the hepatic lesions and intracellularly in infiltrating inflammatory cells. Finally, transmission electron microscopy on formalin-fixed paraffin-embedded liver revealed intraphagocytic (intrahistiocytic) and extracellular ~0.7–1.7 µm coccobacilli (expected size for *Francisella* spp.) in the foci of necrotizing hepatitis. Details of the diagnostic investigation conducted in this case were recently published.[5]

The *Francisellaceae* family comprises gram-negative coccobacilli and four genera are currently recognized: *Francisella*, *Allofrancisella*, *Pseudofrancisella*, and *Cysteiniphilum*, of which only *Francisella* is currently considered of clinical relevance. *Francisella tularensis* is the most studied species because it causes tularemia, a highly transmissible, potentially life-threatening, zoonotic disease, also considered a potential bioterrorism agent.[8] Tularemia occurs over almost the entire Northern Hemisphere but is rarely reported in the Southern Hemisphere, where the only published cases have occurred in Australia.[4]

Although *F. tularensis* has a broad host range, sheep have been considered the only livestock species affected by epizootics of tularemia and have been implicated in disease transmission to sheep industry workers.[7,10] The abortifacient effects of *F. tularensis* in sheep have been described in the United States, and tularemia has been regarded as an overlooked syndrome in sheep.[10]

From a pathologic viewpoint, necrotic foci in the liver, spleen, or lungs in late term aborted ovine fetuses are characteristic of tularemia and should raise suspicion, although gross lesions can be absent even in cases with typical histologic inflammatory and necrotizing lesions.[10] Contrary to most bacterial abortifacients of sheep, *F. tularensis* is not visible upon histopathologic examination of tissues stained with hema

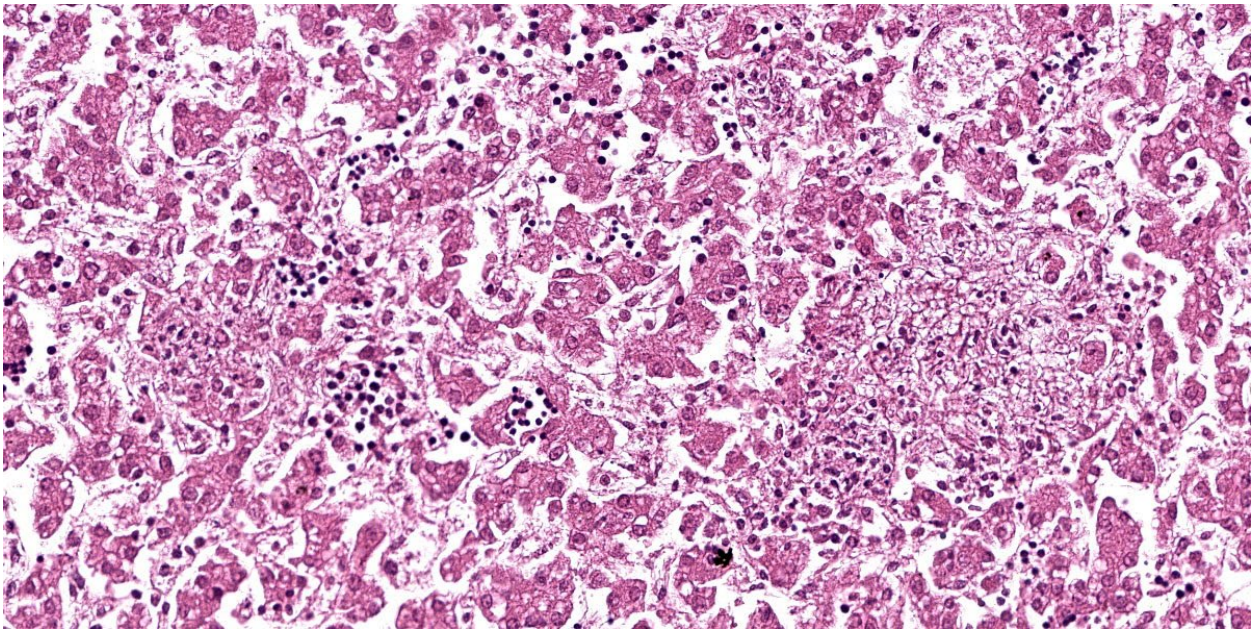

Figure 3-3. Liver, ovine abortus. Hepatocellular plates are disassociated. There are randomly scattered areas of lytic necrosis within the parenchyma which exhibit hepatocellular loss and stromal collapse, with few infiltrating neutrophils. Surrounding hepatocytes contain cytoplasmic lipid vacuoles. (HE, 314X)

toxylin and eosin, Steiner silver stain or Gram stain, even in tissues that have a high bacterial burden demonstrated by IHC.[3,10] The diagnostic investigation of any case of ovine abortion with fetal lesions indicating a bacterial etiology should include ancillary testing to identify *F. tularensis* and rule out other abortigenic pathogens.[3]

