Vol. 132: 37–48, 2018
https://doi.org/10.3354/dao03303

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published December 11

Diagnosis and treatment of multi-species fish mortality attributed to *Enteromyxum leei* while in quarantine at a US aquarium

Michael W. Hyatt[1,5,*], Thomas B. Waltzek[2], Elizabeth A. Kieran[3,6], Salvatore Frasca Jr.[3], Jan Lovy[4]

[1]Adventure Aquarium, Camden, New Jersey 08103, USA

[2]Wildlife & Aquatic Veterinary Disease Laboratory, University of Florida College of Veterinary Medicine, Gainesville, Florida 32611, USA

[3]Aquatic, Amphibian and Reptile Pathology Service, Department of Comparative, Diagnostic, and Population Medicine, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32610, USA

[4]Office of Fish & Wildlife Health & Forensics, New Jersey Division of Fish & Wildlife, Oxford, New Jersey 07863, USA

[5]*Present address:* Wildlife Conservation Society, New York Aquarium, Brooklyn, NY 11224, USA

[6]*Present address:* Arizona Veterinary Diagnostic Laboratory, University of Arizona, Tucson, Arizona 85705, USA

ABSTRACT: *Enteromyxum leei* is an enteric myxozoan parasite of fish. This myxozoan has low host specificity and is the causative agent of myxozoan emaciation disease, known for heavy mortalities and significant financial losses within Mediterranean, Red Sea, and Asian aquaculture industries. The disease has rarely been documented within public aquaria and, to our knowledge, has never been confirmed within the USA. This case report describes an outbreak of *E. leei* in a population of mixed-species east African/Indo-Pacific marine fish undergoing quarantine at a public aquarium within the USA. Four of 16 different species of fish in the population, each of a different taxonomic family, were confirmed infected by the myxozoan through cloacal flush or intestinal wet mount cytology at necropsy. Clinical and histopathological findings in this case are similar to previous findings describing myxozoan emaciation disease, e.g. severe emaciation, cachexia, enteritis, and death. Sequence analysis of the 18S rDNA of intestinal samples from a powder blue tang *Acanthurus leucosternon* and an emperor angelfish *Pomacanthus imperator* confirmed the parasite to have 99–100% identity with other *E. leei* sequences. Spore morphology and ultrastructure were consistent with previous reports of *E. leei*. Treatment of clinically affected fish by oral administration of the coccidiostats amprolium and salinomycin led to reduction of mortalities and resolution of clinical signs. This case report highlights the importance of thorough examination and surveillance of fish during quarantine, particularly with respect to enteric myxozoans.

KEY WORDS: *Enteromyxum leei* · Myxozoan · Myxosporean · Emaciation disease · Amprolium · Salinomycin

1. INTRODUCTION

Enteromyxum leei (Cnidaria: Myxosporea) is an enteric myxozoan parasite affecting a wide range of fish species. This parasite causes myxozoan emaciation disease resulting in outbreaks of heavy mortality and significant financial losses within Mediterranean, Red Sea, and Asian aquaculture industries (Diamant et al. 1994, Rigos et al. 1999, Alvarez-Pellitero et al. 2008, China et al. 2014, Özer et al.

*Corresponding author: mhyatt@wcs.org

2014). Common species reportedly affected in aquaculture include sea breams *Diplodus* (*Puntazzo*) *puntazzo*, *Sparus aurata*, and *Pagrus major* (Diamant 1997, Athanassopoulou et al. 1999, Alvarez-Pellitero et al. 2008, Yanagida et al. 2008), turbot *Scophthalmus maximus* (Branson et al. 1999, Sekiya et al. 2016), flounder *Paralichthys olivaceus* (Yasuda et al. 2005, Sekiya et al. 2016), tiger puffer *Takifugu rubripes* (Tun et al. 2002, Ishimatsu et al. 2007), and grouper *Epinephelus malabaricus* (China et al. 2013). *E. leei* has rarely been reported in public aquaria (Padrós et al. 2001, Katharios et al. 2011, 2014) or in the wild (Özer et al. 2014). To our knowledge, *E. leei* has not been confirmed within the USA, other than a possible case in a tomato clownfish *Amphiprion frenatus* in 1999 (Kent 1999), which was based on histology alone and not confirmed with spore morphology or molecular identification, and an outbreak within a population of yellow tangs *Zebrasoma flavescens* in a public aquarium in 2008 (M. W. Hyatt unpubl. data), which was never confirmed by molecular identification. The yellow tangs presented with clinical signs as described below and heavy mortalities with cytological and histological evidence of an enteric myxozoan similar to other published descriptions of *E. leei*. Unlike most myxozoan parasites that are species-specific and have an indirect life cycle requiring an oligochaete or polychaete alternate host, *E. leei* has low host specificity and is reported to infect over 50 species of fish through direct transmission with no evidence of an alternate actinosporean reproductive stage (Padrós et al. 2001, Katharios et al. 2014, Özer et al. 2014, Sekiya et al. 2016).

Transmission of *E. leei* can occur horizontally through cohabitation and coprophagy, ingestion of infected tissue, and waterborne contamination (Diamant 1997, Alvarez-Pellitero et al. 2008, Katharios et al. 2011). Infection develops when the pre-sporogonic stage is ingested and invades intestinal enterocytes, predominantly in the distal intestine. Enteromyxosis occurs when plasmodia develop within enterocytes causing an inflammatory response, desquamation of the mucosal epithelium, and villous atrophy (Tun et al. 2002, Estensoro et al. 2014). *E. leei* has rarely been reported to infect the liver (Le Breton & Marques 1995), gall bladder (Athanassopoulou et al. 2004, China et al. 2013, Özer et al. 2014), and other tissues (Diamant et al. 1994, Alvarez-Pellitero et al. 2008, Sekiya et al. 2016). Enteromyxosis causes a catarrhal enteritis, which prevents nutrient absorption and disrupts osmoregulatory function (Alvarez-Pellitero et al. 2008, Katharios et al. 2011). Fish usually present with severe emaciation, enophthalmia, cachexia, and death (Tun et al. 2002, Yasuda et al. 2005, Ishimatsu et al. 2007, Alvarez-Pellitero et al. 2008, Katharios et al. 2011, 2014, Sekiya et al. 2016). Successful treatment protocols have not been established (Sitja-Bobadilla et al. 2004, Quiroga et al. 2006, Ishimatsu et al. 2007, China et al. 2014), but Athanassopoulou et al. (2004) and Golomazou et al. (2006) tested several anti-coccidial drugs against *Myxobolus* sp. and *E. leei* for safety and efficacy, with promising results.

As molecular and phylogenetic technologies advance, taxonomic classification of myxozoans continues to be reorganized. Recently, it was confirmed that myxozoans belong within the Cnidaria, rather than their own former Phylum Myxozoa (Chang et al. 2015). Taxonomic classification of myxozoans was extensively reviewed by Fiala et al. (2015). In general, myxozoans are classified by spore morphology. The myxospore is composed of shell valves that are joined by a suture and enclose 1 or more sporoplasms and 1 or more polar capsules, in various arrangements depending on species. Aids for species-level identification are based on spore and polar capsule dimensions and other fine details of myxospore structure, such as the number of turns of the polar filament, the presence of ribs, ridges, and striations on the spore valves, presence or absence of a mucus envelope, and the numbers of sporoplasms and their nuclei (Fiala et al. 2015). In some cases, morphological characteristics are too similar, and identification to the genus and species level requires DNA sequencing and analysis.

This case report describes an outbreak of enteromyxosis in a population of mixed-species east African/Indo-Pacific marine fish undergoing quarantine at a public aquarium. Further, the molecular identification, histopathology, spore morphology, and successful treatment of *E. leei* are described.

