Joint Pathology Center

Veterinary Pathology Services

WEDNESDAY SLIDE CONFERENCE 2016-2017

C o n f e r e n c e 25

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CASE I: WSC 1617 Conf 25 Case 1 (JPC 4065818).

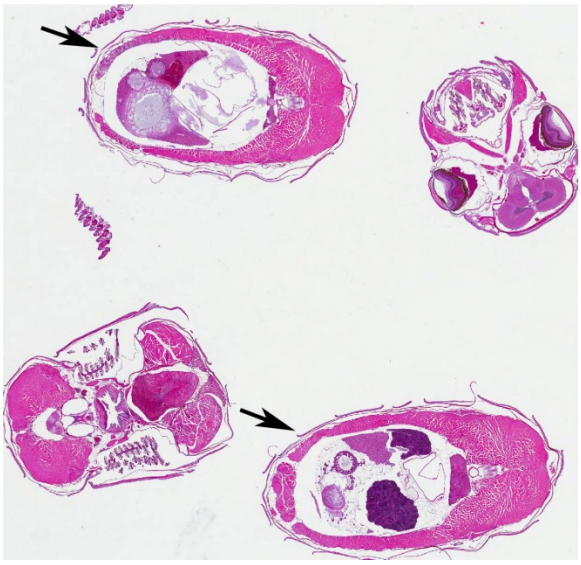
Signalment: 13-month-old male zebrafish (*Danio rerio*).

History: This fish was from a breeding colony of zebrafish. Offspring produced from the breeding colony were either used in genomics research or were used to replace aged members of the breeding colony. Because the breeding colony had been maintained as a "closed colony" for several years, there was concern about inbreeding. Therefore, a group of adult zebrafish was purchased from a commercial vendor to be used to breed with the established colony and thus provide some genetic diversity.

After an uneventful 90-day quarantine period, one of the female fish from the recently-purchased group was bred with a male fish from the long-established colony. The resultant offspring were raised in several small groups up to the age of one month and then the groups were combined and maintained together in one large group in an 85-gal (~ 322 L) tank. At 13 months of age, eight fish from this group were selected at random for routine health assessment. These fish were euthanized by immersion in a solution of tricaine methanesulfonate (MS-222) and then the carcasses were fixed *in toto* in 10% neutral-buffered formalin. The fixed carcasses were then submitted for histopathologic examination.

The submitted slide contains whole-body transverse sections from one of the eight fish.

This fish was part of a research project conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory



Skeletal muscle, zebrafish. Four transverse sections of a zebrafish are submitted for examination. At subgross inspection, there is mild hypercellularity of the musculature of the abdominal wall in multiple sections. (HE, 5X).

Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

Gross Pathology: NA

Laboratory results: NA

Histopathologic Description: There are four transverse sections of intact whole body at different levels on this slide. Within two of these sections, there are multifocal to coalescing degeneration and necrosis of skeletal muscle fibers accompanied by infiltrates of low to moderate numbers of macrophages and fewer lymphocytes. Numerous protozoa-like micro-organisms in various stages of development are present within the cytoplasm of affected myocytes. The different developmental stages of the organisms include: 1) 5-15 µm diameter round to oval basophilic uni-nucleate organisms with an eosinophilic refractile wall; 2) 15-30 µm round cyst-like structures with an eosinophilc refractile wall and containing multiple basophilic nuclei with poorly-defined borders; and 3) 25-40 µm cyst-like structures containing multiple approximately 3 X 5 µm oval refractile spores with basophilic to clear cytoplasm. One or more spores are also occasionally present with macrophages.

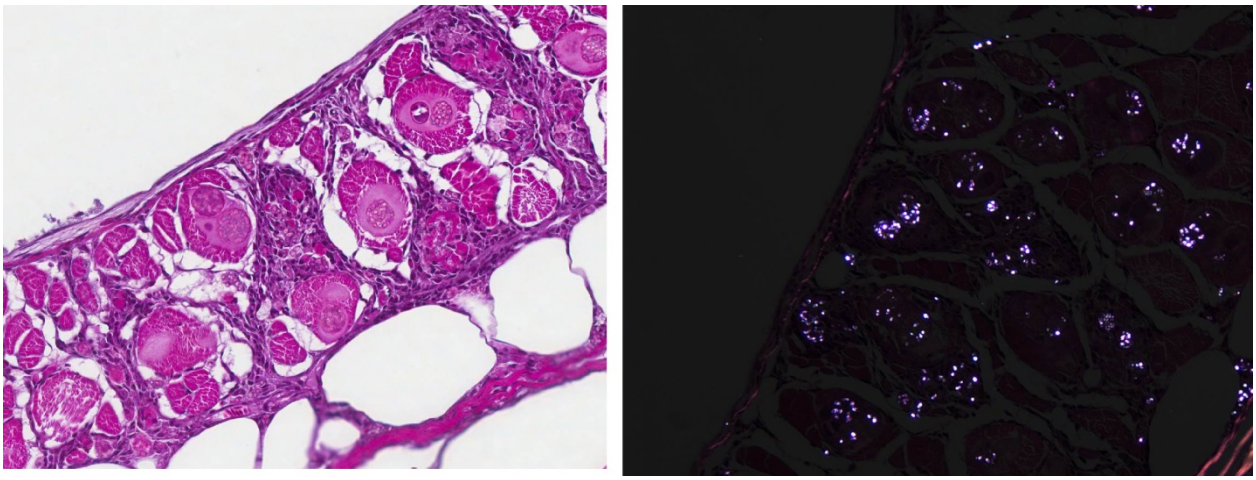
Other than autolysis, there are no significant changes in other organs.

Contributor's Morphologic Diagnosis: Skeletal muscle; multifocal to coalescing myodegeneration, moderate, with necrosis, lymphohistiocytic inflammation and numerous intracytoplasmic protozoa-like organisms at various stages of development (including spores).

Contributor's Comment: The lesions in the muscles of this fish were unexpected findings. These had not been seen previously in this fish colony and a review of the available literature at that time did not reveal descriptions of similar lesions in zebrafish.

Several special stains were done in an attempt to better characterize the organisms. These stains revealed that the spores often had annular bands and a PAS-positive polar granule (Figure 6). The more mature spores were Gram-positive but immature spores were Gram-variable (Gram-negative or did not stain with Gram's stain). These findings are characteristics of microsporidia.[2,3,6]

Further studies conducted by personnel at Oregon State University and the Zebrafish International Resource Center demonstrated that the species of microsporidia in this zebrafish is *Pleistophora hyphessobryconis*.[7] *P. hyphessobryconis* is a common pathogen of neon tetras (*Paracheirodon innesi*) and other species of ornamental tropical fish.[5,6] *P. hyphessobryconis* is so common in neon



Skeletal muscle, zebrafish. Left: Higher magnification of affected skeletal muscle demonstrates the presence of uninucleate sporoblasts as well as larger sporonts containing numerous mature spores in affected myocytes. Degenerate myocytes are fragmented and infiltrated by numerous histiocytes, which also expanded the perimysium. Right: Mature microsporidian spores exhibit birefringence.(HE. 256X) (Photo courtesy of: US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702-5011)

tetras that the disease it causes is simply called "neon tetra disease".[5,6] Although some older reports state that *P. hyphessobryconis* has been seen in zebrafish in the commercial pet trade, this case was the first time it was recognized in a laboratory research colony.[7] Subsequent to the discovery of this index case, other cases of *P. hyphessobryconis* infection in zebrafish have been seen in two other research colonies.[7]

