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# Epitheliocystis hyperinfection in captive spotted eagle rays *Aetobatus narinari* associated with a novel *Chlamydiales* 16S rDNA signature sequence

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ABSTRACT: This report details 2 cases of epitheliocystis in spotted eagle rays *Aetobatus narinari* associated with a novel *Chlamydiales* 16S rDNA signature sequence. Epitheliocystis is a common disease of variable severity affecting >50 species of wild and cultured freshwater and marine teleosts. Disease in elasmobranchs is rarely reported and descriptions are limited. Occurring in gill and skin epithelium, lesions are characterized by large hypertrophied cells with basophilic inclusions containing Gram-negative, chlamydia-like bacteria. Acute lethargy, labored respiration, and abnormal swimming developed in a captive spotted eagle ray following an uneventful quarantine period, and mild epitheliocystis lesions were found microscopically. Three months later, a second animal exhibited similar signs. A gill clip revealed myriad spherical bodies identical to the previous case, and treatment with chloramphenicol and oxytetracycline was initiated. Despite therapy, respiration became irregular and euthanasia was elected. Histologically, epitheliocystis inclusions up to 200 µm filled approximately 80% of lamellar troughs. Multifocal mild hypertrophy and hyperplasia of lamellar tips was accompanied by mild to moderate infiltrates of granulocytes and lymphocytes. Electron microscopy revealed a homogeneous population of elongate chlamydia-like bacterial forms similar in size and morphology to the primary long cells described in teleosts. Immunohistochemical staining with a polyclonal anti-chlamydial lipopolysaccharide antibody was positive. Sequence analysis of a unique 296 bp *Chlamydiales* signature sequence amplicon isolated from the rays showed greatest homology (85 to 87%) to '*Candidatus* Piscichlamydia salmonis'.

KEY WORDS: Elasmobranch · Epithelium · Lesion · Hypertrophy · '*Candidatus* Piscichlamydia salmonis' · Immunohistology · Diagnosis



## INTRODUCTION

Epitheliocystis is a well-known disease in teleost fish, affecting the gills, skin, and occasionally other external epithelial surfaces of >50 marine and freshwater species (Nowak & LaPatra 2006). Infection has also been reported in a chondrostean, the white sturgeon *Acipenser transmontanus* (Groff et al. 1996). Reports in elasmobranchs are limited. Presumptive lesions have been observed in gills of wild spiny and smooth dogfish *Squalus acanthias* and *Mustelus canis*, respectively (Borucinska & Frasca 2002). An unreferenced mention of compatible lesions (Nowak & LaPatra 2006) and epitheliocystis associated with a novel *Chlamydiales* (Polkinghorne et al. 2010) have also been reported in the leopard shark *Triakis semifasciata*.

Originally named mucophilosis, the disease was first recognized in the common carp *Cyprinus carpio* (Plehn 1920). Hoffman et al. (1969) coined the term

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epitheliocystis, based on appearance of the cyst-like epithelial cell inclusions, and made the first association with a rickettsial or chlamydia-like agent while describing lesions in bluegill *Lepomis macrochirus*. Although no piscine agents have been isolated in culture and Koch's postulates remain unfulfilled, there is compelling molecular and ultrastructural evidence to indicate that the disease is caused by intracellular Gram-negative bacteria belonging to the order *Chlamydiales* (Hoffman et al. 1969, Groff et al. 1996, Nylund et al. 1998, Ossewaarde & Meijer 1999, Draghi et al. 2004, 2007, Meijer et al. 2006, Karlsen et al. 2008). Most recently, '*Candidatus* Renichlamydia lutjani' has been identified from the spleen and kidneys of blue-striped snapper *Lutjanus kasmira* (Corsaro & Work 2012).

*Chlamydiae* are obligate intracellular parasites of vertebrates, a few arthropods, and free-living amoebae, all sharing a common developmental cycle involving an infectious elementary body and intracellular vegetative reticulate body (Corsaro & Greub 2006). Crespo et al. (1999) described 2 developmental cycles in sea bream *Sparus aurata*, one typical of chlamydia, with cysts containing elementary, reticulate, and intermediate bodies, all possessing distinct nucleoids. Occurring in small fingerlings, these cysts were reported to be granular, Machiavello (stain)-negative, and associated with minimal inflammation. In contrast, the second cyst type was more rickettsia-like, containing primary and intermediate long cells, as well as coccoid small cell forms, all with indistinct nucleoid regions. These cysts, typical of larger fish that had survived earlier epizootics, were described as amorphous, Machiavello-positive, and capable of inducing a proliferative host response. Cells characteristic of both cycles have been found in the same fish (Paperna et al. 1981, Crespo et al. 1999).