The etiologic diagnosis in our case was reached by the immunohistochemical demonstration of abundant intralesional antigen by a specific monoclonal antibody raised against *F. tularensis* LPS. IHC has proven useful for identifying *F. tularensis* in diagnostic settings.[6,10] *F. tularensis* LPS is a main specific antigen and virulence factor and differs from the LPS of other gram-negative bacteria.[9] According to the manufacturer, the primary antibody used in this case for the IHC does not cross-react with *Francisella novicida*, *Yersinia pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica*, *Vibrio cholerae*, *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Brucella abortus*, *B. suis*, *B. ovis*, *B. melitensis*, or *B. neotomae*. We tested the IHC in cases of ovine abortion caused by *Campylobacter jejuni* and *C. fetus* but observed no cross-reactivity with these pathogens. Although cross-reaction with other members of *Francisellaceae* cannot be completely ruled out, *F. tularensis* is currently the only species of this family recognized as an ovine abortifacient. Definite species and subspecies identification requires bacterial isolation and DNA analysis, which we were unable to perform in this case. The ultrastructural demonstration of intracellular gram-negative coccobacilli of the expected size in phagocytic and inflammatory cells in tissues with lesions, as in our case, aids in the diagnosis, but is by no means confirmatory.

The lack of historical reports of tularemia outside endemic areas of North America and Eurasia has been puzzling.[4] Recently, tularemia emerged in Australia and reemerged in the Northern Hemisphere.[8,15] South America has been considered free of tularemia, which seems to be based solely on the lack of disease reporting.[15] However, tularemia might have been undiagnosed because of limitations in disease surveillance systems in the region. No clinical disease caused by *Francisella* spp. in mammals in the Americas south of Mexico has been described. This case raised concerns about the possible occurrence of tularemia in South America.[5]

The source of infection in this sheep remains unknown; however, *F.tularensis* has a broad range of animal reservoirs, including arthropods, rodents, lagomorphs, and marsupials.[4,12] Brown hares (*Lepus europaeus*), a species that plays a primary role in the ecology of tularemia in Europe, have been introduced to Uruguay and are frequently seen around the affected farm.[6] In addition, *F. tularensis* can be transmitted by ticks, including *Amblyomma* spp., *Haemophysalis* spp., and *Ixodes* spp. ticks, which are endemic in Uruguay. Of note, a gammaproteobacterium related to *Francisella*-like organisms, but different from *F. tularensis*, was identified in Uruguay in *Amblyomma triste* ticks, the most prevalent tick species reported in human tick bites in the country.[14]

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JPC Diagnosis:

1. Liver: Hepatitis, necrotizing, multifocal and random, moderate.
2. Liver: Extramedullary hematopoiesis, diffuse, moderate.

JPC Comment:

Francisella tularensis is poorly staining gram-negative coccobacillus that is nonmotile, aerobic, and is a facultative intracellular pathogen. There are three subspecies that vary in their geographic distribution and virulence. *F. tularensis* subsp. *tularensis* accounts for the majority of clinical infections in domestic animals, is highly pathogenic, and is found in North America and Europe.[11] Two less virulent strains, *F. tularensis* subsp. *holartica* and *F. tularensis* subsp. *mediasiatica* occur in Europe/North America and Asia, respectively. Interestingly, subsp. *holartica* is frequently linked to aquatic mammals such as beavers and muskrats, and its reservoir is considered to be a protozoal organism rather than a mammal.[11]

In vivo, the organism enters host macrophages, where it arrests the maturation and acidification of the phagolysosome.[11] The organism then escapes the phagosome and replicates freely in the cytoplasm, where it can trigger either caspase 3-mediated apoptosis or an inflammatory form of host cell death called pyroptosis, resulting in release of bacteria that can initiate another round of host cell infection.[2,11] The ability of *F. tularensis* to survive the phagosome is dependent on the gene products of a 30kb stretch of bacterial DNA, known as the Francisella Pathogenicity Island (FPI), that encodes 18 genes, 14 of which are required for growth within and escape from macrophages.[2] Among the virulence factors en-