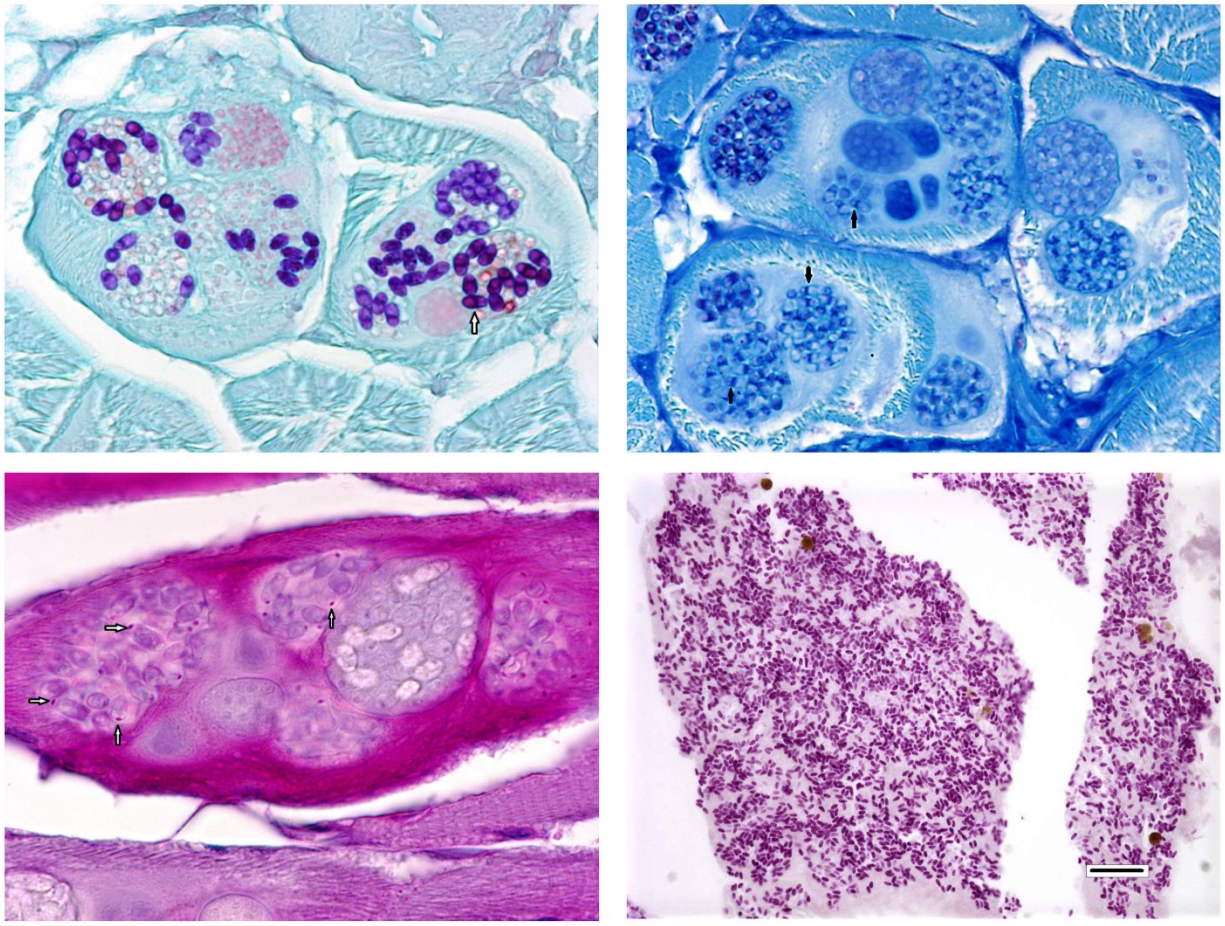
For many years, microsporidia were taxonomically classified as protozoa. However, the results of more recent studies have caused them to be re-classified as fungi.[2,4] Microsporidial spores are the infectious stage and each spore contains a coiled structure called a polar filament or polar tube.[1] When a spore enters a suitable host (usually by ingestion of the spore), the polar filament uncoils, emerges from the spore, and penetrates a host cell. The sporoplasm is then injected through the polar filament into the host cell.[2] The parasite proliferates asexually inside the host cell, first through merogony and then sporogony.[3,6] The meronts of *Pleistophora* spp. (which at first are uninucleate and eventually become multinucleate) are surrounded by a membrane derived from the host cell and are said to reside within a parasitophorous vacuole.[2,5] The meronts of *Pleistophora* spp. mature into sporogonial plasomodia that undergo fission to produce sporoblasts which ultimately develop into spores; multiple spores are contained within each thick-walled sporophorous vesicle.[2,5]

Fish typically become infected by *P. hyphessobryconis* by ingesting spores released into the environment from necrotic muscle fibers of other infected fish or by scavenging dead, infected fish. It is not known how the fish of this report was exposed to *P. hyphessobryconis* spores. This animal had been raised in a laboratory setting without any exposure to neon tetras or other fish species. The water used to house this fish colony is a mixture of deep-well water and municipal water that has undergone reverse-osmosis filtration; thus, it is highly unlikely that this water was the source of infection. The food used to feed the fish is a commercial flake-food that contains ground fish meal. However, the

processing procedures used to produce this food should have inactivated any micro-sporidia spores that were accidentally present.

The mother of this fish had been purchased from a commercial vendor and thus may have been exposed to infected fish before her arrival at the research facility. Although the mother fish was clinically normal, it is possible that she was harboring a low-grade infection and thus may have shed spores into the water during breeding. However, the embryos produced from this breeding were treated with bleach before being transferred to fry-raising tanks, and this bleaching process should have inactivated any micro-sporidia spores present in the water. It is also worth noting that the affected fish was euthanized 13 months after conception and this would be a long time to harbor a subclinical infection.

When this infected fish was detected, all of the fish that had been purchased from the commercial vendor had been removed from the colony months previously. Thus, it was not possible to examine them to determine if they had been the source of infection.



Skeletal muscle, zebrafish. Color plate showing various special stains. Top left: Spores stain strongly with Gram stains. (Lilly Twort, 400X). Top right: A Giemsa stain demonstrates annular bands (arrows). (Giemsa, 200X). Bottom left: A periodic Schiff stain demonstrates the polar granule. (PAS, 400X). Bottom right: A Luna mast cell stain is also an excellent choice for highlighting spores. (Luna, 100X) (Photo courtesy of: US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702-5011)

After this fish was found to be infected, the remaining 135 fish in its sibling co-hort were euthanized and examined grossly (98 fish) and/or histologically (37); none of these were found to be infected and there have not been any other cases in this colony since then.

Note: Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

JPC Diagnosis: Skeletal muscle: Myocyte deneneration and necrosis, multifocal, mild with numerous intrasarcoplasmic meronts, sporoblasts, and spores with histiocytic inflammation, zebrafish, *Danio rerio*.

Conference Comment: Zebrafish are of increasing importance as a research model for the study of infectious diseases, developmental biology, cancer, and toxicology. Their small size, ease of husbandry, and *ex-vivo* transparent embryonic development make them a highly sought after laboratory species with a burgeoning transgenic and specific pathogen free industry to support the increase in laboratory use.[8] This case represents infection by a microsporidian organism that may easily spread within a colony and could potentially confound experimental results; thus making this and other zebrafish diseases of particular concern for investigators and laboratory animal pathologists. Conference participants discussed the importance of proper animal acquisition, good husbandry, routine disease and pathogen monitoring, and a robust sentinel animal program as the cornerstones of laboratory animal disease prevention in any species.

The most commonly encountered infectious diseases of laboratory zebrafish include mycobacteriosis and microsporidiosis.[7,8] As mentioned by the contributor, based on phylogenetic analysis and the presence of chitin within the spore, microsporidia are classified as fungal-like organisms rather than protozoans, as previously thought. *Microsporidia* are a large group of obligate-intracellular eukaryotic parasites that infect a wide range of animal species.[1,3] Their relatively simple life cycle consists of two developmental stages: merogony and sporogony.[1,2,3,5,7,8] After infection, meronts multiply within the host cell, skeletal muscle in this case, eventually forming thick-shelled spores. Spores are released from the ruptured host cell and reach the environment through various bodily secretions. The infectious spores have a thick, chitinous shell and are resistant to environmental stress; thus allowing the infective spores to remain viable in the aquatic environment for a prolonged period of time. As mentioned by the contributor, infective spores can also be ingested through predation.[7,8]

The most commonly identified micro-sporidian parasite affecting zebrafish is *Pseudoloma neurophila*.[7,8] As is implied by the name, *P. neurophila* have tropism for the central and peripheral nervous system and is associated with encephalomyelitis and polyneuritis. Small groups and individual spores can be seen in a variety of extraneural organs, including smooth and skeletal muscle. Spores released from peripheral nerves and skeletal muscle typically cause severe inflammation.[7,8] In contrast, *Pleistophora hyphessobryconis* primarily develops within skeletal muscle with multiple developmental stages (meronts, sporoblasts, and spores) present within the sarcoplasm. The conference moderator noted that the two *Microsporidia* species can be easily distinguished histologically due to the presence of *P.hyphessobryconis'* intra-sarcoplasmic developmental stages and

thick-walled sporophorous vacuoles laden with spores.[7,8] Additionally, spores of *P. hyphessobryconis* are associated with only a mild inflammatory response, as seen in this case. In the areas of mild histiocytic inflammation in the skeletal muscle of the abdominal wall, birefringent spores are present within phagocytes. In photographs provided by the contributor, wet-mount preparations of skeletal muscle from infected fish show individual spores as well as sporophorous vacuoles containing spores with a prominent posterior vacuole and coiled polar filament, typical of *Microsporidia* spp.[1,7,8] Another microsporidium parasite of veterinary importance is *Encephalitozoon cuniculi*, which causes torticollis and phaecoclastic uveitis in the highly susceptible dwarf rabbit.[1] Readers are encouraged to review WSC 2015 Conference 12 Case 4 for an outstanding example of phaecoclastic uveitis caused by *E. cuniculi* in a double-maned lionhead rabbit.