Chlamydial systematics have been reorganized and expanded to include 4 families (*Chlamydiaceae*, *Parachlamydiaceae*, *Simkaniaceae*, and *Waddliaceae*), created to accommodate an increasing number of species, including emerging pathogens of human and veterinary medical significance. Molecular analysis of 16S and 23S rRNA genetic sequences has also identified novel sequences belonging to unclassified lineages from animals and the environment, indicating greater diversity and host range within the order *Chlamydiales* than previously known (Everett at al. 1999, Horn & Wagner 2001, Corsaro & Greub 2006). Two candidate species associated with epitheliocystis have been described in fish: '*Candidatus* Piscichlamydia salmonis' in Atlantic salmon *Salmo salar* (Draghi et al. 2004), Arctic charr *Salvelinus alpinus* (Draghi et al. 2010), and brown trout *Salmo trutta* (Schmidt-Posthaus et al. 2012); and '*Candidatus* Clavochlamydia salmonicola' in Atlantic salmon and brown trout (Karlsen et al. 2008, Mitchell et al. 2010); as well as a *Neochlamydia*-like bacterium in Arctic charr (Draghi et al. 2007), and a number of additional unclassified and novel lineages (Groff et al. 1996, Meijer et al. 2006, Polkinghorne et al. 2010).

Epitheliocystis lesions are characterized by hypertrophied epithelial cells containing a single, basophilic, intracytoplasmic, membrane-bound inclusion, or cyst, up to 400 µm in diameter that displaces cytoplasmic contents to the cell periphery. Epithelial hypertrophy may lead to pressure necrosis of adjacent cells, but infections are often described as benign and self-limiting, with minimal host response. In other instances, hyperinfection may occur, with or without lethal epithelial proliferation (Hoffman et al. 1969, Wolke et al. 1970, Paperna 1977, Paperna et al. 1981, Rourke et al. 1984, Bradley et al. 1988, Wolf 1988, Lewis et al. 1992, Groff et al. 1996, Nylund et al. 1998, Crespo et al. 1999, Draghi et al. 2004, 2007, 2010, Nowak & LaPatra 2006).

Disease prevalence, lesion severity, and mortality were highly variable among the multi-species data summarized by Nowak & LaPatra (2006). In general, when compared to wild counterparts, morbidity and mortality were highest in cultured fish, with losses up to 100%. Deaths were most common in early life stages, where lesions tended to be non-proliferative. In contrast, proliferative epithelial changes were more commonly observed in older fish. Furthermore, mortalities could be exacerbated by culture conditions and the influence of environmental stressors.

Compared to teleost fish, relatively few specific disease entities or agents are recognized among the Chondrichthyes (Terrell 2004). The present report details clinical, pathologic, and electron-microscopic findings in 2 captive spotted eagle rays *Aetobatus narinari* with histopathological lesions consistent with epitheliocystis disease. Unique 16S rDNA signature sequence molecular data is also presented, further illustrating broad genetic diversity among the order *Chlamydiales*.

## MATERIALS AND METHODS

### Fish and case history

The 2 spotted eagle rays were part of a group of 8 animals collected off the Florida (USA) coast in March 2009, retained for 3 wk at an inshore hold-

