



WEDNESDAY SLIDE CONFERENCE 2012-2013

Conference 12

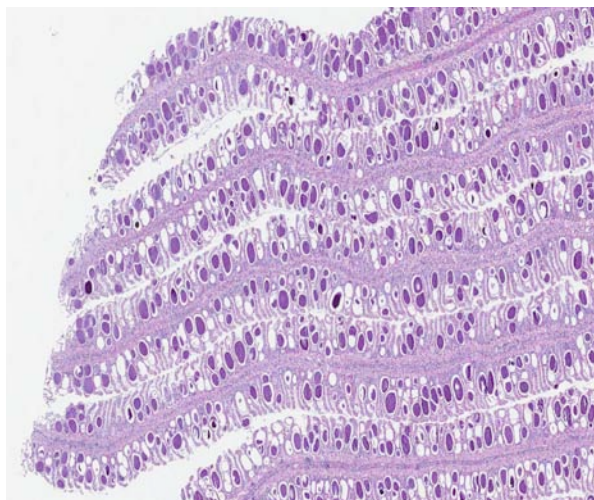
23 January 2013

CASE I: A10-5331 (JPC 3167830).

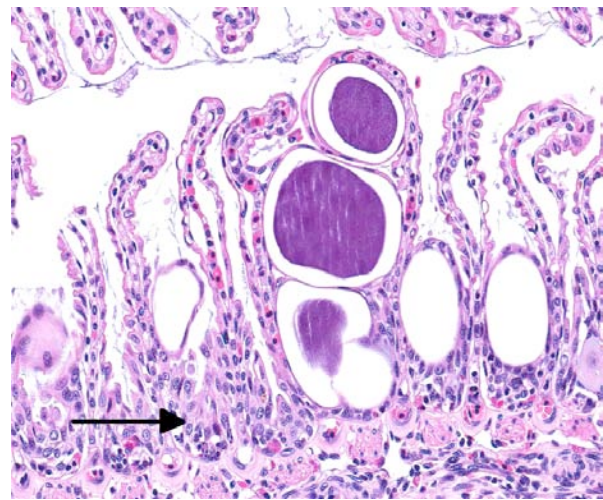
Signalment: Female, age unknown, spotted eagle ray (*Aetobatus narinari*).

History: This 11.6 kg, 90.8 cm disc width female had been collected 5 months earlier off the Florida coast and had completed an uneventful 45-day quarantine period prior to display in a public

aquarium. Acute onset of an intermittent rolling swimming pattern and “flashing,” typically a sign of external parasitism, was followed by progressive lethargy. Supportive treatment was initiated and a gill clip performed. Wet mount preparations of the clip revealed large numbers of large amorphous spherical bodies free and within gill lamellae. Despite treatment with oxytetracycline and chloramphenicol, the animal’s



1-1. Gill, spotted eagle ray: Multifocally, numerous lamellar epithelial cells are expanded by granular basophilic intracytoplasmic inclusions. (HE 24X)



1-2. Gill, spotted eagle ray: Multifocally lamellar epithelial cells are expanded up to 150 μ m by granular intracytoplasmic inclusions composed of myriad bacteria, and multifocally, there is hyperplasia of lamellar epithelium with bridging and fusion of lamellae (arrow). (HE 200X)

condition continued to deteriorate and euthanasia was elected.

Gross Pathologic Findings: Gross necropsy findings were minimal. External changes were limited to mild pallor and mottling of the gills. Internally, the gastrointestinal tract was devoid of contents. The liver was unusually small, firm and tan for an elasmobranch species and failed to float in formalin.

Laboratory Results: Aerobic cultures of liver, spleen and kidney were negative. PCR using pan-chlamydial primers for the 16S rRNA gene sequence produced a 276 bp product with 84% identity to *Candidatus Piscichlamydia salmonis*.

Histopathologic Description: Approximately 60-80% of lamellar troughs were occluded by one or more well delineated, 50-200 µm, amorphous, pale basophilic inclusions that markedly distended lamellar epithelial cells. Additional changes included widespread mild to occasionally moderate epithelial cell hypertrophy and hyperplasia, with patchy multifocal lamellar fusion. Multifocal infiltrates of small to moderate numbers of mixed inflammatory cells, predominated by lymphocytes and granulocytes, were located primarily in gill filaments and at the bases of lamellar troughs.

Contributor's Morphologic Diagnosis: Gill: Multifocal, mild, epithelial hypertrophy and lamellar fusion, with widespread intraepithelial amorphous basophilic inclusions and subacute, mild to moderate, filamental bronchitis.