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US Army Medical Research Institute of Infectious Diseases
Pathology Division
Fort Detrick, MD
http://www.usamriid.army.mil/

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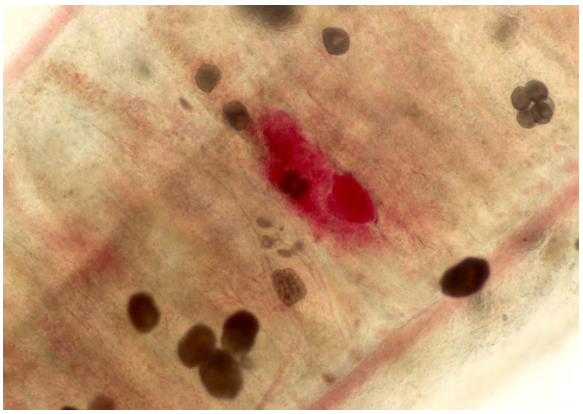
CASE II: 15-3213 (JPC 4084010).

Signalment: Adult female Northern puffer fish (*Sphoeroides maculatus*).

History: This fish was presented to the CVMDL for euthanasia and necropsy after a one-week history of anorexia and labored breathing. Reddish discoloration had been noted around multiple fins. Twelve individuals in two exhibits from this private aquatic education center exhibited the same clinical signs, and several fish from affected tanks had died over a one-month period. The



Gill, pufferfish. Wet mount of gill clip gill reveals tomonts and trophonts (presumptive trophonts of Amyloodinium ocellatum). (Photo courtesy of Department of Pathobiology and Veterinary Science, Connecticut Veterinary Medical Diagnostic Laboratory, Connecticut http://www.pathobiology.uconn.edu/

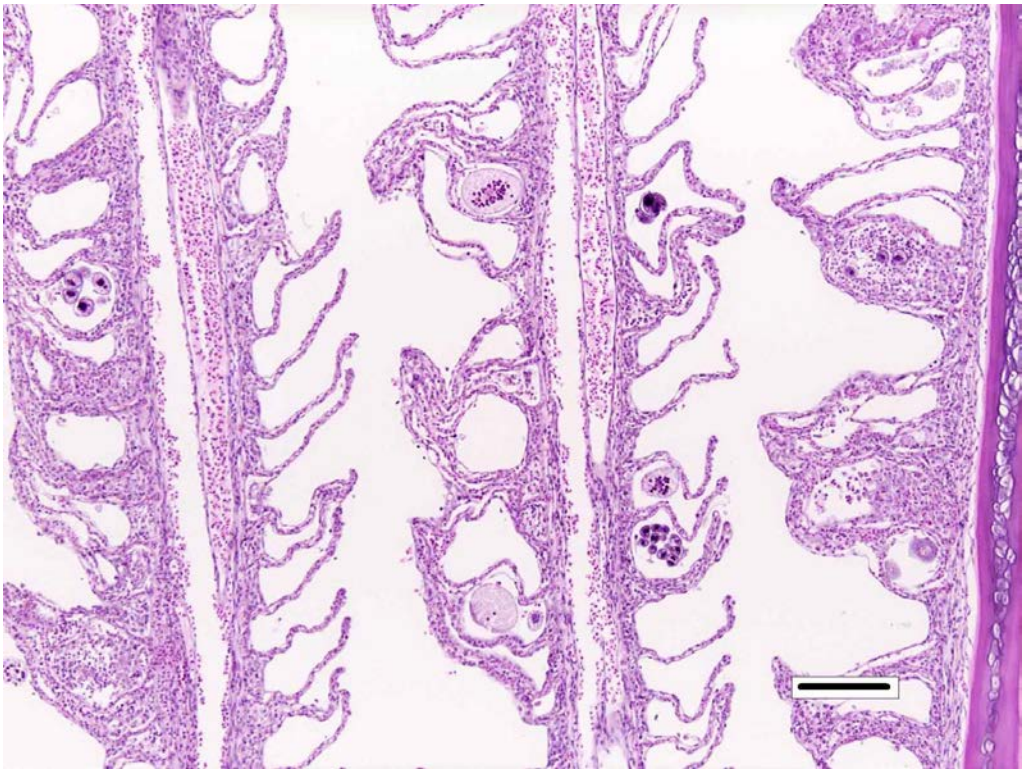
tank was treated with copper and metronidazole. Slight improvement was noted after the first treatment, but clinical signs then worsened with lethargy and lack of appetite. Additional treatment with increased iodine and copper had no effect. The submitted fish was euthanized with an overdose of MS-222 and necropsied immediately.

Gross Pathology: The fish was in poor body condition with scant coelomic adipose stores. External examination revealed slight reddening of the dorsal and pectoral fins that was more severe at the proximal aspect, and the pectoral fins had some indistinct, cloudy stippling. A skin scrape of the body was negative. The gills had disseminated, faint dark-red stippling. The liver was diffusely yellowish-tan and floated when placed in formalin. The gallbladder was moderately dilated with thin, green, watery bile. The gastrointestinal tract was empty except for a small amount of mucus.

Laboratory results: Cytology: Wet mount of the left pectoral fin and gill revealed few and numerous, respectively, round to ovoid, brown structures ranging in size from approximately 50 to 200 microns in diameter (presumptive trophonts of *Amyloodinium ocellatum*). Often smaller round forms were seen in organized aggregates of two to 16 organisms (dividing forms; tomonts).

Histopathologic Description: Gill: There is generalized mild to moderate hypertrophy and hyperplasia of lamellar epithelial cells that often results in fusion along the lengths of some lamellae or at the lamellar tips (synechiae). Pseudocystic spaces formed by synechiae frequently contain sloughed cellular debris, macrophages, numerous rod-shaped bacteria and occasional dinoflagellate organisms (consistent with *Amyloodinium* sp.) in varying stages of development. Present along the lamellar surfaces are dinoflagellate trophonts that are circular to ovoid, range in size from 25 to 100 microns in diameter, and have a single, round, deeply basophilic nucleus and wispy eosinophilic cytoplasm containing several 3-5 micron diameter, circular, brightly eosinophilic bodies and birefringent granules and spicules under polarized light. Tomonts are occasionally trapped in pseudocysts at varying stages of subdivision into 2-20 dinospores, which have a thin refractile and birefringent wall and are round and roughly 20-30 microns in diameter with granular eosinophilic cytoplasm and one to two irregularly shaped, stippled, deeply basophilic masses of chromatin. Lamellae are variably thickened by moderate to marked congestion, occasional hemorrhage and low to moderate numbers of inflammatory cells consisting predominantly of macrophages with fewer lymphocytes and rare granulocytes. There are a few, scattered areas of lamellar necrosis. The cytoplasm of a few lamellar epithelial cells is markedly expanded by an intracytoplasmic, discrete,

round, finely granular basophilic inclusion (bacterial inclusion of epitheliocystis).



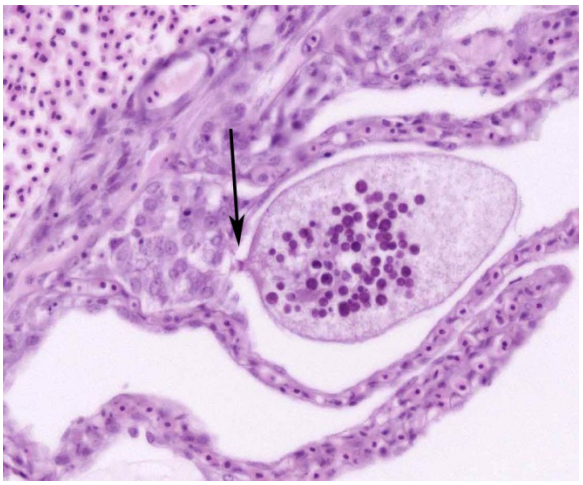
Gill, pufferfish. There are multifocal synechia of secondary lamellae with formation of pseudocysts, as well as secondary lamellae. Pseudocysts occasionally contain variable amounts of cellular debris and multiple trophonts and tomonts of Amyloodinium.

Contributor's Morphologic Diagnosis: Gill: severe diffuse, proliferative and histiocytic branchitis with lamellar fusion, synechiae, and intralesional dinoflagellate organisms, compatible with *Amyloodinium ocellatum*, gram-negative bacteria, and rare epitheliocystis inclusions.

Contributor's Comment: Although there were several etiologic agents seen microscopically in sections of gill that could have contributed to the extensive pro-liferative branchitis, the primary pathogen is believed to be a heavy infestation with the obligate ectoparasitic marine dinoflagellate, *Amyloodinium ocellatum*, the causative agent of marine velvet disease or oodiniosis.