2. MATERIALS AND METHODS

2.1. Fish

In July 2017, a population of mixed-species, wild-caught, east African/Indo-Pacific tropical marine fish undergoing quarantine at Adventure Aquarium (Camden, NJ, USA) began developing clinical signs of emaciation with several mortalities. The starting population of 228 fish, comprising 16 different species (belonging to 3 orders and 6 families), arrived into quarantine in late April from a Kenyan fish collector and wholesaler with direct distribution. The

fish spent an unknown period of time within the collector's facility prior to shipment. During quarantine, the fish were evenly distributed between a divided 11 600 l (3050 gal), three 7150 l (1880 gal), and one 3515 l (925 gal) systems based on expectations of typical conspecific behavior to minimize conspecific aggression. Life support systems (LSS) were basic with bio-towers and rapid sand filters (Hayward). Systems were maintained at 23–25°C (74–77°F) and 35 g l^{-1} salinity. Fish were fed a wide variety of food items, including krill (*Euphausia superba* and *E. pacifica*), clam, squid, capelin and eggs, *Artemia* sp., and Omnivore Aquatic gel (5ML6, Mazuri). Two months after arriving into quarantine, fish in all systems developed *Cryptocaryon irritans* infections. Systems were successfully treated with citrated copper with cytological resolution by the end of July. Despite completing the routine quarantine period, quarantine was continued longer to observe for possible recurrence of *C. irritans* prior to moving on exhibit. While under copper treatment, fish began developing clinical signs of emaciation in the beginning of July, which was originally believed to be related to the treatment. Occasional mortalities occurred during copper treatment with no evidence of *C. irritans*. An increase in mortalities of emaciated fish at the beginning of August prompted further investigation that led to the finding of an enteric myxozoan. An amprolium and salinomycin (Huvepharma) drug combination was selected, as it holds promise as an efficacious and safe anti-myxozoan therapeutic in fish based on experimental infection work (Athanassopoulou et al. 2004, Golomazou et al. 2006, M. W. Hyatt pers. obs.). The drugs, dosed at 100 mg amprolium kg^{-1} of estimated biomass and 70 mg salinomycin kg^{-1} of estimated biomass, were added to the gel based on feeding at an estimated 2% biomass twice daily. The percentage of active drug from the bulk products was taken into account, as the amprolium was only 20% active, but salinomycin was 100% active.

2.2. Molecular identification

Intestinal samples from a powder blue tang *Acanthurus leucosternon* and an emperor angelfish *Pomacanthus imperator* were freshly frozen at −20°C for submission for molecular identification (Wildlife & Aquatic Veterinary Disease Laboratory, University of Florida College of Veterinary Medicine, Gainesville, FL, USA). DNA was extracted from the intestinal samples using a QIAcube (Qiagen) and a DNeasy Blood and Tissue Kit (Qiagen) using the manufacturer's protocol for animal tissues. The extracted DNA was resuspended in 100 µl AE Buffer and stored at −80°C. The sample was amplified by PCR with the primer pairs ERIB1-ACT1R and MYXGEN4f-ERIB10 that generate overlapping fragments of approximately 1000 and 1200 bp, respectively, to obtain the complete 18S rDNA sequence (Barta et al. 1997, Hallett & Diamant 2001, Diamant et al. 2004). Reaction volumes were 50 µl and consisted of 0.25 µl of Platinum *Taq* DNA Polymerase (Invitrogen), 5.0 µl of 10× PCR Buffer, 2.0 µl of 50 mM $MgCl_2$, 1.0 µl of 10 mM dNTPs, 2.5 µl of 20 µM forward and reverse primers, 32.25 µl of molecular grade water, and 4.5 µl of DNA template. An initial denaturation step of 94°C for 5 min was followed by 36 cycles of a 94°C denaturation step, a 55°C annealing step, and a 72°C extension step, each step run for 1 min, and a final extension step at 72°C for 5 min. PCR products were subjected to electrophoresis in 1% agarose gel stained with ethidium bromide. Amplified products were purified using a QIAquick PCR Purification Kit (Qiagen). The concentration of purified DNA was quantified fluorometrically using a Qubit® 3.0 Fluorometer and dsDNA BR Assay Kit (Life Technologies). Purified DNA was sequenced in both directions using the primers described above on an ABI 3130 platform (Applied Biosystems). The sequence data were assembled and edited with CLC Genomics Workbench 7.5 software (Qiagen). BLASTn analysis was performed with generated sequence data compared against known sequences maintained in GenBank.

2.3. Histopathology

Formalin-fixed tissues from an emperor angelfish and another powder blue tang were received by the Aquatic, Amphibian, and Reptile Pathology Service of the Veterinary Diagnostic Laboratories of the University of Florida (Gainesville, FL, USA) for processing. Skin, skeletal muscle, bone, cartilage, stomach, pyloric ceca, intestine, swim bladder including gas gland, mesentery, liver, pancreas, spleen, kidney, and gill of the emperor angelfish were processed and examined microscopically. The intact gastrointestinal tract was prepared by transversely cutting the stomach and pyloric ceca. The intestine was cut into thirds of approximately equal length, and each segment was in turn cut to generate samples for histologic processing. Tissue samples were processed routinely and embedded in paraffin, then sectioned at a

thickness of 3 µm. Histologic sections of all submitted tissues were stained using hematoxylin and eosin, then examined by light microscopy. Additional histologic sections of stomach and pyloric ceca were stained using the periodic acid-Schiff and Giemsa techniques.

2.4. Light and transmission electron microscopic characterization

Mature *Enteromyxum* myxospores were collected from fecal material of the emperor angelfish and suspended in Karnovsky's fixative in a 50 ml centrifuge tube. The fixed suspension was maintained at 4°C until further processing at the Office of Fish and Wildlife Health and Forensics (Oxford, NJ, USA) for light and transmission electron microscopy (TEM). Light microscopic observations and measurements were made from wet mounts of the fixed mature spores. Spores naturally settled to the bottom of the tube; approximately 20 µl of the spore suspension was put on a positively-charged glass slide and coverslipped. Spores were examined using differential interference contrast microscopy under a 100× oil immersion objective using a Zeiss Axioplan-2 research microscope with a mounted Jenoptik ProgRes Speed XT core 3 digital camera. Measurements of spores were made using the Jenoptik ProgRes CapturePro Imaging software 2.9.0.1 (www.jenoptik.com). Measurements are reported based on the mean of 50 individual measurements of fixed mature spores and polar capsules.

For TEM, the fixed spore suspension was transferred to a 1.5 ml centrifuge tube. Washing was done twice by suspending the spores in phosphate buffer, centrifuging at 800 × *g* for 5 min, discarding the supernatant, and resuspending the pellet in fresh buffer. After pelleting the spores, secondary fixation was done in 1% osmium tetroxide in phosphate buffer for 1 h at room temperature. After removal of the osmium tetroxide, the pellet was suspended into 3% low melting point agar and allowed to solidify. The agar-embedded pellet was cut into small pieces and transferred into 2 changes of distilled water. Dehydration was then achieved by 2 changes each in a series of ascending ethanol concentrations, including 50, 70, and 95% for 15 min each, followed by 2 changes in 100% ethanol for 20 min each. Clearing was done with 2 changes of propylene oxide (PO) for 15 min each, followed by infiltration with EMBED 812 resin (Electron Microscopy Sciences) with a 3 h incubation in a resin-PO mixture at 1:1, 2 h incubation in a 2:1 mixture, followed by pure resin overnight in a vacuum desiccator. Embedding was done in pure resin in flat capsules and polymerized at 60°C for 48 h. Trimming and ultrathin sectioning (70 nm) was done using a Leica Ultracut-UCT ultramicrotome. Ultrathin sections were mounted onto 100 mesh copper grids, stained with 1% uranyl acetate in 50% ethanol for 30 min, washed in distilled water, stained in Sato's lead stain for 2 min, and washed in distilled water. TEM was done using a Philips CM12 TEM with a mounted AMT-XR11 digital camera housed at the Department of Pathology, Robert Wood Johnson Medical School, Rutgers University, Piscataway, NJ, USA.