Contributor's Comment: Microscopic findings were consistent with epitheliocystis disease and supported by PCR results indicating 16s rDNA homology with other piscine chlamydia-like agents associated with the condition. *Epitheliocystis* was first observed by Plehn in 1920 and the first connection made with a chlamydial or rickettsial-like agent by Hoffman in 1969. The disease is now known to affect the skin and gills of over 50 freshwater and marine teleosts, but is unusual in elasmobranch species. Although lesions are not uncommon in wild fish, they are rarely associated with mortalities and the condition is considered primarily a disease of aquaculture. Under intensive culture conditions mortalities can reach 100%, probably due to respiratory compromise, but are generally age

dependent, with young fingerlings showing the greatest susceptibility. Losses are also influenced by stress factors typical of aquaculture, such as high stocking densities and suboptimal water quality conditions.^{3,7}

Lesions are characterized by hypertrophied epithelial cells containing intracellular, spherical, membrane bound inclusions. Localization in the gill, as well as proliferative and inflammatory responses to the bacteria are highly variable. By electron microscopy two distinct developmental cycles exhibiting different histochemical and immunohistochemical staining properties have been identified in fish, but their significance is unclear. Cycle I is typical of chlamydia, involving reticulate, intermediate, and infectious elementary bodies that all possess distinct compact nucleoid regions. Microscopically, this cycle has been associated with granular inclusions in young fish during epizootics. Cycle II involves elongate forms lacking nucleoids and is reported to be more common in older animals.³

Growing molecular evidence indicates wider genetic biodiversity and host range among the *Chlamydiales* than previously recognized. This is reflected in the recent expansion of the order to include a number of new families (*Simkaniaceae*, *Parachlamydiaceae*, *Waddliaceae*) and reorganization of the family *Chlamydiaceae*.² Concomitant with this, several specific agents have been described for the first time in association with epitheliocystis, including *Candidatus Piscichlamydia salmonis* in Atlantic salmon, *Candidatus Clavochlamydia salmonicola* in multiple salmonid species, and a *Neochlamydia* sp. in Arctic char.^{4,5,6} To date, however, no piscine chlamydial agents have been isolated in culture and Koch's postulates remain unfulfilled. Diagnosis is based largely on histopathology.

JPC Diagnosis: 1. Gill: Lamellar epithelial hyperplasia and hypertrophy with multifocal lamellar fusion, numerous intraepithelial intracytoplasmic bacilli, and subacute branchitis.
2. Gill: Lamellar telangiectasia with multifocal thrombosis.

Conference Comment: The contributor provides a very good summary of epitheliocystis disease in fish. In addition to the characteristics and causes of this disease, conference participants discussed the presence and role of alarm cells in the sections

examined. Alarm cells (aka alarm substance cells) in fishes in the superorder *Ostariophysi* (minnows, characins, catfishes etc) and the similar club cells in non-ostariophysans (perch, walleye, saugers and darters) are specialized cells found in superficial epidermis. When damaged by predatory attack or other traumatic insult, their contents are released and serve as a chemical alarm to warn neighboring fishes of danger. The pheromone released from alarm cells is referred to as “Schreckstoff” which means “fear substance” in German. Recent studies have suggested that in addition to acting as a warning system, alarm cells may also provide protection against pathogens, parasites and UV radiation that compromise the integrity of the epidermis.¹ In the sections of gill from this spotted eagle ray there were numerous cells that appeared similar to alarm cells (large, round to oval cells with centrally placed nuclei with one to two prominent nucleoli). Due to the lack of a control, participants were unable to determine if these alarm cells were present in normal numbers, or were increased in numbers as a response to chronic gill pathology. Interestingly, the chlamydial colonies appear to be infecting the alarm cells as well as the lamellar epithelium.

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aurata: morphological evidence for two distinct chlamydia-like developmental cycles. *Diseases of Aquatic Organisms*. 1999;37:61-72.

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CASE II: F-09-044 (JPC 3149419).

Signalment: Approximately six months old, no gender noted, green grouper (*Epinephelus coioides*).

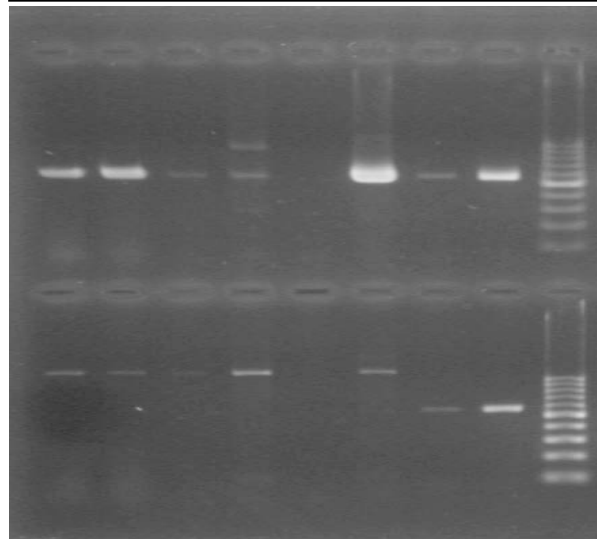
History: Eleven thousand green grouper (*Epinephelus coioides*) were imported from Taiwan in October 2008 and delivered to nine cages in Hong Kong SAR for culture. In mid-June 2009, gradual mortalities began to occur. Up to 70% of the fish displayed clinical signs of lethargy and whirling swimming movement. There were no external lesions noted. Microscopic examination in the field revealed gill parasites. Seven fish were submitted for pathological examination.