This organism is considered an important parasite in aquarium fish and in tropical and subtropical brackish and marine fish culture, and it can infect both elasmobranch and teleost fish.[5] Natural epidemics have also been documented.[3] *Amyloodinium ocellatum* prefers warm water from 17 to 30 degrees Celsius (63-86 F) with potentially greater virulence in high temperatures.[2] Isolates vary in salinity tolerance with ranges from 3 to 45 ppt.[5] Certain species of fish are resistant to infection; these fishes typically produce a thick mucus, possibly preventing attachment of trophonts, or tolerate low oxygen levels.[5]

The life cycle of *A. ocellatum* is direct and triphasic. The parasitic, feeding stage, or trophont, attaches to the host's epithelium by a root-like structured called a rhizoid.[5] After a period of feeding on the host, the trophont detaches from the host, retracts its rhizoid and becomes the encysted, dividing tomont stage in the substrate.[5] The final stage is the free-swimming, infectious dinospore that is released from the dividing tomont (up to 256 dinospores can come from a single tomont) and has flagella to

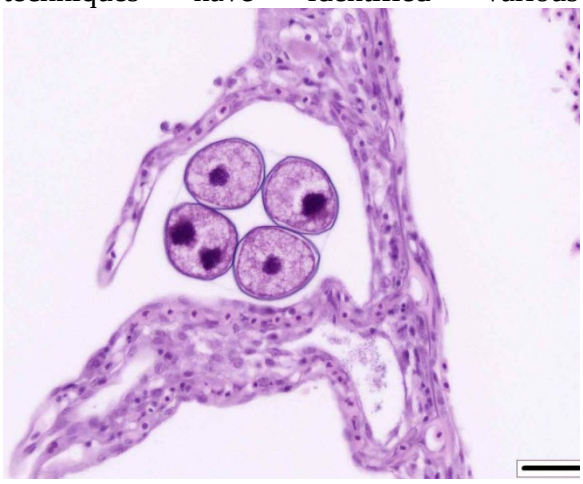


Gill, pufferfish. Amyloodinium trophont with stomatopode . (HE, 400X).

facilitate swimming to find a suitable host.[5] *Amyloodinium ocellatum* causes severe physical damage to the host cells through attachment of the trophont to epithelium, with the gill typically being the primary site of infestation.[5,6] In cases with heavy parasite burdens, the skin and eyes can also be infected, sometimes producing the dusty appearance of the skin that accounts for the name "velvet disease."[5] In some reports infestation of larval fish affected only the skin rather than the gill.[6] The gill was the tissue most severely affected in this puffer fish, although a few of the organisms were seen on a wet mount of the fin; cutaneous changes were mild in histologic sections. Trophonts may also be seen in the pseudobranch, branchial cavity, nasal passages, and gastrointestinal tract (if swallowed).[5]

Microscopic lesions of severely affected gill include epithelial hyperplasia, often with lamellar fusion and distortion, mild inflammatory infiltrates, hemorrhage and epithelial degeneration and necrosis,[3,5,6] as seen in this case. The feeding activity and the detachment of large numbers of organisms can damage epithelium, and potentially result in mortality as a consequence of hypoxia, osmoregulatory imbalance and secondary bacterial infections.[5] There are several modalities of treatment available, most of which only target the dinospore stage, which can make eradication of parasites difficult.[5]

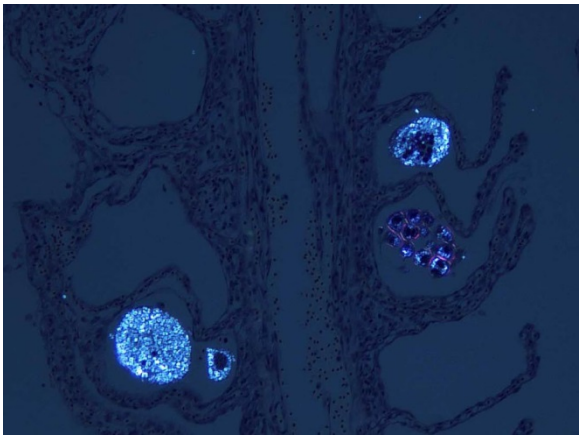
Although not considered the primary disease in this case, epitheliocystis was also noted in this fish. Characterized histologically by large, granular, basophilic, intracytoplasmic, bacterial inclusions within enlarged branchial lamellar epithelial cells, this type of infection is caused by a group of obligate, intracellular, gram-negative, chlamydia-like organisms.[5] Recent advances in molecular techniques have identified various



Gill, pufferfish. Amyloodinium tomont with four divisions. There is a synechia between adjacent secondary lamellae. (HE, 400X).

epitheliocystis agents as belonging to the order Chlamydiales, the first of which was *Candidatus Piscichlamydia salmonis* in the Atlantic salmon.[1,7] Infections of gills, pseudobranch, and rarely the skin by these organisms have been described in many freshwater and marine species of fish.[7] Pathogenicity is dependent upon the host response and the chlamydiales bacterium, and microscopic lesions can range from none to severe epithelial hyperplasia with associated inflammation.[7]

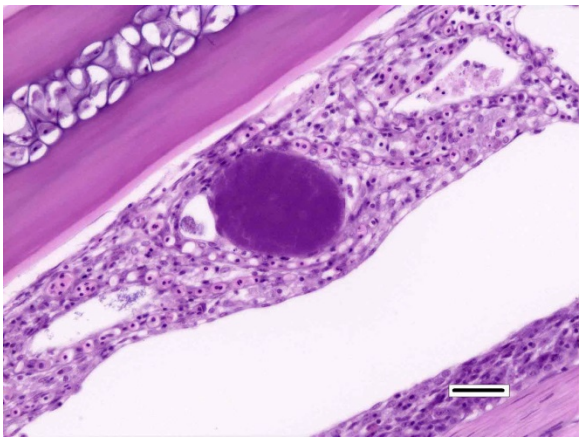
JPC Diagnoses: 1. Gill: Branchitis, proliferative and necrotizing, multifocal, moderate with marked lamellar adhesions (synechiae) and fusion, and numerous dinoflagellate ectoparasites, Northern puffer fish, *Sphoeroides maculatus*.
2. Gill, lamellar epithelial cells: Chlamydial inclusions, multiple.



Gill, pufferfish. Bifringence of multiple life stages of Amyloodinium. (HE, 100X)

Conference Comment: *Amylodinuym ocellatum* is one of the most important pathogenic parasites of both marine and estuarine fish. It is one of the few organisms that can infect the gills and skin of both teleost fish (bony ray-finned fish) and elasmobranchs (cartilaginous fish, ex. sharks).[2,5] This dinoflagellate parasite has the capacity for rapid reproduction and severe infections, such as in this case, can cause devastating disease and high mortality in a wide range of fish species.[2] As nicely summarized by the contributor, the life cycle is simple and requires no intermediate hosts. The parasite has three different developmental stages. The trophont stage is the adult stage that feeds directly on the host and attaches via the rhizoid; the tomont stage is detached from the fish and divides to the third dinospore stage. Dinospores are the free swimming infectious flagellated phase.[2,5] The simple life cycle and free swimming infectious dinospore stage contribute to severe epizootics in both wild and cultured fish, especially when crowded. *A. ocellatum* is a major concern to the aquaculture industry because outbreaks have an acute onset, spread rapidly, and have a high mortality. There are many nice examples of all three life stages in this case, including several trophonts with prominent rhizoids.[2,5] This case is an excellent example of the gill as the primary site of infection for this parasite. Predominant antemortem clinical signs will most often be disturbances in the respiratory system, including increased respiratory rate (indicated by rapid movement of the gill opercula) and gathering at the surface or in other areas of increased oxygen concentration. The skin is also infected and fish can develop white or brown "velvety" appearance.[2] This case also contains excellent examples of epitheliocystis with large inclusions composed of colonies of *Chlamydia*-like organisms within the cytoplasm of lamellar epithelial cells. Epitheliocystis is often considered an incidental finding, and most conference participants agreed that its presence in this case is unrelated to the significant gill pathology caused by *Amylodinuym ocellatum*.[7]

Prior to the conference, the moderator briefly reviewed the normal anatomy and histology of the gill. The gill arch is a series of bony or cartilaginous curved structures that support double rows of paired filaments, also called the primary lamellae. Each filament is composed of numerous perpendicularly-arranged secondary lamellae.[4] The gill arch is covered by epidermis and at the origin of the primary lamellae the epidermis is thicker and contains many mucous cells and subjacent lymphoid tissue. The primary lamellae are covered by a mucoid epidermis which contains a pale-staining, eosinophilic, salt-



Gill, pufferfish. Occasional epithelial cells contain large bacterial inclusions characteristic of Epitheliocystis. (HE, 400X)

secreting chloride cells that function in ionic transport and detoxification. The surface of the secondary lamellae consists of a single layer of interdigitating squamous epithelial cells supported by pillar cells and lamellar blood channels.[4] The surface of the lamellar epithelium has numerous microvilli which serve as a substrate for cuticular mucus and aid in gas exchange and defense against infection and trauma. Thus, gills are important for gas exchange, ionic balance, and the excretion of the nitrogenous wastes. As a result, damage or infection of the gill often results in serious systemic consequences to the fish.[4]

Conference participants also discussed the difference between lamellar fusion and lamellar adhesion, both prominent features in this case. Lamellar fusion is most often associated with inflammation and is a consequence of marked epithelial proliferation. In contrast, lamellar adhesions (also known as lamellar clubbing or synechiae) occur when there is adherence of two or more secondary lamellae forming a pseudocyst, usually in the absence of epithelial proliferation or inflammation. Both lamellar fusion and lamellar adhesions are present in this case.