3. RESULTS

3.1. Fish

Despite good appetites, emaciation progressed in several species of fish, including powder blue tang, lavender tang *Acanthurus nigrofuscus*, emperor angelfish, clown coris wrasse *Coris aygula* and blue-stripe snapper *Lutjanus kasmira*, with an increase in mortalities at the beginning of August (Table 1). Over the course of 3 d, 5 mortalities within 4 different systems occurred. Necropsies on the fish revealed marked emaciation and muscle atrophy (Fig. 1). Internally, specimens were moderately autolyzed. Direct wet mount cytology of the intestine revealed a heavy burden of myxozoan semi-circular spores and round sporoblasts with divergent polar capsules free within the contents. Intestinal myxozoans were confirmed cytologically on all 5 mortalities.

As 4 of the 5 quarantine systems were confirmed to contain myxozoan-infected fish and all fish came from the same collector, treatment was initiated in all 5 quarantine systems. Treatment consisted of the Omnivore Aquatic gel diet compounded with the anti-coccidial medications amprolium and salinomycin. Most fish readily ate the gel, but an emaciated emperor angelfish was anorexic prior to initiating therapy and was being tube fed a krill and capelin based gruel every 3 d. Amprolium and salinomycin were added directly to the gruel once the diagnosis was confirmed in other fish. During one of the tube feedings, while the fish was anesthetized, a cloacal flush was performed using a 5fr red rubber catheter and a small volume of sterile saline. Cloacal flush cytology revealed a large number of the same myxospores. Shortly after, this fish died and was submitted

Table 1. Taxonomic classification of fish species undergoing quarantine and their clinical outcome. Data presented include the total population of fish, the mortalities that occurred during the outbreak of *Enteromyxum leei*, the observed clinical signs of affected fish, and the number of fish with an enteric myxozoan infection identified on necropsy. Note that not all mortalities were evaluated for myxozoans

Fish species		Population	Mortalities	Clinical signs	Myxozoan confirmed
Lutjanidae					
Bluestripe snapper	*Lutjanus kasmira*	161	7	Emaciation	1
Acanthuridae					
Powder blue tang	*Acanthurus leucosternon*	11	5	Emaciation	2
Lavender tang	*Acanthurus nigrofuscus*	13	0	Emaciation	
Eyestripe surgeonfish	*Acanthurus dussumieri*	9	0	None	
Short-nosed unicorn tang	*Naso brevirostris*	2	0	None	
Pomacanthidae					
Emperor angelfish	*Pomacanthus imperator*	3	2	Emaciation	1
Halfmoon angelfish	*Pomacanthus maculosus*	1	0	None	
Haemulidae					
Yellowbanded sweetlips	*Plectorhinchus lineatus*	8	0	None	
Labridae					
Clown coris wrasse	*Coris aygula*	5	3	Emaciation	3
Queen coris wrasse	*Coris formosa*	1	1	None	
Goldbar wrasse	*Thalassoma hebraicum*	4	1	None	
Sixbar wrasse	*Thalassoma hardwicke*	4	1	None	
Red coris wrasse	*Coris gaimard*	2	0	None	
Surge wrasse	*Thalassoma purpureum*	1	0	None	
Balistidae					
Titan triggerfish	*Balstoides viridescens*	1	0	None	
Orange-lined triggerfish	*Balistapus undulatus*	2	0	None	

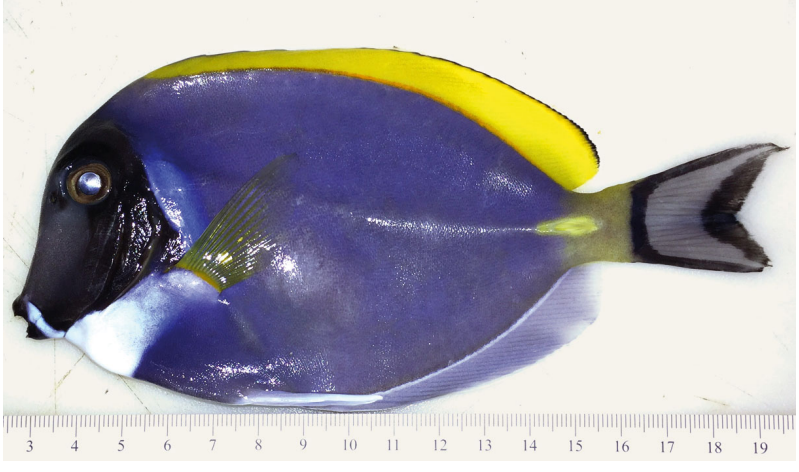

Fig. 1. Powder blue tang *Acanthurus leucosternon* with marked emaciation, muscle atrophy and enophthalmia, suggestive of myxozoan emaciation disease

for necropsy and histopathological examination. Necropsy findings included emaciated body condition, lack of coelomic adipose tissue, atrophied and darkened liver, distended gall bladder, and digesta- and fluid-distended intestines. Representative tissues and spore samples were further evaluated by histology and TEM.

Within days of initiating treatment, mortalities subsided to 1 mortality wk^{-1} (Fig. 2). Subsequent mortalities, though emaciated, showed no evidence of enteric myxozoans, except for 1 severely emaciated clown coris wrasse. This fish died 1 mo after initiation of treatment and still harbored myxozoans. Based on this finding, all systems were continued on the amprolium/salinomycin gel food for another month. During the second month of treatment, 2 mortalities occurred, each with no evidence of enteric myxozoans by cytology and histology. Over the course of the second month of treatment, body condition was improving in the clinically affected fish. To confirm eradication of enteric myxozoans and successful treatment, several fish representing different species from all 5 quarantine systems were anesthetized in buffered MS-222 (Tricaine-S, tricaine methanesulfonate, Syndel [formerly Western Chemical]) for physical examination and cloacal flushes for cytological evaluation, as de-

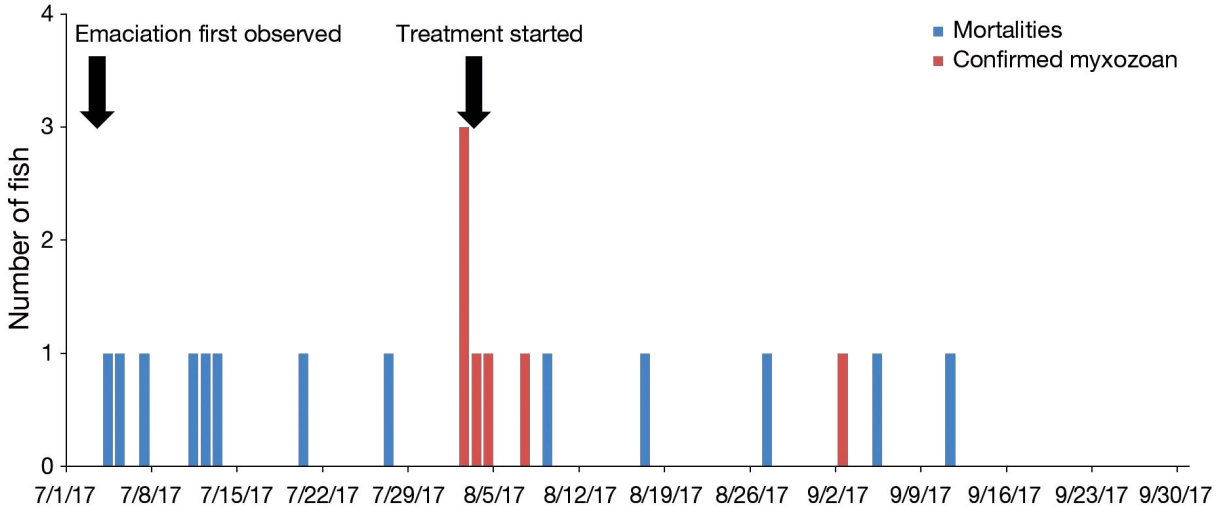

Fig. 2. Timing of mortalities of mixed-species east African/Indo-Pacific fish undergoing quarantine (dates are mo/d/yr). Arrows indicate when clinical signs of emaciation were first observed and when the amprolium/salinomycin combination treatment was first started in relation to the mortalities. Mortalities in red are indicative of fish in which an enteric myxozoan infection was found on necropsy