Gross Pathologic Findings: Moderate levels of parasitic infestation of the gills identified as dactylogyrid flukes. No other significant findings.

Laboratory Results:

Red sea bream Iridovirus detection by gel PCR:

	Gel PCR on Tissue homogenate	Gel PCR on Tissue homogenate
Sample ID	RSIV1 set primers	RSIV4 set primers
Spleen	Pos	Neg
Kidney	Pos	Neg
Brain	Pos	Neg
Eye	Pos	Neg



Histopathologic Description: Spleen: Diffusely, normal splenic architecture is disrupted by the presence of hypertrophic cells that have enlarged nuclei with stippled, faintly basophilic cytoplasm. Small numbers are contracted and densely amphophilic. Some cells measure up to 25 µm in diameter.

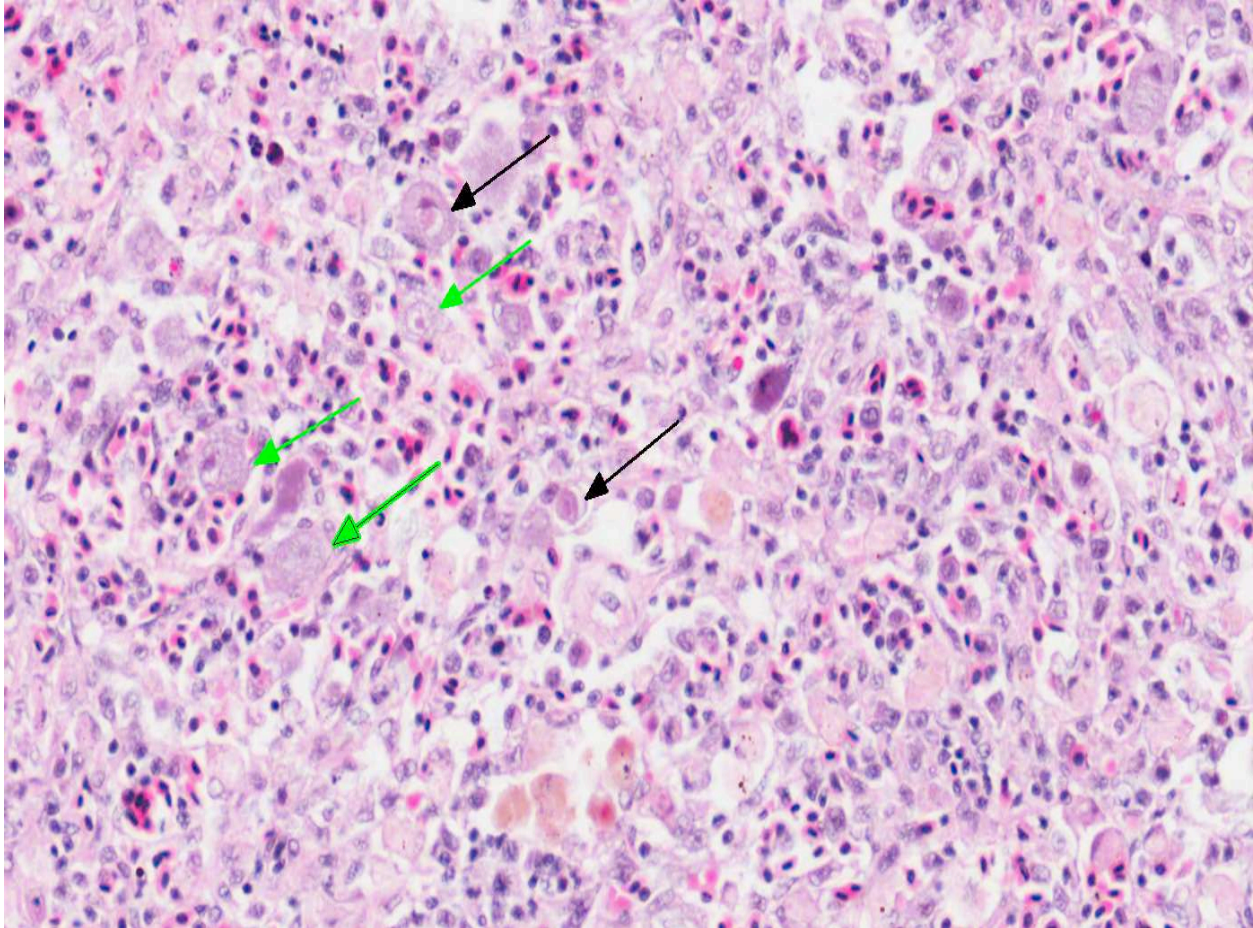
Contributor’s Morphologic Diagnosis: Spleen: Splenitis, severe, diffuse, subacute, cellular hypertrophy consistent with Red sea bream iridovirus disease.