Multifocally, within the secondary lamellae of this case, there are dilated capillaries that contain organizing fibrin thrombi, interpreted by conference participants as examples of telangiectasia. The moderator noted that this vascular change can be a perimortem artifact secondary to capture of the fish or an antemortem change secondary to the disease process. Distinguishing between peri- and antemortem telangiectasis is often difficult, even for those experienced in gill histopathology; however, in this case, the presence of organizing fibrin thrombi is characteristic of antemortem telangiectasia rather than a perimortem capture artifact.

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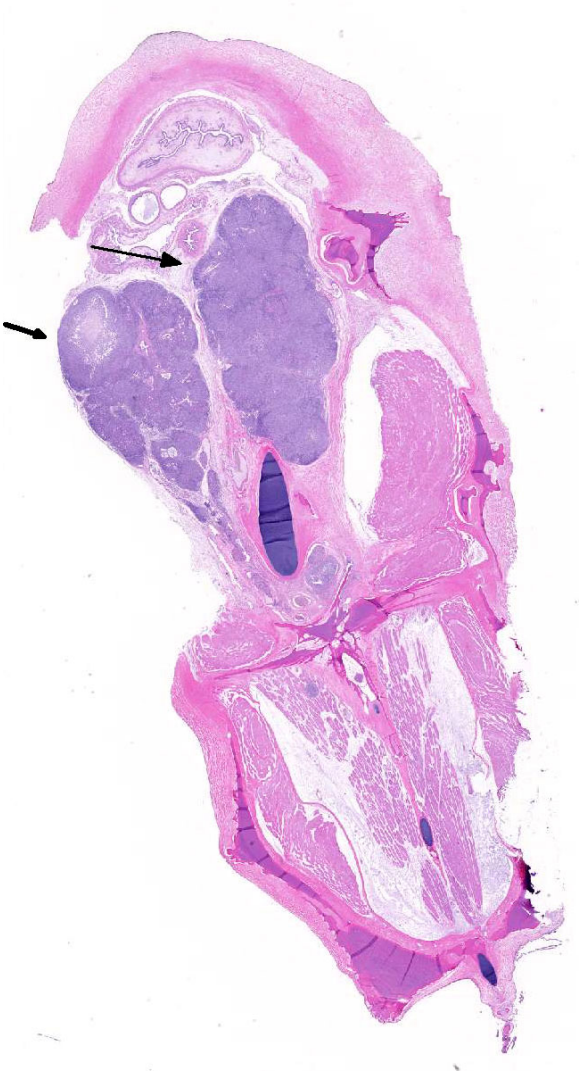
CASE III: A15-25281 (JPC 4084652).

Signalment: Adult male long snout seahorse (*Hippocampus reidi*).

History: This animal was one of 20 seahorses that experienced an epizootic of ulcerative dermatitis shortly after arrival into quarantine facilities of a public aquarium. The 15.4 cm, snout to tail tip, the male became moribund and was euthanized 14 days after shipment.

Gross Pathology: Unlike other animals from the group, there was no evidence of dermatitis. The caudal third of the kidney was enlarged and pale, with an irregularly nodular surface that bulged into the coelomic cavity.



Kidney, seahorse: Renal parenchyma is expanded and architecture is diffusely effaced by granulomatous inflammation. (HE, 5X)

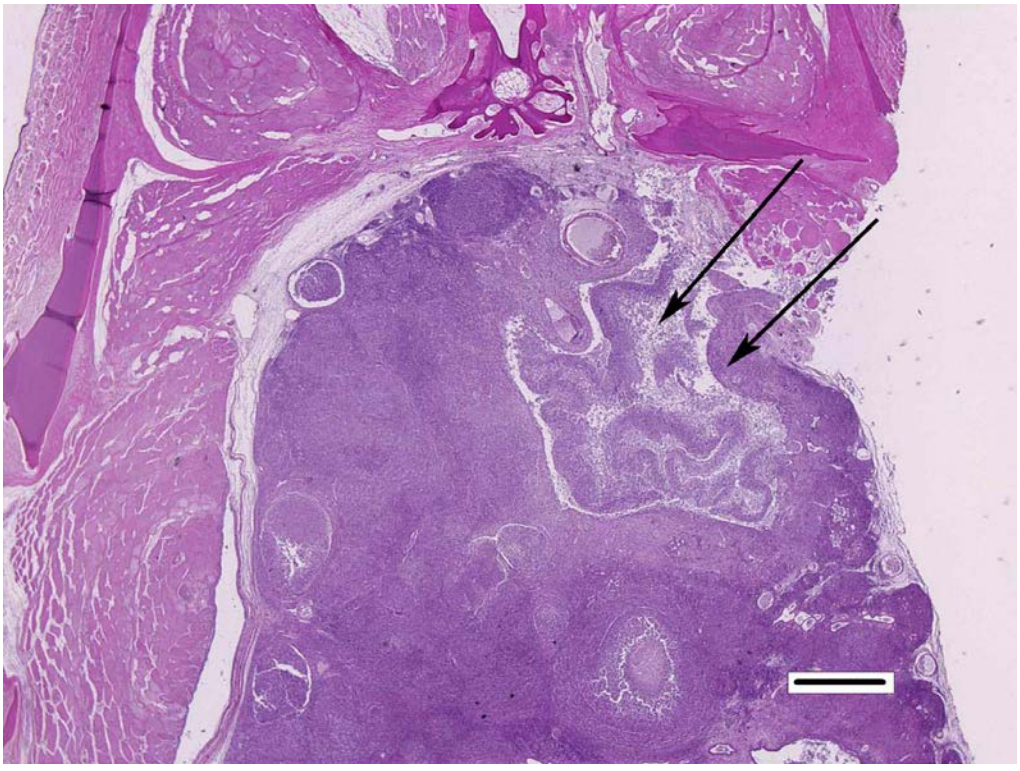
Laboratory results: Cultures of kidney and liver yielded a *Nocardia* sp. PCR amplification of the 16S rRNA gene produced a 524 bp sequence with 100% similarity to *Nocardia nova* (GenBank KP025810.1). Mycobacterial and fungal cultures were negative.

Histopathologic Description: Approximately 75% of the normal renal

parenchyma is severely altered by large numbers of variably-sized, multifocal to coalescing, haphazardly organized sheets and nodular collections of macrophages, with scattered lymphocytes and variable degrees of fibrous encapsulation. Similar streams of macrophages, fibrous tissue and scattered fibroblasts extensively displace and isolate normal hematopoietic tissue and groups of tubules. Discrete, well-organized granulomas are uncommon. Also present are multiple, often large, encapsulated foci with central areas of hemorrhage, necrotic cellular debris and individualized macrophages. On H&E and Ziehl-Neelsen stained sections, rod-shaped and filamentous bacteria can be poorly visualized, particularly in areas of necrosis. With Lillie-Twort stains, bacteria appeared as Gram-positive, coccobacillary forms and beaded filaments. Fite's stain reveals large numbers of elongate, slender, branching filaments.

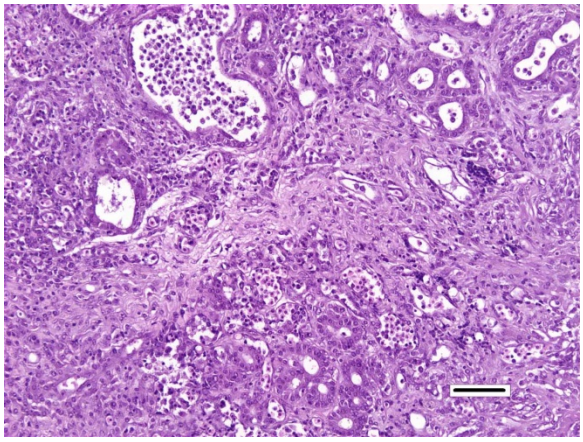
Contributor's Morphologic Diagnosis: Kidney: Granulomatous nephritis, multifocal to coalescing, chronic, severe, with multiple granulomas, multiple foci of necrosis and modified acid-fast branching bacterial filaments.

Contributor's Comment: Microscopic findings are typical of piscine nocardiosis. Changes vary between groups of slides, particularly the extent of extrarenal involvement, which includes connective tissues, muscle, intestine and the coelomic cavity in some sections. Additional findings in some sections include renal calculi, intestinal nematodes, and parasitic granulomas. Smaller lesions were widely distributed in the gills, skeletal muscle, one ocular choroid, and dermal and hypodermal connective tissues. Similar bacteria were present in all lesions. Three additional animals from the group were examined microscopically, but none showed evidence of infection.