ing facility, then transported to the Georgia Aquarium, Atlanta, GA, USA. Physical examinations found the animals to be in good condition. Following 53 uneventful days of quarantine, including routine antiparasitic treatments with praziquantel (Medisca), sulfadimethoxine (Pfizer Animal Health), and diflubenzuron (Crompton Manufacturing Company), the sentinel case became acutely lethargic and exhibited an increased ventilation rate, with intermittent episodes of bottom-sitting and inverted swimming. Despite supportive therapy with oxygen bubbled across the gills, 7.5 ml $kg^{-1}$ shark Ringer's solution containing 2 mEq $ml^{-1}$ Na acetate intracoelomically (Andrews & Jones 1990), and 2 doses of 2 mg $kg^{-1}$ intravenous prednisolone acetate (Central Avenue Pharmacy), the animal expired 12 h later. Similar signs, and 'flashing-like' rubbing on substrate, developed in the second ray 139 d after arrival. Supportive therapy, as described above for the sentinel case, was initiated, and a gill clip revealed myriad spherical bodies, free and in association with lamellae, consistent with epitheliocystis. Treatment with 15 mg $kg^{-1}$ intramuscular oxytetracycline every 7 d (Pfizer Animal Health) and 40 mg $kg^{-1}$ intramuscular chloramphenicol every 48 h (Central Avenue Pharmacy) (Paperna 1977, Goodwin et al. 2005) was administered for 2 d, but with no improvement in the animal's condition, and euthanasia was elected.

### Gross necropsy and histopathology

Gill clips and skin scrapings were performed using standard methods on both rays (Stoskopf 1993). The fish were then placed in dorsal recumbency and the abdominal wall aseptically removed. Samples of liver, spleen, and kidney were collected for aerobic bacterial culture on tryptic soy agar (TSA) with 5% sheep's blood and MacConkey's agar. Plates were incubated at 30°C. An entire gill arch was removed from the second ray and placed in 70% ethanol.

Representative samples of gill, heart, liver, spleen, pancreas, kidney, stomach, intestine, brain, skin, and muscle were fixed for 24 h in 10% neutral buffered formalin. Tissue samples were processed routinely, embedded in paraffin, and sectioned at 5 µm. Prepared slides were stained with hematoxylin and eosin (H&E) (Allen 1992). Selected gill sections were also stained by Giemsa (Sheehan & Hrapchak 1980), Gimenez (Gimenez 1964), and Lillie-Twort (Bancroft & Stevens 1975) procedures.

### Transmission electron microscopy

Approximately 3 mm cubes of gill filaments were fixed overnight in a solution of 2% glutaraldehyde, 2% paraformaldehyde, and 0.2% picric acid in 0.1 M cacodylate-HCl buffer (pH 7.0 to 7.3). Tissues were then post-fixed in 1% $OsO_4$ for 1 h, dehydrated in a series of ethanols, stained 'en bloc' with 0.5% uranyl acetate and embedded in Epon-Araldite (Electron Microscopy Sciences). Ultrathin sections were cut on a Reichert Ultracut S ultramicrotome (Leica), stained with lead citrate and examined on a JEM-1210 transmission electron microscope (JEOL).

### Immunohistochemistry

Staining was performed using monoclonal mouse anti-chlamydial clone ACI and polyclonal rabbit anti-chlamydial 20-CR19 antibodies (Fitzgerald Industries). Slides were flooded for 1 h with primary antibody dilutions of 1:200 (monoclonal) and 1:2000 (polyclonal) prepared in Dako® Antibody Diluent (S0809, Dako). Antibodies were substituted with Universal Negative Controls N1698 and N1699 (Dako) in the 2 protocols, respectively.

Antigen retrieval for the monoclonal stain utilized Proteinase K for 5 min (S3020, Dako). For the polyclonal stain, a heat-induced epitope retrieval (HIER) system using citrate buffer (pH 6.0) (HK086-9K, Biogenex) was used. For both, endogenous peroxidase was blocked by 3% hydrogen peroxide (H312-500, Fisher Scientific). All other blocking was performed using Power Block (HK085-5K, Biogenex).

Biotinylated secondary antibodies consisted of rat-absorbed, horse anti-mouse immunoglobulin G (IgG) (BA-2001, Vector Labs) and goat anti-rabbit IgG (BA-1000, Vector Labs). The tertiary antibody used for both was LSAB® 2 Streptavidin conjugated to horseradish peroxidase in phosphate-buffered saline (PBS) (K1016, Dako). The substrate-chromogen system was diaminobenzidine (DAB) (K3466, Dako). Sections were counterstained with Gills II hematoxylin.