Cause: Infectious spleen and kidney necrosis virus.

Contributor’s Comment: Red sea bream iridovirus disease (RSIVD) is caused by a particular strain of Red sea bream Iridovirus (Ehime-1) as well as by other genotypes of Red sea bream Iridovirus (RSIV). Infectious spleen and kidney necrosis Virus (ISKNV) has also been proven to cause RSIVD.³

Iridoviridae gets its name from *Tipula* iridescent virus, which grows in the haemocoel of the crane fly (*Tipula* sp.) and makes the fly iridescent. The iridoviruses may have an envelope derived from the host plasma membrane. This membrane may not be critical for infectivity but enhances it. The iridoviruses are large isometric viruses with icosahedral symmetry, 130-133 nm in diameter, and comprise a spherical nucleoprotein core surrounded by a membrane consisting of lipid modified by protein subunits. The genome consists of a single linear molecule of double stranded DNA. Transcription and DNA synthesis are nuclear. Virion assembly is cytoplasmic.⁴ Replication occurs in two stages: the first stage is in the nucleus and second stage is in the cytoplasm.¹

Piscine iridoviruses belong to the genera *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus*. In recent years, the genotypical variation between newly found iridovirus strains included in the genus *Ranavirus* has been studied in this viral family. However, the properties of and variation between iridovirus species have not been well characterized except for a few iridoviruses isolated from amphibians. Members of the *Megalocytivirus* genus produce characteristic basophilic inclusion bodies in the enlarged cells of host fish organs, which have



2-1. Spleen, green grouper: Myeloid precursors are enlarged up to 40 μm with a large nucleus and a prominent nucleolus (green arrows) and variably distinct, basophilic, irregular intracytoplasmic viral inclusion bodies (black arrows). (HE 400X)

been collected from mass mortalities occurring in wild and cultured fish species. Many other piscine iridoviruses have been reported in Asian countries from more than 100 different species, including freshwater and marine fish.⁴

In this case, gel PCR was performed on tissue homogenate by using two sets of primers according to the recommended World Organization for Animal Health (OIE) protocol. RSIV1 primers revealed virus in all tissues tested (i.e., spleen, kidney, brain, and eye). RSIV1 (Forward & Reverse) primers are used for the amplification of the gene sequence (570 bp) of both RSIV DNA and ISKNV DNA. RSIV4 primers were used for amplification of DNA gene sequence (open reading frame, ORF) (563 bp); however, all results were negative.³ RSIV4 primers (Forward & Reverse) amplify RSIV, but not ISKNV DNA. These results indicated that the virus detected in these tissues is ISKNV, one of

the causative agents of Red sea bream Iridovirus Disease.

The first outbreak of RSIVD was recorded in 1990 on Shikoku Island, Japan. Since that time, the disease has also been found widely in East and Southeast Asian countries, including Chinese Taipei, People's Republic of China, Hong Kong, Republic of Korea, Malaysia, Philippines, Singapore and Thailand. Transmission is horizontal via the water. Vertical transmission of RSIVD has not yet been investigated. Carrier states of the agents have also not yet been investigated.³

As in this case (which occurred in July 2009), disease outbreaks seem to occur most often during the summer months when water temperatures are 25°C or above.³ Diseased fish are typically lethargic, show severe anemia, petechiae of the gills and hypertrophic spleens.⁶ The susceptibility of juveniles is generally higher than adults.³ The

only clinical sign seen in this case was lethargy. Histological preparations characteristically show enlarged cells of the spleen, kidney, liver and gill.⁴ Hematopoietic tissue is located in the stroma of the spleen and the interstitium of the kidney in teleosts. Therefore, histopathological observations are consistent with the anemia and splenomegaly observed in fish with RSIVD.⁶ Not all of the fish in this group had histopathological changes and many had no lesions in any organ examined.

We found some inconsistencies in the terminology from the literature regarding the enlarged cells seen in RSIVD. Some texts and journals refer to the hypertrophied cells as having inclusion bodies, while others distinctly refrain from using that term.

JPC Diagnosis: 1. Spleen, leukocytes: Cytomegaly, diffuse, marked, with intracytoplasmic viral inclusions.
2. Mesentery: Peritonitis, subacute, mild.