Kidney, seahorse: Higher magnification of the kidney, demonstrating poorly demarcated, coalescing granulomas with a necrotic core. (HE, 100X)

Nocardiosis in fish has been summarized in multiple sources.[3,7] *Nocardia asteroides* was first reported from neon tetra in 1962 and has

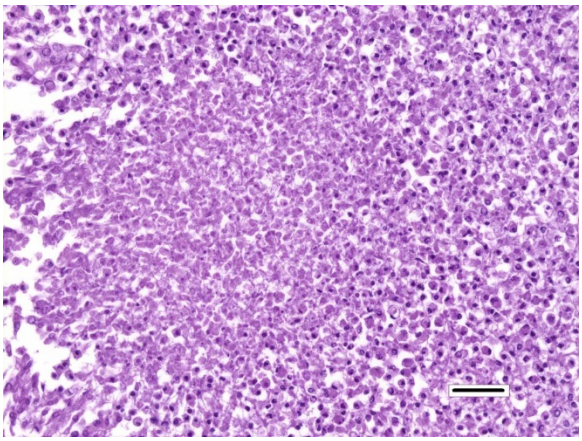


Kidney, seahorse: Higher magnification of affected kidney with marked loss of tubules. Remaining tubules are ectatic and contain numerous sloughed epithelial cells and cellular debris. (HE, 400X)

caused epizootics primarily in freshwater fish. *N. seriolae* is a major pathogen of marine fish worldwide, particularly in Asian mariculture.[2,9] *Nocardia salmonicida* has received little attention since its first isolation from sockeye salmon.[1] The systemic granulomatous disease is characterized by progressive lethargy and emaciation. Infections can be confused both clinically and grossly with those of mycobacteriosis, which is extremely common in captive seahorses. Gross lesions can include skin ulcers, muscle necrosis, and organomegaly, with small nodules in parenchymal organs and the gills. Microscopically, nodules are variously described as granulomas and abscesses, frequently with necrotic centers, peripheral macrophages, lymphocytes, possibly giant cells, and variable fibrous encapsulation.[7] The filamentous branching bacteria, stain weakly and irregularly gram-positive, poorly or not at all with the Ziehl-Neelsen acid-fast stain, and are best visualized with modified acid-fast stains, such as Fite's.

Although contaminated feed has been suggested as a source of infection, the pathogenesis of natural disease is poorly understood. Transmission has been demonstrated experimentally by injection, dermal abrasion, immersion, feeding and cohabitation.[6] Losses of 15-17% from *N. seriolae* have been reported in cultured sea bass and yellow croaker, respectively.[2,9] While microscopic lesions are frequently described, many reports only include bacterial identification to the genus level.[5] Identification of *Nocardia nova* in this case suggests greater species diversity of *Nocardia* could be involved in fish disease.[1]

JPC Diagnosis: Kidney: Nephritis, necro-granulomatous, diffuse, severe, with numerous extracellular filamentous bacilli, long snout seahorse, *Hippocampus reidi*.

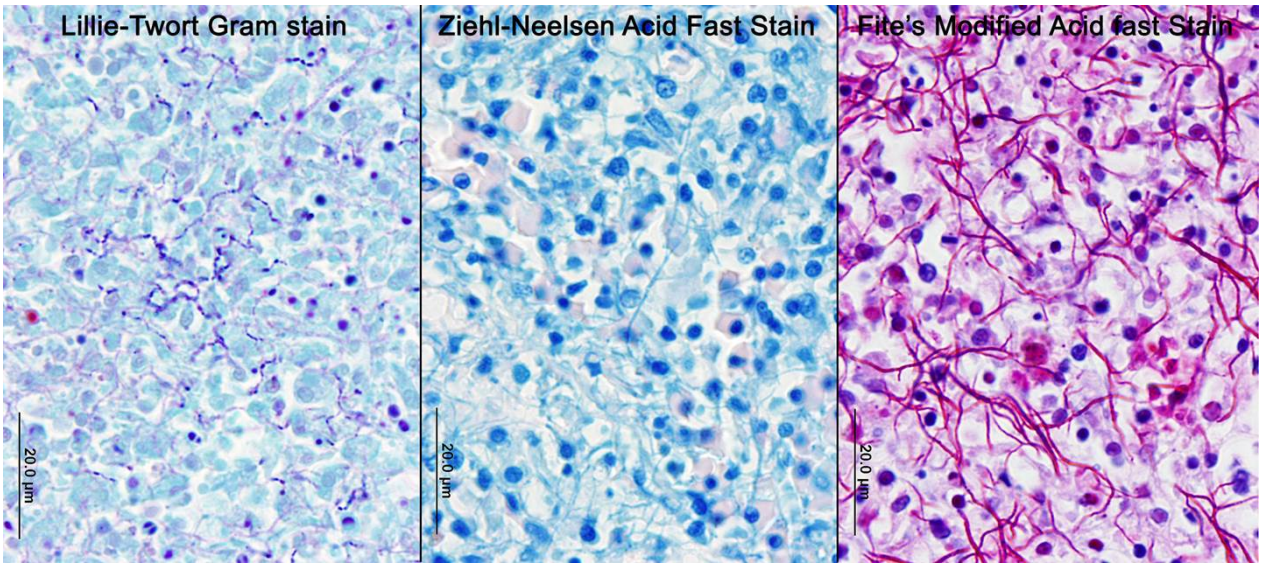


Kidney, seahorse: Necrotic core of one of the granulomas. (HE, 400X)

Conference Comment: *Nocardia* spp. are ubiquitous, saprophytic, gram-positive, higher order bacteria that are associated with both opportunistic and primary infections in a variety of terrestrial and aquatic species worldwide. Morphologically, the bacteria are long, thin, beaded filaments with frequent right-angle branching resembling Chinese letters.[8] They can be seen on standard H&E stained sections; however, they are best visualized by the histochemical stains Gomori methenamine silver (GMS), gram stains, and modified acid-fast stains, such as Fite-Faraco, as demonstrated by the

outstanding photographs provided by the contributor.[8]

Although generally thought to cause low mortality in fish, *Nocardia* spp. can induce severe chronic granulomatous systemic disease.[1,7] In this case, the normal renal architecture is almost completely effaced by multifocal to coalescing necrotizing and granulomatous inflammation. Although



Lung, penguin: Special stain panel demonstrating numerous gram-positive and acid-fast bacilli within the necrotic cores of the granulomas. (Photo courtesy of: University of Georgia College of Veterinary Medicine, Department of Pathology, 501 DW Brooks Drive, Athens, GA 30602, http://www.vet.uga.edu/VPP)

there is some slide variability, in several examined tissue sections, inflammation extends into the adjacent coelomic cavity and skeletal muscle. As mentioned by the contributor, the primary differential diagnosis for piscine nocardiosis includes infection with the much more common *Mycobacteria* sp., which produces nearly identical gross and histological lesions in fish.[7] Gross lesions include cachexia, ascites, dermal ulceration, multifocal skeletal muscle necrosis, and small white well-circumscribed granulomas in the kidney, spleen, heart, and liver.[7] As a result, nocardiosis can be easily misdiagnosed as mycobacteriosis. Positive differentiation requires isolation and identification of the infectious organism and examination of histologic sections of infected organs. Histologically, *Mycobacteria* sp. are non-filamentous, non-branching, gram-positive, strongly acid-fast bacteria and are easily differentiated from *Nocardia* with the special histochemical stains previously mentioned.[1,7,8] Additionally, virulent *Mycobacteria* spp. are obligate intracellular pathogens that replicate within host macrophages. In contrast, pathogenic *Nocardia* spp. are facultative intracellular bacteria that have complex cell wall lipids that allow for resistance to phagocytosis by host macrophages.[7,8] In this case, the vast majority of bacteria are extracellular.

In a small number of tissue sections, conference participants noted a focal granuloma within the intestinal wall centered on a degenerate larval cestode characterized by a 2 um eosinophilic tegument, a lacy, fibrillar eosinophilic parenchymatous body cavity, scattered 5 um diameter, basophilic, calcareous corpuscles, and birefringent hooks in some sections.[4] The

pathologic significance of the parasitic granuloma in the intestinal wall, in this case, is unclear; however, it may indicate that this seahorse was immunocompromised, allowing for the proliferation of various concurrent opportunistic pathogens.

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CASE IV: R1 or R2 (JPC 4085971).

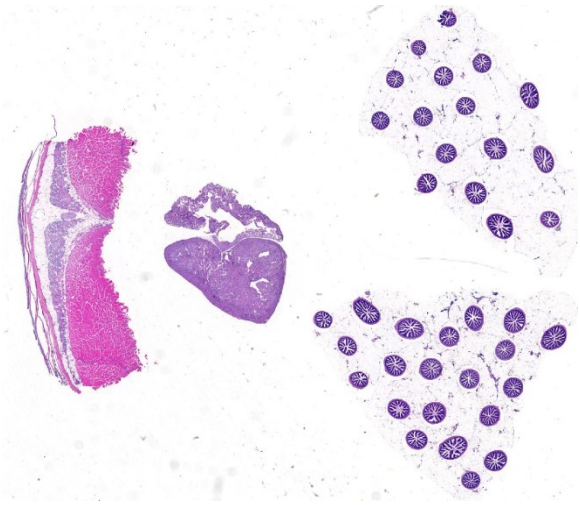
Signalment: Post-smolt, unknown gender, Atlantic salmon (*Salmo salar*).

History: This case was submitted as part of a sample of five fish from a seawater tank in which fish were presenting with loss of appetite and lethargy.

Gross Pathology: There was no digesta/ingesta in the gastrointestinal tract with small amounts of soft yellow material (fecal casts) in the distal intestine.