### PCR

Genomic DNA was extracted from ethanol-fixed gill of the second ray using the small sample volume

protocol of a DNeasy extraction kit (Qiagen). Chlamydial DNA was amplified using *Chlamydiales*-specific 16S rRNA signature sequence primers 16SIGF (5′-CGG CGT GGA TGA GGC AT-3′) and 16SIGR (5′-TCA GTC CCA GTG TTG GC-3′) (Everett et al. 1999) synthesized by Integrated DNA Technologies. *Chlamydophila psittaci* genomic DNA (parakeet strain 6BC, National Veterinary Services Laboratories [NVSL]) was used as a positive control.

PCR reaction mixtures contained 1 µl of DNA, 1 mM of each dNTP, 2.5 µM of each primer, 20 mM $MgCl_2$, 5 U FastStart Taq DNA Polymerase (Roche Applied Science), and nuclease-free water to 10 µl. Amplification was performed in a rapid-cycling protocol using a MyCycler thermocycler (Bio-Rad Laboratories), with 40 cycles each of denaturation at 94°C for 0 s, annealing at 45°C for 0 s, and extension at 72°C for 15 s. PCR products were visualized by UV transillumination (254 nm) following electrophoresis and separation in a 2.0% agarose gel stained with ethidium bromide. Results were recorded digitally using a GelDoc XR instrument (Bio-Rad Laboratories).

### DNA sequencing and analysis

PCR products were purified from gels or directly from PCR reactions using a QIAquick Gel Extraction Kit or QIAquick PCR Purification Kit (Qiagen), respectively, and sequenced by SeqWright DNA Technology Services. Consensus 16S target sequence contigs were constructed using Sequencher 5.0 sequence-alignment software (Gene Codes Corporation). A 16S rDNA consensus sequence was constructed from reverse and forward primers (Everett et al. 1999) and compared in a nucleotide basic local alignment search tool (BLAST) search of the National Center for Biotechnology Information databases (www.ncbi.nlm.nih.gov). A phylogenetic tree illustrating relationships between the sequence isolated from the eagle rays and those of selected *Chlamydiales* was constructed using neighbor-joining (Saitou & Nei 1987). Bootstrap testing was performed to demonstrate associated taxa (Felsenstein 1985), and evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004). Distances in composite bias among sequences were considered evolutionary comparisons (Tamura & Kumar 2002). Phylogenetic analyses were conducted using the Molecular Evolutionary Genetics Analysis version 5 (MEGA5) software (Tamura et al. 2011).

## RESULTS

### Gross necropsy

The sentinel case was a female of 15.9 kg weight and 109 cm disc width. Autolysis was mild to moderate. There were variably sized minor skin abrasions and a single 3 × 4 cm shallow ulcer on the ventral pectoral girdle. Gills were pale grayish-red and covered by a heavy layer of mucus. Gill clips revealed rare sessile peritrichs and large numbers of globoid structures with a thin limiting capsule and finely granular golden contents among lamellae. The ventricular epicardium and intestinal serosa was mildly petechiated. Gastrointestinal contents were scant and fluid to mucoid. The meningeal vasculature was mildly congested.

The second animal, also a female, weighed 11.6 kg and had a 90.8 cm disc width. The gills were pale and mottled. Gill clips revealed myriad spherical bodies as described in the previous paragraph, but no other parasites. Skeletal muscle contained multifocal petechiae and firm, white-tan, foci up to 1 × 1.5 cm. The liver was light brown, moderately firm, and did not float in formalin. The gastrointestinal tract was empty. Aerobic bacterial cultures were negative in both animals.

### Histopathology

On H&E sections, the gills of both rays contained multiple lamellar epithelial cells with cytoplasm distended by 50 to 200 µm, and round to ovoid cytoplasmic inclusions. Inclusions were amorphous to finely granular, moderately basophilic, and often surrounded by a thin pale eosinophilic membrane-like structure. Contents were Gram-negative by the Lillie-Twort method, and deep purple and bright magenta with Giemsa and Gimenez stains, respectively.