Conference Comment: Participants noted that, although cells displaying advanced cytoplasmic effects (i.e., degeneration and necrosis) are seen in the tissues examined, the more prominent feature in these sections is cytomegaly, which is typical of *Megalocytivirus* infections.

In addition to RSIVD, proficiently described by the contributor, participants discussed other viruses in the family *Iridoviridae* that affect commercial fish production. Specifically, *Lymphocystivirus* spp., including lymphocystis disease virus 1 (LCDV-1) and LCDV-2, cause lymphocystis disease in many species of freshwater and marine fish.² These viruses infect fibroblasts in the skin, gills, and internal connective tissue and arrest cell division but not cell growth, resulting in markedly hypertrophied cells (i.e., “lymphocysts”) that can reach up to 100,000 times their normal size.² Microscopically, lymphocysts have the following distinct features: a large nucleus, a hyaline-like capsule, and bizarre and segmented cytoplasmic inclusions. Grossly they appear as raised, pearl-like lesions. Lymphocystis is generally a self-limiting disease and is rarely fatal, as fish usually slough the external lymphocysts; however, the virus does impact the fish industry due to the cosmetic effects in fish sold for food or as ornamental fish. Additionally, the lesions may provide portals of entry for secondary pathogens.²

Conference participants briefly discussed the nomenclature for green grouper. In addition to green grouper, *Epinephelus coioides* is known by several common names, including orange spotted grouper, estuary cod, and estuary rock cod.⁷

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7. www.arkive.org

CASE III: 11-8273184 (JPC 4004721).

Signalment: Adult blacklip abalone (*Haliotis rubra*).

History: Approximately twenty sick and dying abalone were found off Tyinga, near Baird's Bay on the west coast of South Australia. Post-mortem changes were significant and the cause of death was unclear. Due to an outbreak of abalone viral ganglioneuritis (AVG), a notifiable disease of abalone in South Australia occurring in Tasmania, the case was regarded as urgent and treated as a possible emergency disease.

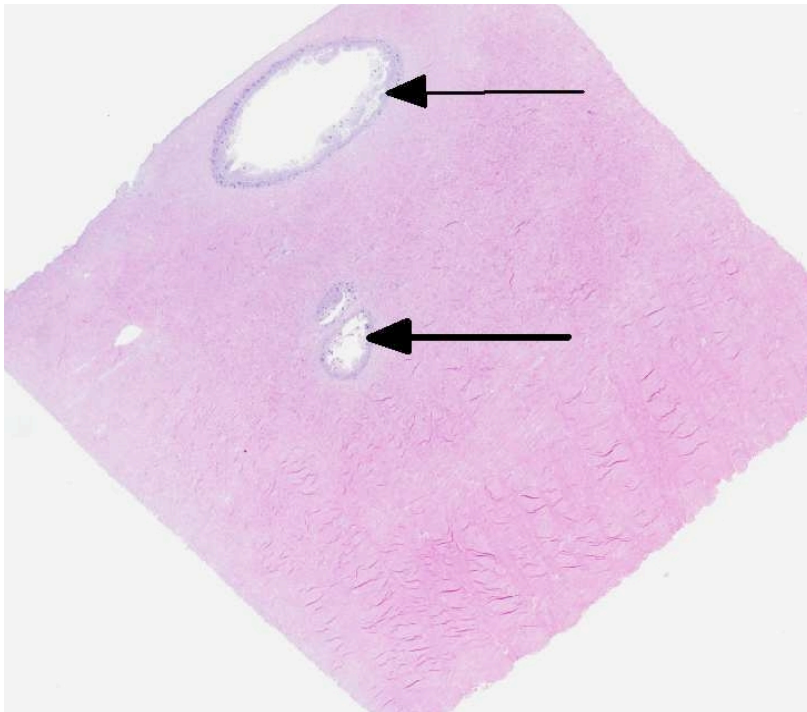
Samples of mouthparts and a part of the foot and surrounding mantle from 3 affected blacklip abalone, 1, 2, and 3 were submitted in 10% buffered formalin for microscopic examination as well as a part of the foot with pedal nerves in alcohol for referral to Australian Animal Health Laboratory for PCR for abalone ganglioneuritis (ABG).

The cystic nodules contained flocculent yellow or yellow-brown watery fluid. Lesions were most numerous in abalone 2 (from which these sections have been prepared).

Laboratory Results: PCR was negative for ABG.

Histopathologic Description: Pedal tissue: Moderate post mortem changes are present. There are single (in some sections) or multiple rounded cystic lesions containing large numbers of ovoid vacuolated or dark-staining unicellular organisms 10-17 µm in diameter and multicellular rounded organisms 12-30 µm in diameter amongst moderate numbers of infiltrating haemocytes. The lesions are well delineated but non-encapsulated. In occasional sections, similar lesions are seen abutting onto, but not infiltrating into, pedal nerves.