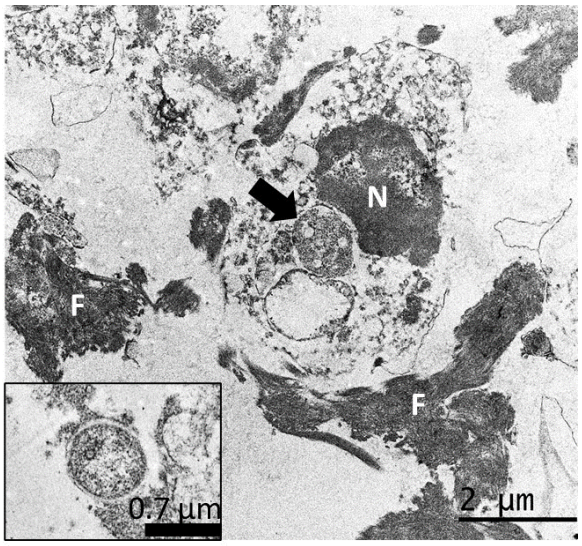

Figure 3-4. Liver, ovine abortus. An infiltrating phagocyte (center), presumably a macrophage, contains intracytoplasmic debris, including debris of a gram-negative bacterium within a phagolysosome (arrow) and is surrounded by extravasated fibrin (F). N: phagocyte nucleus, bar = 2 µm. Inset: A preserved gram-negative bacterium of the expected size for *Francisella* spp. is seen in an adjacent extracellular location, bar = 0.7 µm. Photos courtesy of Dr. Aníbal G. Armién (California Animal Health and Food Safety Laboratory, University of California, Davis, CA, USA).

coded on the FPI is a recently discovered Type VI secretion system which is required for macrophage escape and intracytoplasmic replication.[1] *F. tularensis* organisms that are deficient in key pieces of this secretion nanomachine are rendered essentially avirulent in mouse models.[1]

If all is working well, however, *F. tularensis* can cause fulminating, fatal disease in wildlife species, domestic animals, and humans. In most species, the disease is characterized by fever, depression, inappetence, and manifestations of septicemia. Outbreaks have been reported in sheep, horses, and young pigs, while adult pigs, cattle, and dogs appear to be relatively resistant to infection.[11]

Cats are the domestic animal most associated with disease, with clinical forms (typhoidal, respiratory, ulceroglandular, and oropharyngeal) mirroring those described in humans.[11] Grossly, tularemia in cats is characterized by sizeable (2mm diameter or more), miliary white foci in the liver, spleen, and lymph nodes.[13] Histologically, lesions are identical to those presented here, with focal areas of severe necrosis with bacteria that are difficult to visualize on H&E section.[13]

Conference discussion focused largely on the typical presentations and pathogenesis of tularemia in various species. From a diagnostic standpoint, it is important to remember that, unlike most infectious differentials for hepatic necrosis, *F. tularensis* is, rather unhelpfully, not visible on H&E, Steiner, or Gram stains, even in tissues with high bacterial burdens demonstrated with immunohistochemical staining. Dr. Travis also discussed documented human cases of tularemia contracted from "face snuggling" domestic dogs ("if we die, we die") and from aerosolized infective material liberated from wildlife carcasses during yard work.

Despite the absence of demonstrable organisms, this case provides an excellent example of the hepatic manifestation of gram-negative sepsis. Participants noted multifocal areas of extramedullary hematopoiesis within sinusoids, which was felt substantial enough to include in the morphologic diagnoses.

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CASE IV:

Signalment:
Adult female Zebrafish (*Danio rerio*)

Figure 4-1. Presentation, zebrafish. There are ulcers on the right abdominal wall and gill plate. (*Photo courtesy of:* Laboratory of Comparative Pathology, Memorial Sloan Kettering Cancer Center, The Rockefeller University, Weill Cornell Medicine, http://www.mskcc.org /research/comparative-medicine-pathology-0)

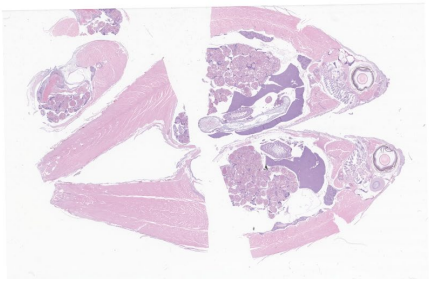

Figure 4-2. Whole body, zebrafish. Two longitudinal and two cross sections are presented for examination. At subgross magnification, there is a hole in the body wall on one transverse section and internal pathology is evident. (HE, 6X)

History:
The animal presented with an ulceration on the right caudal abdomen, but appeared otherwise bright, alert, and responsive and was in good body condition.