Small to occasionally moderate numbers of inclusions were scattered throughout lamellae of the first case, but elicited no significant inflammatory or proliferative responses. In contrast, hyperinfection involving approximately 80% of interlamellar spaces markedly altered normal gill architecture in the second ray, where long series of spaces were often filled by 3 to 4 closely apposed cyst-like inclusions (Fig. 1A). Tissue changes included patchy mild hypertrophy and hyperplasia of lamellar tip epithelium, with variable widespread fusion of adjacent to extensive groups and of successive lamellae. Affected lamellae were infiltrated by minimal numbers of granulocytes,

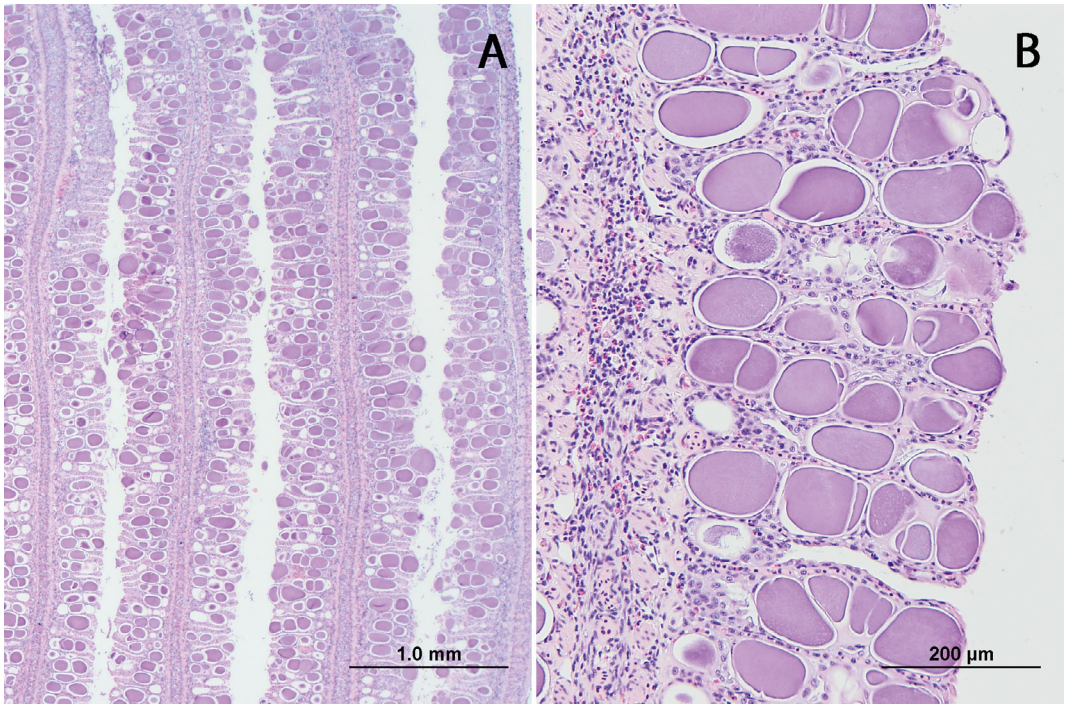


Fig. 1. *Aetobatus narinari*. (A) Low-magnification image of gill filaments illustrating epitheliocystis hyperinfection. (B) Lamellar troughs are filled by multiple epitheliocystis inclusions, with resultant fusion of a long series of adjacent lamellae. Filamental tissue at lamellar bases is infiltrated by small mixed populations of granulocytes and lymphocytes. Hematoxylin and eosin (H&E) staining

lymphocytes, and plasma cells, with small to occasionally moderate numbers of similar infiltrates in filaments at lamellar bases (Fig. 1B).

Additional findings common to both rays included multifocal mild interstitial fibrosis in skeletal muscle, accompanied by small mixed infiltrates of granulocytes and lymphocytes. Some myofibers contained intrasarcoplasmic plasmodia filled by myxospores suggestive of *Kudoa hemiscylli* (Gleeson et al. 2010). Hepatocytes were diffusely small and closely apposed, with cytoplasm devoid of typical lipid vacuoles, but frequently contained small hyaline droplets and golden ceroid granules. Livers and epigonal organs contained small random foci of hemorrhage.

### Transmission electron microscopy

Inclusions were filled by a relatively homogeneous population of elongate bacterial forms averaging 2.53 ± 0.41 × 0.65 ± 0.11 µm (n = 20), with rounded ends (Fig. 2A). The granular cytoplasm of individual bacteria was frequently confined to the cell periphery, creating irregular areas of central clearing with no electron-dense nucleoid. Inclusions were delineated from host cell cytoplasm by a bilaminated membrane (Fig. 2B).