scribed previously. Cloacal flush direct wet mount cytological examinations revealed adequate fecal sample collection with no evidence of any myxospore stages in all fish sampled. Based on these results, treatment was discontinued. Fish were monitored for another month for evidence of clinical recurrence. At this point, all fish appeared clinically healthy and in good body condition, thus the fish were transported from quarantine and released onto exhibit. At the time of publication, there was no evidence of recurrent disease involving fish within the exhibit.

3.2. Molecular identification

PCR yielded fragments of the expected sizes. The powder blue tang and emperor angelfish intestinal samples yielded identical 1630 bp contiguous sequences (2–4× coverage), excluding primer sequences and regions of low-confidence base calling at both the 5′ and 3′ ends. BLASTn analysis revealed 100% nucleotide identity (100% coverage) to an *Enteromyxum leei* associated with a life-threatening emaciation disease in farmed olive flounder *Paralichthys olivaceus* in Korea (Sekiya et al. 2016). Nearly identical *E. leei* matches (>99% nucleotide identity, 91–94% coverage) were also detected in the gastrointestinal tracts of red sea bream *Pagrus major* cultured in Japan (GenBank accession no. AB243447), cultured spotted knifejaw *Oplegnathus fasciatus* (DQ139796), olive flounder cultured in Japan (DQ127230), sheepshead bream *Diplodus puntazzo* cultured in Greece (AF411334), gilthead seabream *Sparus auratus* cultured in Israel (DQ 448298), and Malabar grouper *Epinephelus malabaricus* cultured in Japan (KF056890). The *E. leei* sequence generated in this study from the powder blue tang intestinal sample has been submitted to GenBank (MH465674).

3.3. Histopathology

Myxozoan developmental stages were observed in the cytoplasm of enterocytes in the pyloric ceca and intestine (Fig. 3). Rare pyknotic nuclei and karyorrhectic debris were scattered within the affected mucosal epithelium. Individual and aggregated myxozoan cells were located within membrane-bound vacuoles in the cytoplasm. Myxozoan stages were observed in the lumen of the stomach but were not recognized in cells of the gastric mucosa. In some sections, the density of infection of the mucosal epithelium was subjectively high. Low to moderate numbers of inflammatory cells including lymphocytes and macrophages were dispersed throughout the lamina propria and submucosa, most prominent in sections of the pyloric ceca (Fig. 4). There was marked, diffuse atrophy of hepatocytes with intracytoplasmic accumulation of iron in hepatocytes and histiocytes, consistent with hemosiderosis. Mild, multifocal, skeletal myofiber necrosis and degeneration were observed.

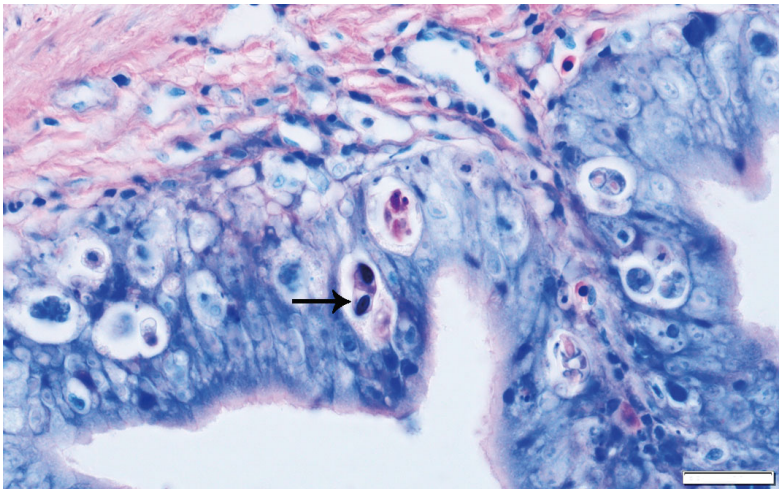

Fig. 3. Myxozoan developmental stages, including spore-forming stages with prominent polar capsules (arrow), are located within membrane-bound vacuoles in the cytoplasm of enterocytes. Giemsa stain. Scale bar = 20 µm

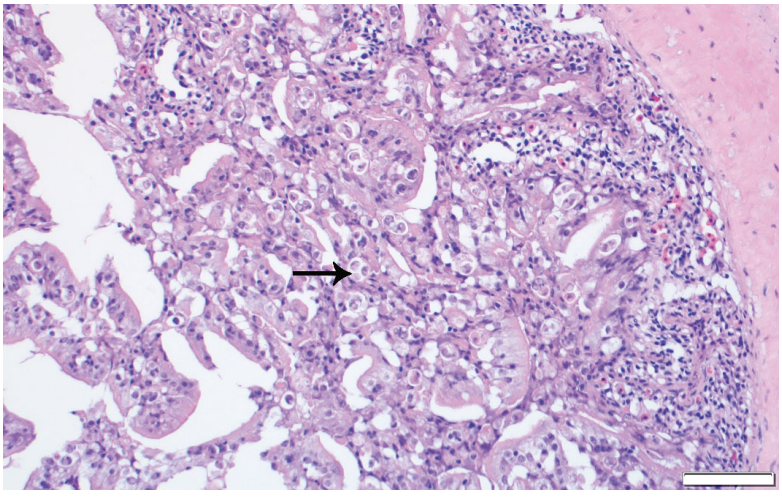

Fig. 4. Myxozoan developmental stages (arrow) are present in the cytoplasm of a large proportion of enterocytes in this section. Low numbers of lymphocytes and macrophages are dispersed throughout the lamina propria and submucosa in this image. Hematoxylin and eosin stain. Scale bar = 50 µm

A powder blue tang was euthanized 1 mo into the treatment period following a poor recovery from anesthesia for cloacal cytology collection. Tissues submitted for histopathological evaluation showed severe and widespread deposition of globular or crystalline proteinaceous material of unknown identity in multiple organs, primarily the liver. Associated with the deposition and accumulation of this material in the liver was disruption of the hepatic architecture and hyperplasia of biliary epithelial cells, which replaced the normal parenchyma in some areas. Of note, matching the cloacal cytology, there was no histological evidence of enteric myxozoans in this fish.

3.4. Light microscopy and TEM characterization

Light microscopic observations showed that the spores were roughly hemispherical, semi-rounded on one side with 2 outwardly projecting polar capsules (PCs) opening toward both lateral ends of the spore (Fig. 5). The central part of the spore between the PCs contained a sporoplasmic cell. The side opposite the rounded portion of the spore was composed of 2 valve (VV) cells with the nuclei creating 2 bulges (Fig. 5A). Fixed spore length, i.e. the longest point measured between both tips of the opposing polar capsules, was 15.9 ± 0.61 µm (mean ± SD) (range: 14.7–17.1 µm). The spore width, oblique to the length, was 8.6 ± 0.43 µm (range: 7.5–9.4 µm). Polar capsules had a length of 6.6 ± 0.43 µm (range: 5.7 to 8 µm), and a width of 3.2 ± 0.39 (range: 2.4–4 µm). In wet mount preparations of fixed spores, individual spores were most frequently observed, although occasionally mature spores occurred in pairs within a sporocyst (Fig. 5B).