Laboratory results: Positive for salmonid alphavirus (SAV) by RT-PCR

Histopathologic Description: Pyloric caeca/pancreas: one section is examined consisting of multiple cross sections of



Viscera, Atlantic salmon. Left-right: Skeletal muscle, heart, and pyloric ceca and mesentery are presented for evaluation. (HE, 5X)

pyloric caeca and adjacent adipose tissue. There is extensive loss of exocrine pancreatic tissue. Multifocally, there are small to moderate numbers of mononuclear inflammatory cells (lymphocytes and macrophages). Remaining exocrine epithelial cells have reduced numbers of zymogen granules, and the islets of Langerhans remain intact.

Heart: There are degenerative changes of the cardiomyocytes in the spongy and compact layers of the ventricle, characterized by marked cytoplasmic hypereosinophilia, with variable vacuolation and occasional shrunken nuclei. In these areas, there are low to moderate numbers of lymphocytes and macrophages. Multifocally, there is increased cellularity, prominence and occasional karyomegaly of endocardial endothelium (hyperplasia). Multifocally, the epicardium is expanded by mild to moderate numbers of lymphocytes and macrophages.

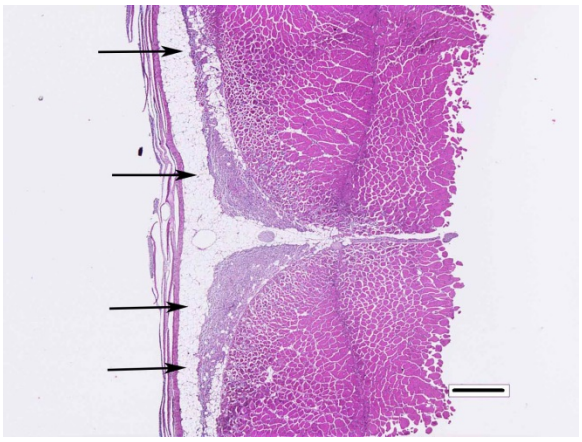
Skeletal muscle: Multifocally, there is marked loss of myofibres in the red muscle. The majority of the remaining myofibres are shrunken with densely eosinophilic sarcoplasm, surrounded by moderate numbers of mononuclear inflammatory cells. In the white muscle, there are scattered necrotic and degenerating myofibres, with swollen fragmented, eosinophilic sarcoplasm, central migration of nuclei, and sarcoplasmic infiltration by macrophages. Affected myofibres are surrounded by low numbers of lymphocytes and macrophages.

Contributor's Morphologic Diagnoses: Exocrine pancreas: Severe, diffuse, pancreatic degeneration and loss

Heart, myocardium: Marked, multifocal to coalescing, cardiomyocyte degeneration and necrosis, with endocardial hyperplasia and hypertrophy, and mild to moderate, multifocal, lymphohistiocytic epicarditis

Skeletal muscle (red myofibres): Moderate to marked, chronic, multifocal to coalescing, necrotizing myositis

Skeletal muscle (white myofibres): Mild to moderate, chronic, multifocal, necrotizing myositis



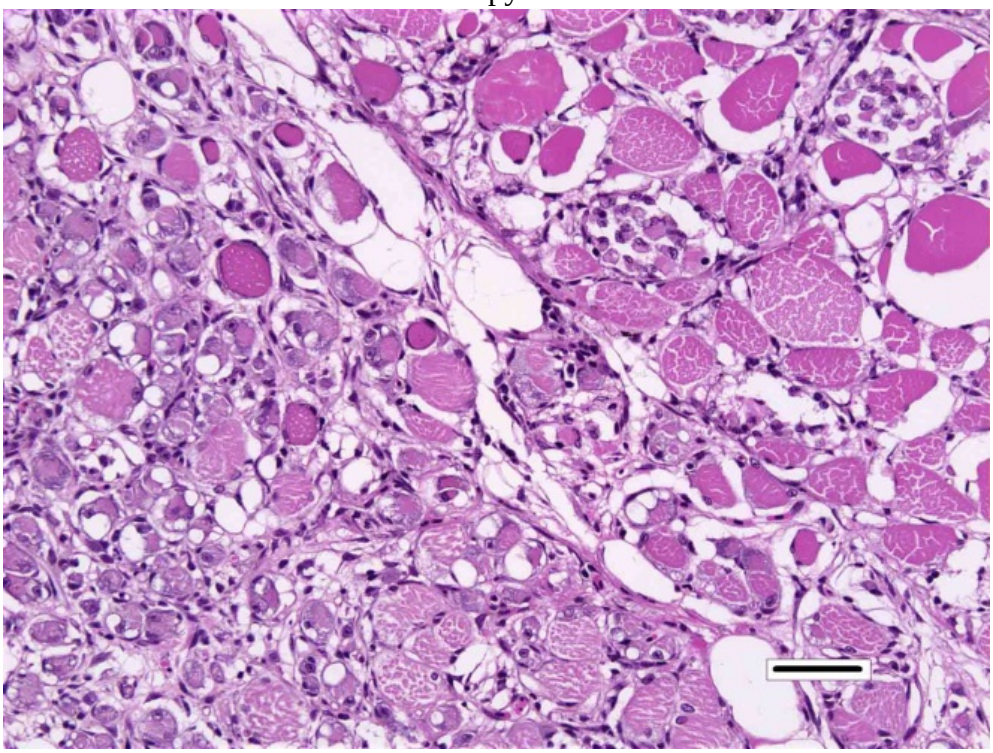
Skeletal muscle, lateral body wall, Atlantic salmon. There is marked degeneration of the overlying red muscle (arrows). There is milder degeneration and atrophy of the exterior portion of the underlying white muscle. (HE, 40X)

Contributor's Comment: The microscopic features of this case are consistent with previously published findings for pancreas disease (PD) in farmed Atlantic salmon, and the histopathological diagnosis was confirmed by RT-PCR. PD is caused by salmonid alphavirus (SAV), which was first described in farmed Atlantic salmon from Scotland in the mid-seventies.[16] It has subsequently been responsible for major economic losses to the Atlantic salmon farming industries in Scotland, Ireland, and Norway. At present, six closely related subtypes have been identified for SAV[4,5], which differ in host specificity, geographical location[7,10] and aquatic environments (Table 1). SAV-2 is the only subtype commonly detected in freshwater systems, causing sleeping disease in freshwater trout. Current research also suggests there may be differences between strains in the infection dynamics[7] as well as minor differences in prevalence and severity of the tissue damage[8]. Naturally occurring outbreaks of PD in farmed Atlantic salmon have only been reported in the seawater phase of production.[15] To date, there has been no evidence of vertical transmission of the disease[11] with horizontal transmission being by far the most important means of spreading the virus[1,19], with shedding of mucus and feces described as transmission routes for SAV.

Table 1. Summary of Salmonid alphavirus subtypes, their geographical distribution and species susceptibility

SAV Subtype	Species	Production Phase	Country
SAV-1	Atlantic salmon (*Salmo salar*)	Seawater	Ireland and Scotland
SAV-2	Rainbow trout (*Oncorhynchus mykiss*)	Freshwater	France, England, Scotland, Spain, Croatia and Germany
SAV-2 (Marine)	Atlantic salmon (*Salmo salar*)	Seawater	Scotland and Norway
SAV-3	Atlantic salmon (*Salmo salar*) Rainbow trout (*Oncorhynchus mykiss*)	Seawater	Norway
SAV-4	Atlantic salmon (*Salmo salar*)	Seawater	Ireland and Scotland
SAV-5	Atlantic salmon (*Salmo salar*)	Seawater	Scotland
SAV-6	Atlantic salmon (*Salmo salar*)	Seawater	Ireland

Gross findings at postmortem examination during the early stages of the disease may include the absence of food in the gut and the presence of fecal casts in the hindgut.[15] Occasionally, petechial hemorrhages can be detected over the surface of the pyloric caeca and surrounding visceral fat. During the chronic stages of the disease, non-feeding fish with low body condition and minimal internal body fat (runts) tend to be more common due to the failure of the pancreas to recover[20]; these fish are more susceptible to parasitic and secondary bacterial infections, which may present concurrently. Apparently healthy fish may also die suddenly due to cardiac and skeletal muscle damage and exhaustion; this manifestation of the disease appears to be more common when older grower fish are infected during their second year at sea.