### Immunohistochemistry

Immunohistochemical stains of inclusions using polyclonal anti-chlamydial antibody 20-CR19 were strongly positive (Fig. 3), while the monoclonal antibody ACI was negative.

### DNA sequencing and analysis

The 296 bp *Chlamydiales* signature sequence was submitted to GenBank (accession no. KC454358). The amplicon isolated from the eagle ray branched separately from select other members of the *Chlamydiales* in a phylogenetic tree, but demonstrated greatest (85 to 87%) similarity with '*Candidatus* Piscichlamydia salmonis'. The signature sequences isolated from the ray and and from leopard shark (GenBank accession no. FJ001668) branched more distantly (Fig. 4) and shared only 80% similarity.

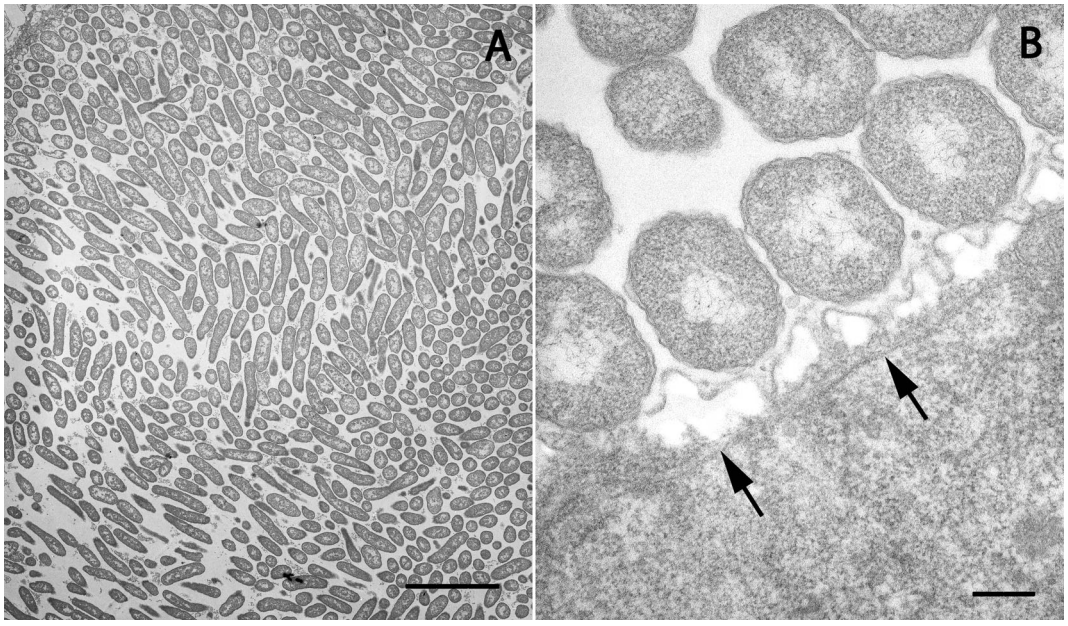


Fig. 2. *Aetobatus narinari*. Transmission electron micrographs of epitheliocystis inclusions. (A) Inclusions were filled by a relatively homogeneous population of elongate cells. Scale bar = 4 µm. (B) Bilaminate membrane-delineated inclusions from host cell cytoplasm (arrows). Individual bacteria had granular cytoplasm and lacked electron-dense nucleoid regions. Scale bar = 100 nm

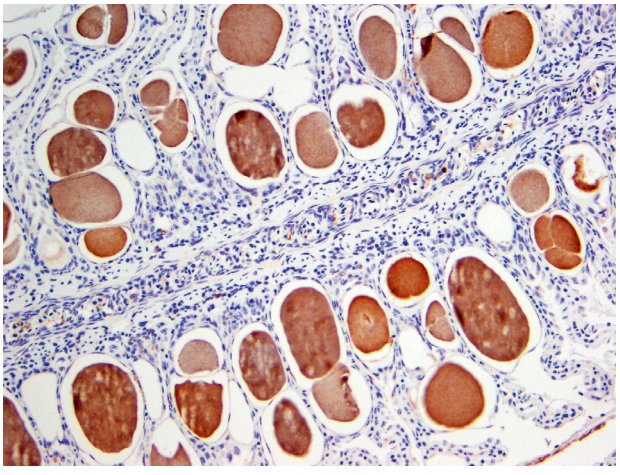

Fig. 3. *Aetobatus narinari*. Positive immunohistochemical staining of epitheliocystis inclusions by a polyclonal anti-chlamydial lipopolysaccharide antibody