There were no histopathological lesions of ABG in the mouthparts (sections not presented).



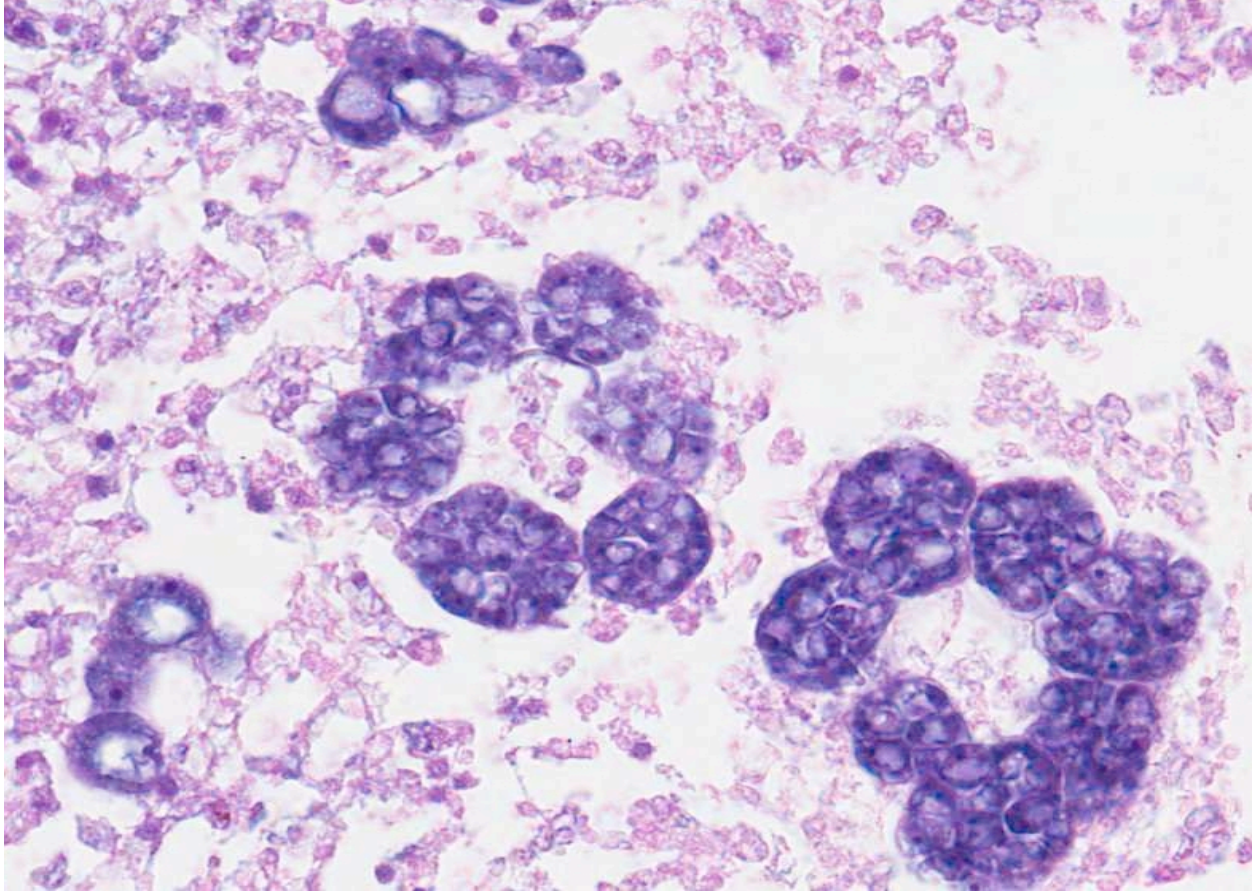
3-1. Foot, abalone: Within the skeletal muscle of the foot, there are multifocal areas of necrosis measuring up to 5 mm in diameter (arrows). (HE 40X)

Contributor's Morphologic Diagnosis: Multifocal necrosis and cavitation with numerous intralesional protistal organisms consistent with *Perkinsus* spp. and haemocyte infiltration, pedal tissue.

Contributor's Comment: *Perkinsus* spp. is a group of protists in the phylum *Perkinsozoa*, in the infra-kingdom *Alveolata*, but there remains uncertainty about the status of the genus and there are divergent views regarding its taxonomy. Susceptible hosts include abalone: *Haliotis rubra*, *Haliotis laevigata*, *Haliotis cyclobates* and *Haliotis scalaris*, but a wide range of other molluscs are also susceptible. The known range of *P. olseni* includes Australia, Europe and eastern Asia.