TEM showed VV cells with highly condensed cytoplasm composed of electron-dense granular material and a condensed nucleus (Fig. 6A). The condensed nuclei and surrounding cytoplasm of both VV cells created the 2 bulges observed on the posterior portion of the spores (Fig. 6B). Apposing flanges of the VV cells overlapped and contained a septate junction (Fig. 6C). Occasionally adjacent to the connected flanges of the VV cells were stacked electron-dense membranous elements (Fig. 6D). Polar capsules in the mature spore had a thick wall, ranging from 125–240 nm in thickness depending on the plane of section. The PC wall was made up of 2 granular electron-dense layers surrounding an electron-lucent center (Fig. 6E). Polar capsules contained 4 to 6 coils of the polar filament and granular material (Fig. 6E). Cytoplasm of the sporoplasmic cell was condensed and contained electron dense granular material, lipid, ribosomes, mitochondria, and condensed nuclei. Due to the condensed nature of the cytoplasm, it was difficult to discern the characteristics of the nuclei.

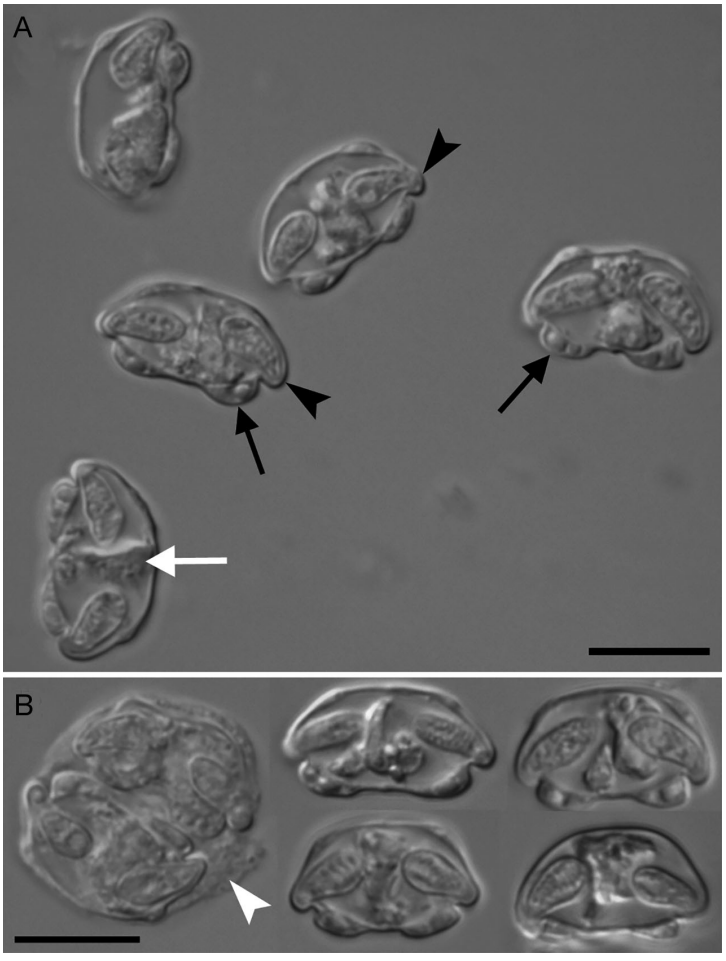

Fig. 5. Wet-mount microscopic preparation of fixed myxospores. (A) Spores have 2 valve cells creating 2 bulges on one side of the spore (black arrows), 2 polar capsules opposite each other and opening to the sides (black arrowheads), and sporoplasmic cells between the polar capsules (white arrow). (B) Disporoblast (white arrowhead) and 4 individual spores. Scale bars = 10 µm

4. DISCUSSION

A mortality event affecting multiple species of wild-caught east African/Indo-Pacific tropical marine fish undergoing quarantine was confirmed to be caused by the enteric myxozoan *Enteromyxum leei*, the causative agent of myxozoan emaciation disease. The clinical and histopathological findings in this case are similar to those published in the literature describing this disease. Further, the 18S rDNA sequence showing 99–100% identity to other *E. leei* sequences and spore morphology consistent for this species corroborate the myxozoan in this case to be *E. leei*. Previously reported myxospore measurements for *E. leei* ranged from 13.2–19 µm in length, 5–11 µm in width, 6.2–9.8 µm in PC length, and 2.5–3.6 µm in PC width (Diamant 1992, 1998, Diamant et al. 1994, Le Breton & Marques 1995, Sakiti et al. 1996, Padrós et al. 2001). All spore measurements from fixed material in the present study were within these reported ranges. Aldehyde fixation is known to cause minimal shrinkage in myxozoan spores (Parker & Warner 1970), although even with minimal shrinkage, the spores in this study were still within the range of previous descriptions for *E. leei*. Ultrastructural findings of mature spores in this study were consistent with the description of mature *E. leei* spores reported by Cuadrado et al. (2008). One noted difference in the mature myxospores from this study was that occasionally, there were stacked, electron-dense membranes adjacent to the valve cell flanges. The significance of these membranous elements near the flanges is unknown. Some differences exist in the distribution of glycogen and lipid reserves within trophozoites of *E. leei* compared to other species in the genus (Cuadrado et al. 2008). Descriptions of proliferative stages of the parasite were not a focus of this current study, thus we were unable to confirm the subtle differences of glycogen and lipid reserves within trophozoites.

The density of myxozoan infection of the mucosal epithelium of the absorptive portions of the alimentary tract, e.g. pyloric ceca and intestine, would help to explain the clinical history of emaciation. Hepatocyte atrophy and iron accumulation could have been consequences of emaciation. Similar to previous reports, *E. leei* in this case showed low host specificity with at least 4 fish species, each of a different taxonomic family, confirmed to be infected. This number may be underrepresented due to mortalities of other species prior to recognition of enteromyxosis in this population. This was due, in part, to the fact that many of the specimens were moderately to severely autolyzed and that necropsies were biased toward evaluating predominantly for *Cryptocaryon irritans* (i.e. external cytologies only). To complicate the diagnosis of *E. leei*, this outbreak occurred after a *C. irritans* outbreak. Initially, mortalities were assumed to be related to *C. irritans*, secondary to copper immersion therapy, or altered water quality related to copper treatment. Some of the mortalities reported in early July may have indeed been related to *C. irritans* or copper treatment. These mor-