Gross Pathology:
The skin of the right caudal abdomen contains a single approximately 1.5-2 mm diameter circular pale tan ulceration. Fin clip and gill clip wet mounts are unremarkable.

Laboratory Results:
Aerobic culture of ulcerated region: Mixed bacterial flora including *Plesiomonas shigelloides* and *Pseudomonas putida.*

Aerobic and anaerobic culture of kidney: Negative

Microscopic Description:
Markedly expanding the coelomic cavity and separating and surrounding viable and degenerate developing eggs within the ovary is a large inflammatory population composed of numerous epithelioid macrophages, granulocytes, and rare multinucleated giant cells. This inflammatory population is admixed with small amounts of necrotic cellular debris, moderate hemorrhage, and fibrin. This inflammatory process extrudes through the extensively ulcerated overlying body wall with loss of all layers including the skin, muscle, and coelomic cavity lining. Few small basophilic rod-shaped bacterial colonies are present near the surface of the ulceration within the degenerate egg material. The myocytes adjacent to this body wall ulceration are multifocally degenerate, with swollen vacuolated sarcoplasm, or necrotic with shrunken, hypereosinophilic, fragmented sarcoplasm. Several macrophages infiltrate between and surround damaged myocytes.

Contributor's Morphologic Diagnosis:
Ovary and coelom: Severe, multifocal to coalescing, chronic granulomatous and granulocytic oophoritis and coelomitis with hemorrhage, degenerating eggs, myocyte loss, degeneration, and necrosis, and severe full thickness body wall, dermal, and epidermal ulceration with multifocal intralesional rod-shaped bacterial colonies.

Contributor's Comment:
Chronic ovarian and coelomic inflammation in zebrafish associated with degenerating eggs is a common condition observed in laboratory facilities and is referred to as Egg Associated Inflammation (EAI). The cause of this condition is uncertain with some individuals describing this as a syndrome of egg retention.[6] A potential link to Mycobacterial infection has been suggested though no causative agents have been identified currently and it is uncertain that Mycobacterium is a primary pathogen in this disease.[2] Acid fast staining of our case was undertaken to rule out Mycobacterial infection and was negative.