## DISCUSSION

Gross and microscopic findings in these eagle rays are typical of branchial epitheliocystis disease in teleost fish. Although epitheliocystis is common in teleosts under a variety of environmental and culture conditions, reports in elasmobranchs are rare. Inclusions in the first ray were relatively low in number, and the role of infection in its death is unclear. However, despite only mild epithelial proliferation, the degree of hyperinfection and lamellar fusion in the second animal is interpreted as sufficient to have resulted in respiratory compromise.

Additional findings included atrophic hepatocytes, devoid of the abundant lipid typically found in elasmobranch livers, suggesting a prolonged catabolic state. No cause-and-effect relationship between gill and hepatic changes could be determined microscopically, i.e. whether gill lesions and resultant hypoxia led to anorexia and consumption of hepatic energy reserves, or if anorexia and debilitation due to unknown causes predisposed hyperinfection. The rare peritrichs in the first case and intrasarcoplasmic *Kudoa* sp. in both rays are considered incidental findings. Aerobic bacterial cultures were negative in both animals, and there were no microscopic changes to suggest systemic infection.

Two distinct pleomorphic biphasic cycles have been described in the development of epitheliocystis inclusions in teleosts (Paperna et al. 1981, Crespo et al. 1999). At the light-microscopy level, bacterial inclusions were largely amorphous, Gram-negative, and Gimenez-positive. Transmission electron microscopy revealed inclusions limited by a bilaminated membrane and filled by elongate forms lacking nucleoid regions. These findings are consistent with