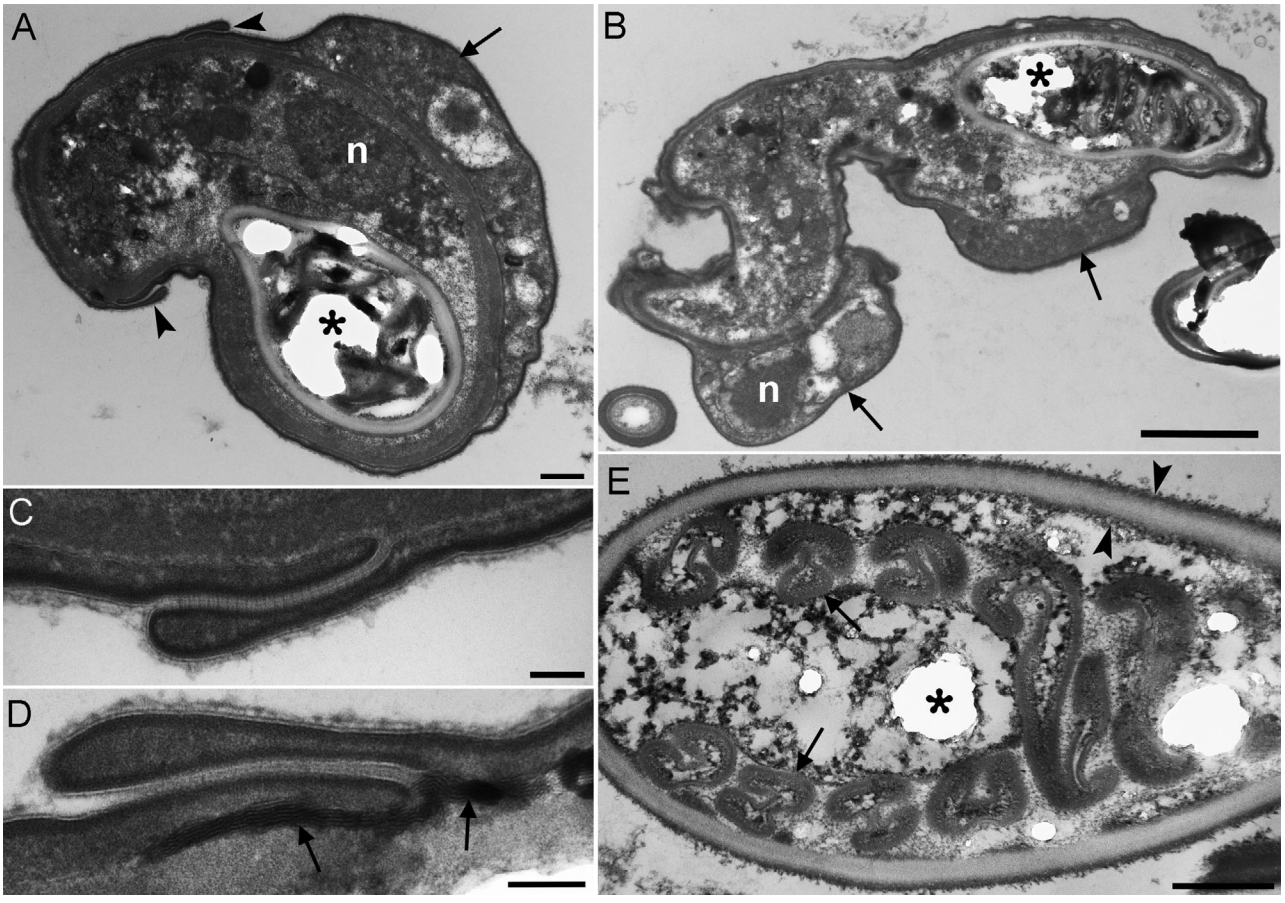

Fig. 6. Ultrastructural details of mature myxospores. (A) Myxospore with a valve cell (arrow), overlapping flanges of 2 valve cells (arrowheads), polar capsule (*), and nucleus of the sporoplasmic cell (n); scale bar = 500 nm. (B) Spore with 2 valve cells creating bulges on one side of the spore (arrows), nucleus (n), and polar capsule (*); scale bar = 2 µm. (C) Septate junction connecting 2 overlapping valve cell flanges; scale bar = 100 nm. (D) Stacked, electron-dense membranes (arrows) adjacent to the joined valve cell flanges; scale bar = 100 nm. (E) Polar capsule (*) with a thick wall (arrowheads) composed of an electron-lucent layer sandwiched between 2 granular electron dense layers. Notice sections of the polar filament (arrows); scale bar = 500 nm

talities were still included, as *E. leei* could not be ruled out given that other species, such as the powder blue tang and the lavender tang, were already showing clinical signs of poor body condition. The few mortalities that occurred toward the end of treatment showed no evidence of enteric myxozoans. This may have been reflective of successful treatment, but resistance to the parasite (Padrós et al. 2001, Alvarez-Pellitero et al. 2008) cannot be ruled out. Notably, the sixbar wrasse *Thalassoma hardwicke* and goldbar wrasse *T. hebraicum* did not develop clinical signs.

Interestingly, some species, such as the powder blue tang, emperor angelfish, and clown coris wrasse, appeared more susceptible to *E. leei* infection and disease, as these species had higher percent mortality with marked clinical signs. In contrast, the lavender tangs developed emaciation but experienced no mortalities, and the bluestripe grunts became emaciated and had slow growth rates, but with only 11% mortality. Differences in host susceptibility have been observed in several studies and case reports, suggesting possible parasite–host relationships involving immune mechanisms, physiological status, and behavioral and environmental factors (Athanassopoulou et al. 1999, Rigos et al. 1999, Padrós et al. 2001, Katharios et al. 2014, Özer et al. 2014). Differences in immune response were observed with short snout sea bream and gilthead sea bream experimentally infected with *E. leei*: the gilthead sea bream had a stronger immune response, produced less severe clinical signs that developed later, and suffered less mortality (Muñoz et al. 2007, Alvarez-Pellitero et al. 2008). Some species of fish, such as the damselfish *Chromis chromis*, have been found to carry the parasite, but not to develop morbidity or mortality, suggesting they may be a natural host or reservoir

(Padrós et al. 2001, Özer et al. 2014). Previous exposure and infection by the parasite may stimulate an acquired immunity, as described in turbot surviving an *E. scophthalmi* epizootic (Sitja-Bobadilla et al. 2004). Adequate nutrition is imperative for proper immune function, reduction in disease susceptibility, and reduction in pathological changes (Trichet 2010, Calduch-Giner et al. 2012, Oliva-Teles 2012). In the case described herein, numerous fish at the end of treatment had unremarkable cytological examinations of cloacal flushes. As some of these fish were in good body condition, they either cleared the infection or were never infected. The cytological results could have produced false negatives if the samples did not produce adequate fecal material or in the absence of myxospore shedding during treatment. Fecal quantity was comparable between positive and negative cloacal flush samples, reducing the likelihood of the technique being a confounding factor. Complete eradication could not be confirmed, as DNA-based testing was not performed to screen for cryptic developmental stages.

Even though the life cycle of *E. leei* has not been conclusively established, previous studies have shown evidence for direct transmission of the parasite, which is not typical for myxozoans (Diamant 1997, 1998, Redondo et al. 2002, Yasuda et al. 2002, 2005, Yanagida et al. 2008). Enteromyxosis was confirmed in all 5 of our quarantine systems, of which all fish arrived from the same fish collector's holding facility. It is possible that the fish became infected while maintained together at the holding facility, and the infected fish continued to shed the parasite after arrival into the different quarantine systems, allowing transmission of the parasite via coprophagy and direct water exposure within the system. Indeed, clinical signs and mortalities were delayed 2 mo from arrival. The pre-patent period of *E. leei* and a closely related myxozoan, *E. scophthalmi*, can be prolonged and is reported to range from 10 to 38 d and 8 to 48 d, respectively, depending on route of infection and water temperature (Redondo et al. 2002, Cuesta et al. 2006); it can even be as prolonged up to 3 mo (Quiroga et al. 2006). Redondo et al. (2002) experimentally infected turbot with *E. scophthalmi* through either cohabitation with waterborne exposure or oral transmission by feeding infected intestinal tissue. The oral route of exposure induced clinical disease with mortalities by 21 d, whereas cohabitation did not induce clinical disease or mortalities until 48 d post infection. Prevalence of disease increased quickly by 62 d. The various routes of experimental exposure and clinical progression fit the delayed timetable in the present case. The infected fish may have been carriers. The stress of quarantine coupled with the *C. irritans* outbreak may have induced recrudescence, followed by direct spread within the quarantine systems. Interestingly, the quarantine system holding the large population of blueline snapper was divided into 2 separate systems sharing the same LSS. Only the population of snapper in 1 of those systems developed clinical signs and mortalities. The fish in the affected system progressively developed emaciation throughout the entire population. Over time, these fish also remained reduced in size compared to those of the unaffected system, which exhibited good body condition and growth. It appears *E. leei*, if shed into the water, was not able to pass through the sand filter and biotower to infect the other system. Quiroga et al. (2006) also observed a similar event with cultured turbot infected with *E. scophthalmi*; those fish held in systems with filtration never became infected, whereas those fish held in systems without filtration became infected. The overall percent mortality of the blueline snapper (4%) may be misleading, as this includes the population that apparently was not exposed. If only considering the affected population, then the mortality would be 11%.