Gross Pathology: Multiple randomly scattered, firm, yellow, raised or centrally cystic nodules, ranging from 2 mm to 8 mm were seen within and along the edges of the foot of all three abalone.

O'Donoghue et al linked *P. olseni* to widespread kills of abalone in the Gulf of St. Vincent side of the Yorke Peninsula, South Australia, and abalone stocks in this area have not recovered



3-2. Foot, abalone: Areas of necrosis contain numerous protozoal trophozoites and multinucleated hypnospores. (HE 400X)

(S. Mayfield personal communication).⁵ In the early 1990's, a significant proportion of *H. rubra* along approximately 500 km of the NSW coastline between Port Stephens and Jervis Bay died in association with *P. olseni* infections.

The route of entry of *Perkinsus olseni* is unknown. It proliferates in tissues but is eventually sequestered by the host and killed or ejected. During sequestration the parasite becomes surrounded by abalone pigment, causing the yellow/brown appearance of the nodules. These reduce market value, causing losses in fishery value. In some severe infections, death occurs without nodule formation, suggesting that the host is unable to mount an effective immune response in some cases. Infection is not always lethal to *H. rubra* and expression of disease is widely associated with environmental variables, primarily temperature, with infections becoming more severe at higher temperatures or when food availability diminishes.⁴ Transmission occurs directly; prezoosporangia develop into

zoosporangia in seawater which then release hundreds of motile, biflagellated zoospores (about 3 by 5 μm) which are infective to abalone and other molluscs.³

JPC Diagnosis: Pedal tissue: Rhabdomyositis, necrotizing, focally extensive with numerous protistal trophozoites and schizonts.

Conference Comment: The contributor provides a good summary of *Perkinsus olseni* infections in mollusks. Conference participants discussed terminology for the life stages of these protists. The life cycle of *Perkinsus olseni* consists of trophozoite, hypnospore and zoospore stages.^{1,6} Hypnospores (prezoosporangia) are dormant, thick-walled cells that develop into motile, flagellated zoospores in aerated seawater. Once ingested by the host, the zoospore develops into a non-motile, multi-nucleated single cell trophozoite. Under anaerobic conditions, such as occurs in necrotic tissue, the trophozoites develop the vegetative hypnospores.^{1,6} Participants also

discussed another *Perkinsus* species, *P. marinus*, which causes Dermo disease in oysters. Dermo is a significant cause of mortality in the Eastern oyster, *Crassostrea virginica*.⁶

<http://shellfish.ifas.ufl.edu/PDFs/Publications/Perkinsus%20Infections%20of%20Bivalve%20Molluscs.pdf>. Accessed online 26 January 2013.

Although this animal did not have abalone viral ganglioneuritis (AVG), participants discussed this disease as well. AVG is caused by a herpesvirus that affects only abalone; other mollusks appear to not be affected. Mortality rates in farmed abalone are up to 100%. The disease has been found to be slowly spreading in wild abalone along the Victorian (Australia) coast as well; however, the exact mortality rate in wild populations is uncertain.²

There was moderate slide variation, with some sections exhibiting more significant necrosis, and others showing cavitation of the pedal tissue.

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CASE IV: 116910 (JPC 4020017).

Signalment: Adult female African clawed frog (*Xenopus (Silurana) tropicalis*).

History: Chronic mortality in the colony, severe weight loss, and in some animals, cutaneous nodular, lesions.

Gross Pathology: Splenomegaly, hepatomegaly and nephromegaly with multiple round, whitish, 1 to 2 mm in diameter nodules. Three cutaneous, whitish and firm, round, ulcerated, 2 to 5 mm in diameter lesions, associated to loss of a claw.

Laboratory Results: Culture and sequencing 500pbb rADN16S: *Mycobacterium gordonae*.

Histopathologic Description: Liver: Over 80% of the parenchyma is affected by multifocal to coalescent lesions replacing normal hepatic parenchyma. These foci are characterized by aggregates of round to polygonal cells, up to 100 µm in diameter, with abundant pale staining or often foamy cytoplasm and an eccentric ovoid nucleus (epithelioid macrophages) admixed with small numbers of lymphocytes and granulocytes. Numerous acid-fast staining intracellular bacilli are often present within the cytoplasm of epithelioid macrophages.