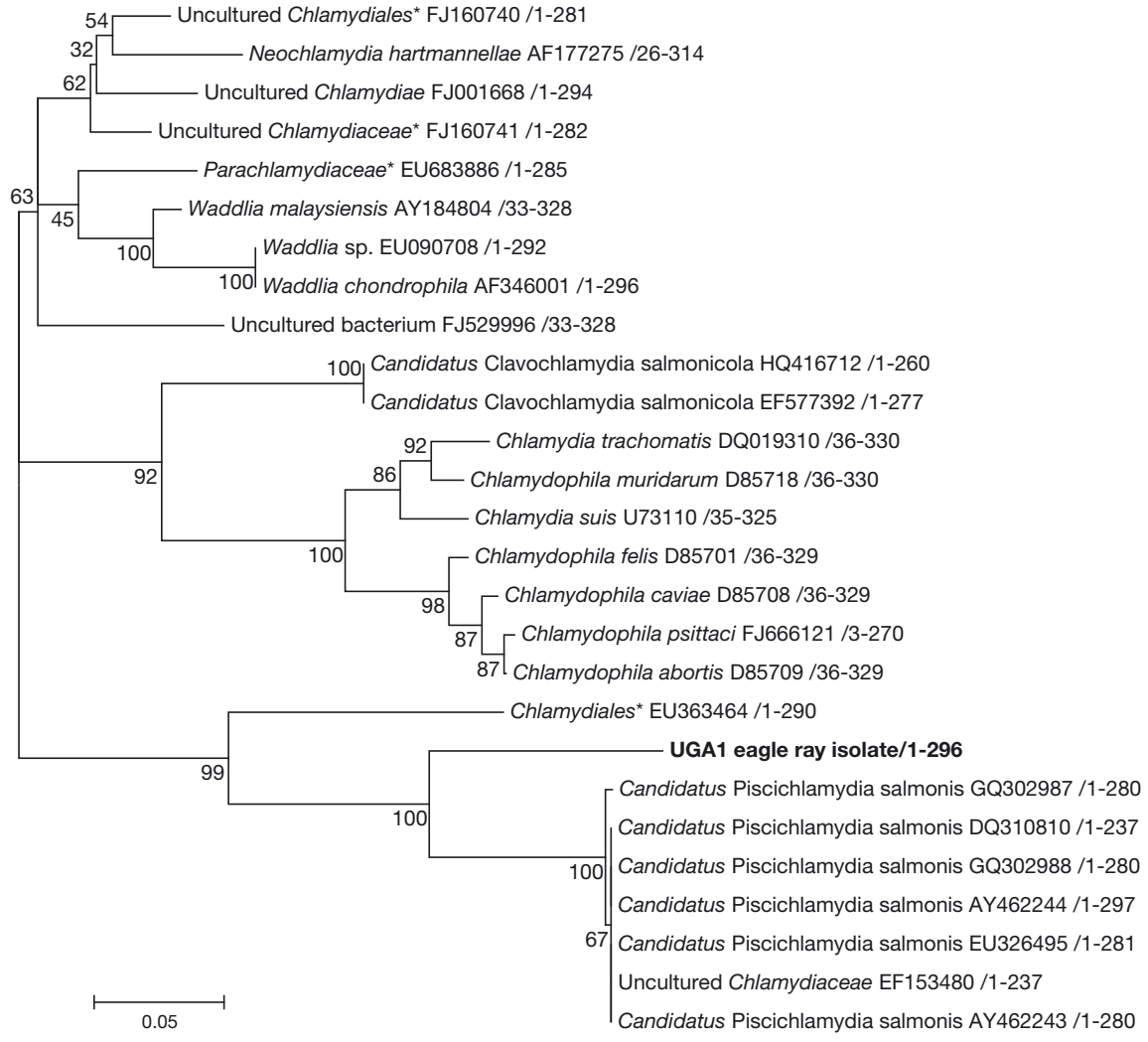


Fig. 4. The evolutionary history of a unique 296 bp *Chlamydiales* signature sequence amplicon isolated from *Aetobatus narinari* was inferred using neighbor-joining (Saitou & Nei 1987). The optimal tree with the sum of branch length = 1.18243288 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using maximum composite likelihood (Tamura et al. 2004) and are in units of the number of base substitutions per site. Differences in composition bias among sequences were considered in evolutionary comparisons (Tamura & Kumar 2002). The analysis involved 27 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 209 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 software (Tamura et al. 2011). Asterisks indicate where only partial sequences were available for analysis

the primary long cells found in amorphous cysts of the rickettsia-like Cycle II described by Crespo et al. (1999) in older sea bream that had survived previous epizootics.

Cellular responses to infection in teleost species are highly variable. Changes here were similar to those described in Atlantic salmon, where encapsulation of the cyst was limited to a thin layer of epithelium and lamellar fusion due to cyst size (Nowak & LaPatra 2006). The hyperinfection and large cysts failed to elicit marked epithelial proliferation or encapsulation, and inflammatory changes were mild (Paperna 1977, Crespo et al. 1999).

Immunohistochemistry using chlamydial anti-lipopolysaccharide (LPS) antibodies has detected antigen in a number of fish species, including white sturgeon, but is negative in most, consistent with molecular data indicating wide taxonomic variation among epitheliocystis agents (Groff et al. 1996, Everett et al. 1999, Nowak & LaPatra 2006). In the rays in the present re-

port, results were strongly positive using a polyclonal anti-chlamydial LPS antibody, but as in the leopard shark, were negative when a monoclonal antibody was used (Polkinghorne et al. 2010). These findings call into question the specificity of the polyclonal antibody, and it is suspected that it may cross-react with LPS of other Gram-negative bacteria.

Pan-chlamydial PCR primer sets, including those used here, have been widely applied to environmental samples and clinical specimens from various animals to identify a growing number of *Chlamydiales* (Everett 2000, Corsaro & Greub 2006). While not suitable for extensive phylogenetic analysis, the 296 bp signature sequence presented in the present report is 80% similar to a novel *Chlamydiales* signature sequence from a leopard shark (Polkinghorne et al. 2010) and showed the greatest similarity to '*Candidatus* Piscichlamydia salmonis', with which it shared 85 to 87% homology. Findings demonstrate the pathogenic potential of epitheliocystis-associated *Chlamydiales* in elasmobranchs and further illustrate the broad genetic diversity within this group.

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