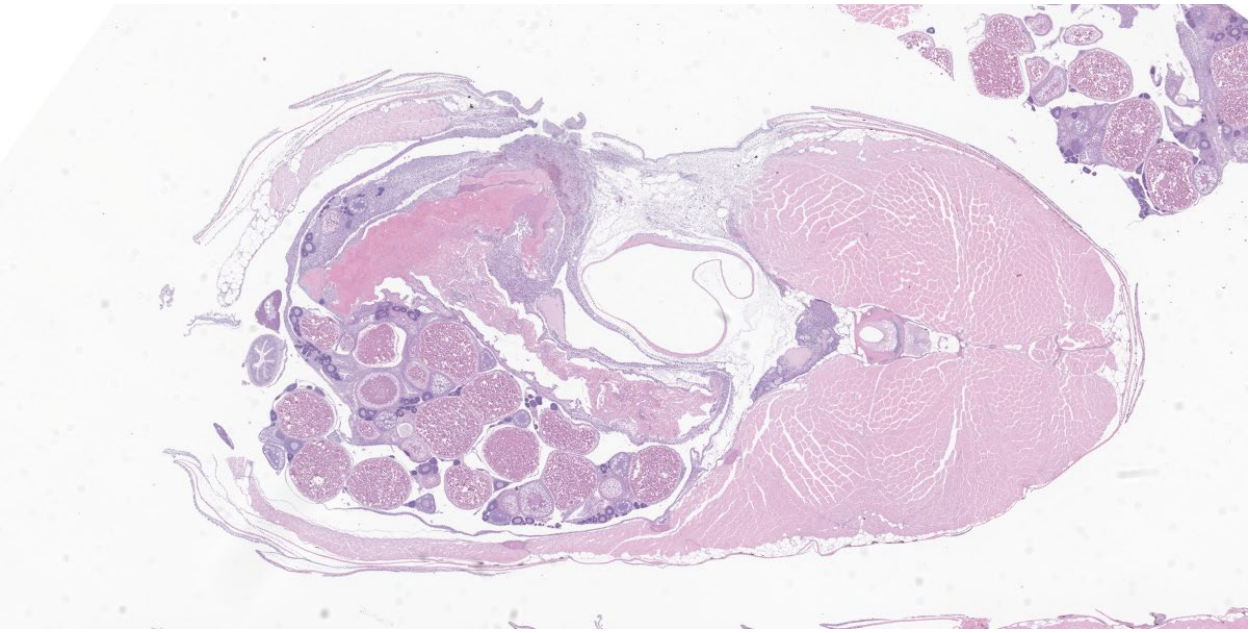

Figure 4-3. Cross section, zebrafish. This one cross section has all the goodies. There is full-thickness necrosis of the body wall which extends into the coelom. There is marked histiocytic inflammation within the ovary centered on degenerating ova and a large mass of brightly eosinophilic protein. (HE, 16X)

Gross examination of these fish reveals a large abdomen that when opened may contain a mass of tissue, possibly adhered to the overlying coelomic cavity lining and body wall. Occasionally, as in our case, a full thickness ulceration may be visible externally, covered or surrounded by a rim of pale tan to white tissue. This ulceration is secondary to the extrusion of tissue through the body wall from the point of underlying coelomic adhesion.

Microscopically, the observed inflammation is often granulomatous with varying numbers of granulocytes and is always associated with various stages of degenerating eggs within the coelom. The inflammatory population may be surrounded and infiltrated by abundant fibroplasia and rarely this may promote the formation of fibromas and fibrosarcomas.[6]

The rod-shaped bacterial colonies noted on H&E-stained sections highlight as gram negative with gram staining. Aerobic culture of the ulcerated region grew *Plesiomonas shigelloides* and *Pseudomonas putida*, both gram negative rod-shaped bacteria. *Plesiomonas shigelloides* is found commonly within the environment and gut of laboratory and wild zebrafish but has been identified in few cases to be pathogenic to zebrafish.[5] *Pseudomonas putida* is another bacterium widely distributed in soil, water, and on the skin of animals.[3] The skin ulceration secondary to EAI likely lead to an optimal situation for these environmental organisms to grow within the damaged tissue.

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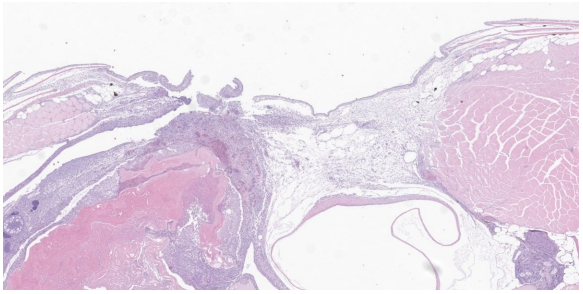

Figure 4-4. Abdominal wall, zebrafish. Higher magnification of the full thickness necrosis of the body wall. (HE, 37X)

JPC Diagnosis:
Body wall, ovary, coelom: Granulomatous inflammation with ovarian follicular degeneration, extracellular bacteria, and body wall perforation.

JPC Comment:
As the contributor notes, egg associated inflammation (EAI), also referred to as Egg Associated Inflammation and Fibroplasia (EAIF) is thought to be associated with egg retention in fish that have not had proper opportunity to spawn.[1] While mild forms of the condition may be clinically inapparent, in many cases female zebrafish present with an enlarged, ulcerated abdomens and superimposed bacterial infections within the fertile, yolky soil of the affected ovary.[6]

The histologic features of this case are typical of the condition. The eosinophilic coagulum represents yolk from atretic follicles and the periphery of this material is often characterized by finely granular basophilic material consisting of lysed inflammatory cells, necrotic cellular debris, and visible bacterial colonies.[1] This material is usually rimmed by an intense granulomatous reaction with epithelioid macrophages and multinucleated giant cells.[1]

EAI is currently considered a non-infectious condition, though that assertion is typically heavy with caveats. As the contributor notes, some cases have been associated with mycobacteriosis; however, infectious agents are not usually found in cases of EAI in the absence of body wall defects, and the granulomatous and inflammatory changes seen in zebrafish with mycobacteriosis are typically found in organs other than ovaries, whereas EAI arises, as the name implies, from the ovaries.[4]

In addition to the detrimental effects of EAI on the individual animal, the condition raises confounding concerns due to the use of zebrafish in experimental interrogations of developmental genetics. Zebrafish are used extensively to investigate the effects of exogenous chemicals, particularly endocrine disrupting compounds, on the vertebrate reproductive system.[1] Follicular atresia is a common non-specific effect of several classes of these compounds and research results may be confounded by the chronic oophoritis that is characteristic of EAI.[1] In females with subclinical EAI, egg production may be reduced, leading to skewed research results in studies that use fecundity endpoints.[1]