Skeletal muscle, lateral body wall, Atlantic salmon. Higher magnification of red (left) and white muscle(right.) There is marked muscle atrophy and numerous degenerate and necrotic fibers. There is prominent hypertrophy of satellite nuclei and expansion of the perimysium with histiocytes and lymphocytes. (HE, 100X)

The acute phase of the disease is relatively short-lived with rapid destruction of the exocrine pancreatic tissue and a variable inflammatory response ranging from no inflammation to moderate mononuclear cell infiltration and/or fibrosis of the periacinar tissue.[15] In recovering fish, regeneration of exocrine tissues can take place as early as four weeks post infection. Acute heart lesions can be observed concurrently or slightly lagging behind the acute pancreatic changes and includes multifocal cardio-myocyte necrosis with affected cells having a shrunken, deeply eosinophilic cytoplasm and pyknotic nuclei. Both the compact and spongy layer of the ventricular myocardium and the atrial myocardium can be affected to varying degrees, ranging from mild focal changes to involvement of the entire heart musculature.[15] Hypertrophy of myocardial nuclei, particularly at the junction of the spongy and compact ventricular myocardium can be evident in the recovery phase of the disease. Skeletal muscle pathology tends to first appear 3–4 weeks after the appearance of pancreatic and heart lesions, and fish sampled in late-phase disease may only have skeletal muscle lesions. The skeletal muscle lesions consist

of hyaline degeneration with swollen fragmented eosinophilic sarcoplasm, central migration of myocytic nuclei and subsequent invasion of the sarcoplasm by phagocytic macrophages.[15]

The differential diagnoses for these cardiac lesions should at least include the two following diseases:

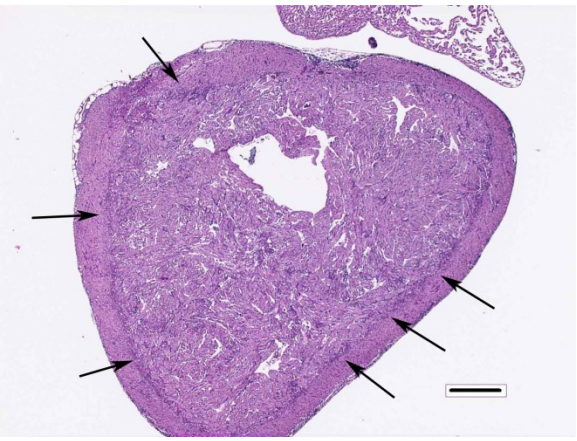
Heart and skeletal muscle inflammation (HSMI) has been reported in farmed Atlantic salmon in Norway, Scotland, and Chile. It typically affects fish 5 to 9 months after sea-transfer. Mortality in affected cages may be negligible but in some cases can reach up to 20%. Histologically, HSMI mainly targets cardiomyocytes and myofibres of red skeletal muscle. Affected myocytes show signs of degeneration, including loss of striation, sarcoplasmic eosinophilia, vacuolation, centralized nuclei (in skeletal muscle), and karyorrhexis. In HSMI inflammation is more prominent than myocyte necrosis in most cases.[11,13,18] Recently piscine reovirus (PRV) has been suggested to be associated with HSMI infection[17,21].

Cardiomyopathy syndrome (CMS) affects harvest-sized Atlantic salmon, 12–18 months after sea-transfer, and mainly affects the heart without involvement of skeletal muscle. Histopathological changes include necrosis and inflammation of the spongy layer of ventricle and atrium, epicarditis, and infiltration of lymphocytes and macrophages, with rupture of atrium or sinus venosus visible macroscopically.[3,18] A *totivirus* (piscine myocarditis virus (PMCV)) is proposed as the causative agent for this syndrome[14,18,19,21]. Both HSMI and CMS are not typically associated with any pancreatic pathology.

Differential diagnosis for pancreatic necrosis should include infectious pancreatic necrosis (IPN), a viral disease that primarily targets the pancreatic tissue and is caused by an aquatic birnavirus (known as IPN virus). However, IPN does not typically cause cardiac and skeletal muscle pathology.

Table 2. Comparison of histopathological lesions in farmed Atlantic salmon with Pancreas disease (PD), Heart Skeletal Muscle Inflammation (HSMI), Cardiomyopathy Syndrome (CMS) and Infectious Pancreatic necrosis (IPN)

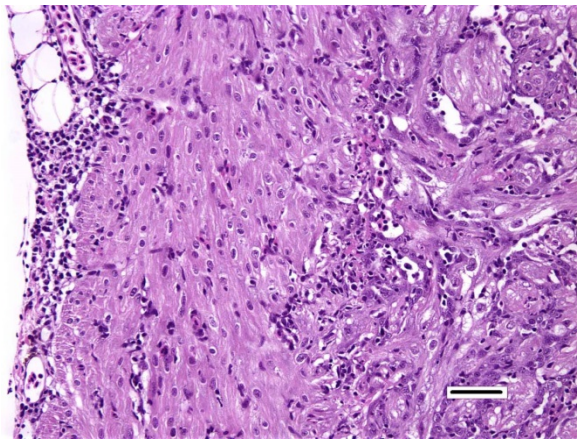
Lesions	PD				HSMI	CMS	IPN
	Acute	Sub-acute	Chronic	Recovery			
Myocardial necrosis	+	+	+	-	+	+	-
Endocardial proliferation	+	+	+	+	+	+	+/-
Cardiomyocyte hypertrophy	-	+/-	+	+	+	+	-
Epicarditis		+	+	+	+		-
Exocrine acinar necrosis	+	+	+	+/-	-	-	+
Myositis	-	-	+	+	+		-



Heart, Atlantic salmon. At subgross magnification, there is a cellular infiltrate within the epicardium and at the junction between the compact and spongy layers of the ventricular myocardium (arrows). (HE, 20X).

JPC Diagnosis: 1. Pancreas: Loss, diffuse, severe with mild lymphocytic pancreatitis, Atlantic salmon, *Salmo salar*.
2. Heart, ventricle: Epicarditis and myocarditis, lymphocytic, diffuse, moderate with multifocal mild myocardiocyte necrosis.
3. Skeletal muscle, red: Degeneration and necrosis, multifocal to coalescing, severe with histiocytic myositis.
4. Skeletal muscle, white: Degeneration and necrosis, multifocal to coalescing, mild to moderate with histiocytic myositis.

Conference Comment: This excellent case demonstrates the prototypical constellation of lesions associated with chronic salmonid alphavirus (SAV) infection. Histological examination of infected fish typically reveals a near complete loss of exocrine pancreatic tissue, epicarditis and myocarditis centered on the ventricle, and skeletal muscle degeneration and necrosis of the white and red muscle.[9,15] Each subtype of SAV listed in Table 1 produces similar morphologic changes and are unable to be distinguished histologically. One of the most difficult tasks for the novice histopathologist is to recognize the complete absence of a normal structure in a tissue section, especially when there is little to no inflammation associated with that loss. Within the multiple cross sections of the pyloric cecae, in this case, conference participants astutely noted a near diffuse loss of exocrine pancreas with only small clusters of remaining exocrine pancreatic cells containing brightly eosinophilic zymogen granules surrounded by mild lymphocytic inflammation that extends into the peripancreatic fat. The islets of Langerhans are mostly unaffected. This diffuse loss of exocrine pancreas with relatively mild inflammation is typical for the chronic phase of this disease. As mentioned by the contributor, the acute phase is associated with a short-lived with rapid necrosis of the exocrine pancreatic tissue and an inflammatory response ranging from no inflammation to moderate mononuclear cell infiltration. The contributor provides an outstanding and thorough review to SAV and includes helpful tables to rule out potential differential diagnoses based on the lesions present in this case.



Heart, Atlantic Salmon. Higher magnification demonstrating the lymphocytic infiltrate in the epicardium (left) and within the myocardium (center). (HE, 200X)

Both pancreas disease (PD) of salmon, seen in this case, and sleeping disease (SD) of rainbow trout is caused by related SAV infection. This virus is in the genus *Alphavirus* and family *Togaviridae*, a group of important enveloped ssRNA viruses.[9,15] Other members of the *Togaviridae* family include eastern, western, and Venezuelan equine encephalitis viruses. Generally, this family of viruses is associated with transmission by insects, usually mosquitoes. An aquatic arthropod vector has not yet been identified for SAV; however, direct horizontal transmission has been well documented.[9,15] The virus can survive for extended periods of time in the water outside of the host with a marked increase in survival in sea water compared to fresh water. The virus can also survive for long periods within the fat of dead fish and leaked fat droplets floating on the surface may contribute to long distance spread of the virus. Vertical transmission has not been shown to be a significant route of infection.[9,15]

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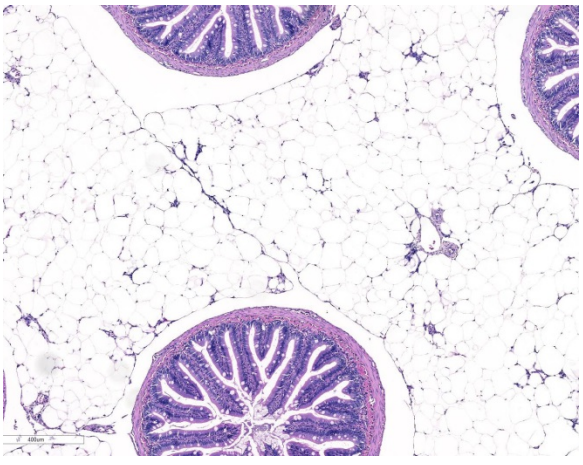

Mesentery and pyloric ceca, Atlantic salmon: The mesentery is devoid of pancreatic tissue. Few pancreatic islets remain. (HE, 30X)

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