Treatment of enteromyxosis was apparently successful through oral administration of the coccidiostats amprolium and salinomycin, compounded within a gel diet. These drugs are commonly used in the poultry industry to treat coccidiosis. Amprolium is a structural analogue of thiamine that competitively inhibits thiamine utilization by parasites. It acts upon the first coccidian generation, preventing differentiation of merozoites and suppresses sexual stages and sporulation of oocysts (Hamamoto et al. 2000, Golomazou et al. 2006). Salinomycin is an ionophore antimicrobial agent characterized as a highly lipophilic polyether that accumulates in cell membranes disrupting potassium homeostasis and killing sensitive microorganisms (Russell & Houlihan 2003, Golomazou et al. 2006). The combination of amprolium and salinomycin had the highest safety and efficacy when compared to other coccidiostats, such as fumigillin and toltrazuril, with no evidence of drug toxicity or mortality (Athanassopoulou et al. 2004, Golomazou et al. 2006). Within days of initiating the medicated feed, mortalities reduced. A similar finding was observed in yellow tangs undergoing the same treatment for suspected *E. leei* enteromyxosis (M. W. Hyatt unpubl. data). Treatment success was based on reduction or cessation of mortalities and improvement in body condition. The lack of cytological and histological evidence of myxospores

prompted discontinuation of the medicated feed. Treatment was continued for a total of 60 d, as there was still evidence of shedding spores at 30 d. This is not unexpected, as Golomazou et al. (2006) did not observe *E. leei* clearance with amprolium and salinomycin until Day 42 of treatment. Yet, natural clearance of the infection cannot be ruled out (Cuesta et al. 2006, Fleurance et al. 2008). A treatment option that was not explored, but should be considered, is water temperature modification. Elevated water temperatures above 25–30°C have been shown to inhibit *E. leei* development, allowing for reduction of mortalities and clearance of the infection (China et al. 2014). The ideal temperature range for *E. leei* to proliferate and induce heavy host mortalities is between 15 and 25°C (Yanagida et al. 2006).

The origin or pathological process that led to deposition of the crystalline proteinaceous material in the liver of the one necropsied powder blue tang remains unresolved. This fish was euthanized 1 mo into a combination amprolium and salinomycin treatment. One possibility is that the injury to the liver was an idiosyncratic drug reaction, which contributed to this fish's acute anorexia and poor recovery from anesthesia. Another possibility is that the accumulation of this crystalline proteinaceous material was precedent to collection.

In conclusion, *E. leei* was confirmed for the first time within a cohort of multi-species east African/Indo-Pacific marine fish undergoing quarantine within a US public aquarium. Given evidence for fish-to-fish transmission and nonspecific infection, this case report highlights the importance of thorough examination and surveillance of fish during quarantine. *E. leei* should be included as a differential diagnosis when evaluating unknown fish mortalities presenting with severe emaciation and enteritis. The combination of amprolium and salinomycin was shown to be an effective treatment for myxozoan emaciation disease caused by *E. leei*. This parasite has the potential to become an emerging disease outside of aquaculture, with worldwide spread due to globalization of the aquarium trade.

Acknowledgements. We thank the fish and invertebrate husbandry staff at Adventure Aquarium for their dedicated care of the fish. We also thank the support staff at the different diagnostic laboratories.

LITERATURE CITED

Alvarez-Pellitero P, Palenzuela O, Sitja-Bobadilla A (2008) Histopathology and cellular response in *Enteromyxum leei* (Myxozoa) infections of *Diplodus puntazzo* (Teleostei). Parasitol Int 57:110–120

Athanassopoulou F, Prapas T, Rodger H (1999) Diseases of *Puntazzo puntazzo* C. in marine aquaculture systems in Greece. J Fish Dis 22:215–218

Athanassopoulou F, Karagouni E, Dotsika E, Ragias V, Tavla J, Christofilloyanis P, Vatsos I (2004) Efficacy and toxicity of orally administrated anti-coccidial drugs for innovative treatments of *Myxobolus* sp. infection in *Puntazzo puntazzo*. Dis Aquat Org 62:217–226

Barta JR, Martin DS, Liberator PA, Dashkevicz M and others (1997) Phylogenetic relationships among eight *Eimeria* species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. J Parasitol 83: 262–271

Branson E, Riaza A, Alvarez-Pellitero P (1999) Myxosporean infection causing intestinal disease in farmed turbot, *Scophthalmus maximus* (L.) (Teleostei: Scophthalmidae). J Fish Dis 22:395–399

Calduch-Giner JA, Sitja-Bobadilla A, Davey GC, Cairns MT, Kaushik S, Perez-Sanchez J (2012) Dietary vegetable oils do not alter the intestine transcriptome of gilthead sea bream (*Sparus aurata*), but modulate the transcriptomic response to infection with *Enteromyxum leei*. BMC Genomics 13:470

Chang ES, Neuhof M, Rubinstein ND, Diamant A, Philippe H, Huchon D, Cartwright P (2015) Genomic insights into the evolutionary origin of Myxozoa within Cnidaria. Proc Natl Acad Sci USA 112:14912–14917

China M, Nakamura H, Hamakawa K, Tamaki E, Miwa S, Meng F, Yokoyama H (2013) Occurrence of the myxosporean emaciation disease caused by *Enteromyxum leei* in cultured Malabar grouper *Epinephelus malabaricus*. Fish Pathol 48:88–96

China M, Nakamura H, Hamakawa K, Tamaki E, Yokoyama H, Masuoka S, Ogawa K (2014) Efficacy of high water temperature treatment of myxosporean emaciation disease caused by *Enteromyxum leei* (Myxozoa). Fish Pathol 49:137–140

Cuadrado M, Marques A, Diamant A, Sitja-Bobadilla A and others (2008) Ultrastructure of *Enteromyxum leei* (Diamant, Lom, & Dyková, 1994) (Myxozoa), an enteric parasite infecting gilthead sea bream (*Sparus aurata*) and sharpsnout sea bream (*Diplodus puntazzo*). J Eukaryot Microbiol 55:178–184

Cuesta A, Muñoz P, Rodríguez A, Salinas I and others (2006) Gilthead seabream (*Sparus aurata L.*) innate defence against the parasite *Enteromyxum leei* (Myxozoa). Parasitology 132:95–104

Diamant A (1992) A new pathogenic histozoic *Myxidium* (Myxosporea) in cultured gilt-head sea bream *Sparas aurata* L. Bull Eur Assoc Fish Pathol 12:64–66

Diamant A (1997) Fish-to-fish transmission of a marine myxosporean. Dis Aquat Org 30:99–105

Diamant A (1998) Red drum *Sciaenops ocellatus* (Sciaenidae), a recent introduction to Mediterranean mariculture, is susceptible to *Myxidium leei* (Myxosporea). Aquaculture 162:33–39

Diamant A, Lom J, Dyková I (1994) *Myxidium leei* n. sp., a pathogenic myxosporean of cultured sea bream *Sparus aurata*. Dis Aquat Org 20:137–141