Contributor's Morphologic Diagnosis: Liver: hepatitis, granulomatous, multifocal to coalescing, chronic, severe, with acid-fast staining

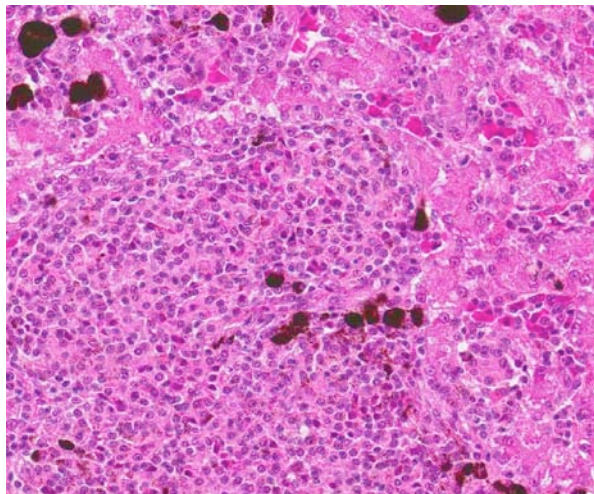
intracellular bacilli, etiology consistent with *Mycobacterium gordonae*.

Contributor's Comment: The African tropical clawed frog, *Xenopus tropicalis*, is an increasingly used vertebrate model system for biological studies. Mycobacteria are acid-fast organisms that are commonly found in aquatic environments.² Amphibian mycobacteriosis has been described as a disease of the integument and/or as a systemic disease with multiple nodules in different organs. Several species of mycobacteria have been isolated in the past from frogs: *M.marinum*, *M.chelonae*, *M.szulgai*, *M. xenopi*, *M.gordonae*, and *M.liflandii*.^{3,4,5,6,10,11,13}

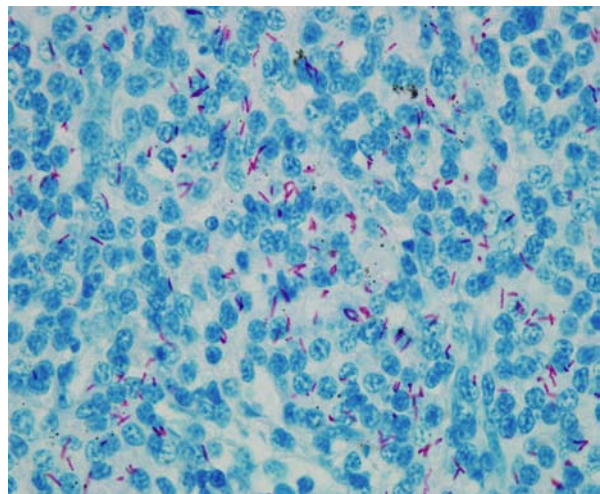
In *Xenopus tropicalis*, *M. szulgai* and *M.liflandii* have been described as a systemic disease and *M.gordonae* as a disease of the integument until now.^{3,11,5,13} *M.gordonae* is considered as an occasional human pathogen, especially in immunocompromised patients.^{1,9} It has been associated with granulomatous skin lesions or with disseminated lesions.^{1,9}

In our case, *Mycobacterium gordonae* has been isolated from internal organs (spleen and liver) and therefore associated with a systemic disease. The source of the infection has not yet been identified.

JPC Diagnosis: Liver: Hepatitis, granulomatous, diffuse, severe, with moderate hepatocellular atrophy.



4-1. Liver, African clawed frog: Multifocally, hepatic sinusoids are expanded by a dense, nodular aggregates of histiocytes which results in hepatocyte compression and atrophy. (HE 260X)



4-2. Liver, frog: Multifocally, within granulomas are numerous 1x4 µm acid-fast bacilli. (Fite-Faraco 1000X)

Conference Comment: Conference participants discussed several aspects of amphibian immune responses, including the tendency for granuloma formation in non-mammalian vertebrates. In this case, there was discussion on the appropriate terminology to describe the immune response, with some participants favoring “granulomatous” and others favoring “histiocytic.” Although the macrophages in this case do not have the expected morphology that typically accompanies a mammalian granulomatous response (i.e., epithelioid and multinucleated macrophages) they do have characteristics of activated macrophages, thus the participants agreed on “granulomatous.” Participants also discussed the role of melanomacrophages in amphibians. Amphibians, like reptiles and fish, have aggregates of phagocytic macrophages within several organs (liver, spleen, and kidney of fish; liver and spleen of amphibians and reptiles).⁷ Systemic inflammation can cause these macrophages to proliferate and develop aggregates in other organs as well, such as the atrium of fish. Macrophages within these aggregates often contain melanin granules, as well as hemosiderin and lipofuscin. While melanomacrophage aggregates are sometimes considered metabolic dumps, the melanin granules are thought to play a role in the production of free radicals and microbial killing.^{8,14} In fish, melanomacrophage aggregates have been shown to function as primitive analogues to lymphoid germinal centers, trapping antigens and immune complexes. In higher fish, amphibians and reptiles, melanomacrophage aggregates may have a capsule.¹⁴

Lastly, participants noted the presence of few hepatocytes with eosinophilic intracytoplasmic vacuoles. These were likened to the postmortem vacuoles described in mice that result from plasma influx into the cytoplasm, as such, they were considered clinically insignificant to this case.⁷

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