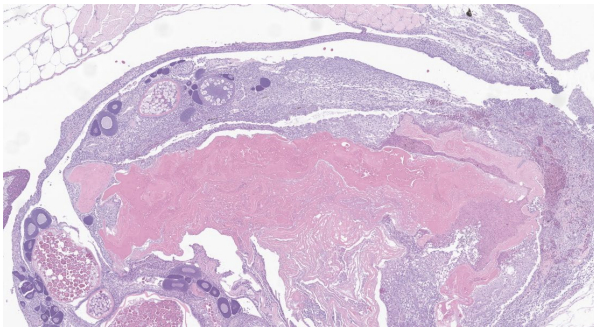

Figure 4-5. Ovary, zebrafish. Profound histiocytic inflammation within the ovary is centered on extruded protein from degenerate eggs. (HE, 50X)

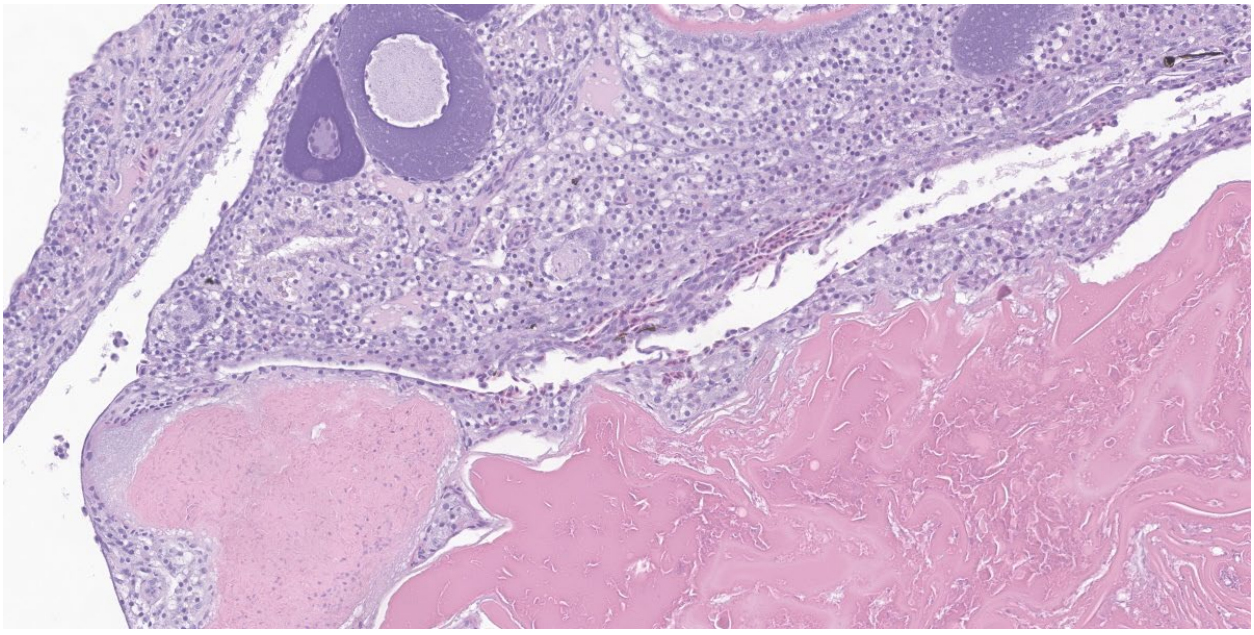

Figure 4-6. Ovary, zebrafish. Higher magnification of the histiocytic inflammation within the ovary. (HE, 60X)

The appearance of these apparently spontaneous degenerative and inflammatory changes in the ovaries of zebrafish highlight the importance of recognizing baseline variations in the histologic morphology of target organs in research animals.[4]

The specifics of this condition are largely unknown, leaving scant fodder for conference discussion. Dr. Travis discussed several factors that have been associated with EAI, including small body size, obesity, overcrowding, water quality issues, sex ratio imbalances, stress, and lack of proper environmental cues (e.g., photoperiod, temperature) in cultured fish. Conference participants also considered sequelae of the profound abdominal distention characteristic of the disease, including organ compression and malfunction, prolapse of the ovary through the genital pore, and dystocia.

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