Diamant A, Whipps CM, Kent ML (2004) A new species of *Sphaeromyxa* (Myxosporea: Sphaeromyxina: Sphaeromyxidae) in devil firefish, *Pterois miles* (Scorpaenidae), from the northern Red Sea: morphology, ultrastructure, and phylogeny. J Parasitol 90:1434–1442

Estensoro I, Mulero I, Redondo MJ, Alvarez-Pellitero P, Mulero V, Sitja-Bobadilla A (2014) Modulation of leukocytic populations of gilthead sea bream (*Sparus aurata*) by the intestinal parasite *Enteromyxum leei* (Myxozoa: Myxosporea). Parasitology 141:425–440

Fiala I, Bartosova-Sojkova P, Whipps CM (2015) Classification and phylogenetics of Myxozoa. In: Okamura B, Gruhl A, Bartholomew JL (eds) Myxozoan evolution, ecology and development. Springer International Publishing, Cham, p 85–110

Fleurance R, Sauvegrain C, Marques A, Le Breton A, Guereaud C, Cherel Y, Wyers M (2008) Histopathological changes caused by *Enteromyxum leei* infection in farmed sea bream *Sparus aurata.* Dis Aquat Org 79: 219–228

Golomazou E, Athanassopoulou F, Karagouni E, Vagianou S, Tsantilas H, Karamanis D (2006) Efficacy and toxicity of orally administered anti-coccidial drug treatment on *Enteromyxum leei* infections in sharpsnout seabream (*Diplodus puntazzo* C.). Isr J Aquacult Bamid 58: 157–169

Hallett SL, Diamant A (2001) Ultrastructure and small-subunit ribosomal DNA sequence of *Henneguya lesteri* n. sp. (Myxosporea), a parasite of sand whiting *Sillago analis* (Sillaginidae) from the coast of Queensland, Australia. Dis Aquat Org 46:197–212

Hamamoto K, Koike R, Machida Y (2000) Bioavailability of amprolium in fasting and nonfasting chickens after intravenous and oral administration. J Vet Pharmacol Ther 23: 9–14

Ishimatsu A, Hayashi M, Nakane M, Sameshima M (2007) Pathophysiology of cultured tiger puffer *Takifugu rubripes* suffering from the myxozoan emaciation disease. Fish Pathol 42:211–217

Katharios P, Rigos G, Divanach P (2011) *Enteromyxum leei* (Myxozoa), a lethal intruder of tropical pet fish: first case in humphead wrasse, *Cheilinus undulatus* (Ruppell, 1835). J Exot Pet Med 20:138–143

Katharios P, Kokkari C, Sterioti A, Smyrli M, Kalatzis PG (2014) *Enteromyxum leei* infection in parrotfish, *Sparisoma cretense*: histopathological, morphological and molecular study. Vet Parasitol 199:136–143

Kent ML (1999) A myxozoan resembling *Myxidium leei* in the anemone fish *Amphiprion frenatus* from the Pacific Ocean. Bull Eur Assoc Fish Pathol 19:42–43

Le Breton A, Marques A (1995) Occurrence of a histozoic *Myxidium* infection in two marine cultured species: *Puntazzo puntazzo* C. and *Pagrus major.* Bull Eur Assoc Fish Pathol 15:210–212

Muñoz P, Cuesta A, Athanassopoulou F, Golomazou H and others (2007) Sharpsnout sea bream (*Diplodus puntazzo*) humoral immune response against parasite *Enteromyxum leei* (Myxozoa). Fish Shellfish Immunol 23: 636–645

Oliva-Teles A (2012) Nutrition and health of aquaculture fish. J Fish Dis 35:83–108

Özer A, Öztürk T, Özkan H, Cam A (2014) First report of *Enteromyxum leei* (Myxozoa) in the Black Sea in a potential reservoir host *Chromis chromis.* Fish Pathol 49:57–60

Padrós F, Palenzuela O, Hispano C, Tosas O, Zarza C, Crespo S, Alvarez-Pellitero P (2001) *Myxidium leei* (Myxozoa) infections in aquarium-reared Mediterranean fish species. Dis Aquat Org 47:57–62

Parker JD, Warner MC (1970) Effects of fixation, dehydration and staining on dimensions of myxosporidian and microsporidian spores. J Wildl Dis 6:448–456

Quiroga MI, Redondo MJ, Sitja-Bobadilla A, Palenzuela O and others (2006) Risk factors associated with *Enteromyxum scophthalmi* (Myxozoa) infection in cultured turbot, *Scophthalmus maximus* (L.). Parasitology 133: 433–442

Redondo MJ, Palenzuela O, Riaza A, Macias A, Alvarez-Pellitero P (2002) Experimental transmission of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot *Scophthalmus maximus.* J Parasitol 88:482–488

Rigos G, Christophilogiannis P, Yiagnisi M, Andriopoulou A, Koutsodimou M, Nengas I, Alexis M (1999) Myxosporean infections in Greek mariculture. Aquacult Int 7:361–364

Russell JB, Houlihan AJ (2003) Ionophore resistance of ruminal bacteria and its potential impact on human health. FEMS Microbiol Rev 27:65–74

Sakiti N, Tarer D, Jacquemin D, Marques A (1996) Présence en Méditerranée occidentale d'une Mixosporidie histozoique pathogène dans les élevages du daurade, *Sparas aurata.* Ann Sci Nat Zool Paris 17:123–127

Sekiya M, Setsuda A, Sato H, Song K, Han JK, Kim GJ, Yeo IK (2016) *Enteromyxum leei* (Myxosporea: Bivalvulida) as the cause of myxosporean emaciation disease of farmed olive flounders (*Paralichthys olivaceus*) and a turbot (*Scophthalmus maximus*) on Jeju Island, Korea. Parasitol Res 115:4229–4237

Sitja-Bobadilla A, Redondo MJ, Macias MA, Ferreiro I, Riaza A, Alvarez-Pellitero P (2004) Development of immunohistochemistry and enzyme-linked immunosorbent assays for the detection of circulating antibodies against *Enteromyxum scophthalmi* (Myxozoa) in turbot (*Scophthalmus maximus* L.). Fish Shellfish Immunol 17: 335–345

Trichet VV (2010) Nutrition and immunity: an update. Aquacult Res 41:356–372

Tun T, Ogawa K, Wakabayashi H (2002) Pathological changes induced by three myxosporeans in the intestine of cultured tiger puffer, *Takifugu rubripes* (Temminck and Schlegel). J Fish Dis 25:63–72

Yanagida T, Sameshima M, Nasu H, Yokoyama H, Ogawa K (2006) Temperature effects on the development of *Enteromyxum* spp. (Myxozoa) in experimentally infected tiger puffer, *Takifugu rubripes* (Temminck & Schlegel). J Fish Dis 29:561–567

Yanagida T, Palenzuela O, Hirae T, Tanaka S, Yokoyama H, Ogawa K (2008) Myxosporean emaciation disease of cultured red sea bream *Pagrus major* and spotted knifejaw *Oplegnathus punctatus.* Fish Pathol 43:45–48

Yasuda H, Ooyama T, Iwata K, Tun T, Yokoyama H, Ogawa K (2002) Fish-to-fish transmission of *Myxidium* spp. (Myxozoa) in cultured tiger puffer suffering from emaciation disease. Fish Pathol 37:29–33

Yasuda H, Ooyama T, Nakamura A, Iwata K, Palenzuela O, Yokoyama H (2005) Occurrence of Myxosporean emaciation disease caused by *Enteromyxum leei* in cultured Japanese flounder *Paralichthys olivaceus.* Fish Pathol 40: 175–180

Editorial responsibility: Dieter Steinhagen, Hannover, Germany

Submitted: June 19, 2018; Accepted: October 10, 2018
Proofs received from author(s): November 23, 2018