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Characterization of Myxozoan Parasites Associated with Catfish Aquaculture in Mississippi with Notes on the Development of H. Ictaluri In Susceptible and Non-Susceptible Catfish Hosts

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Characterization of myxozoan parasites associated with catfish aquaculture in Mississippi with notes on the development of *H. ictaluri* in susceptible and non-susceptible catfish hosts

By

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Myxozoans are cnidarian parasites of primarily freshwater and marine fish, with some being important pathogens of aquacultured fish species worldwide. Their life cycles have waterborne actinospores released from aquatic annelid definitive hosts and myxospore stages in fish intermediate hosts.

In the southeastern United States, catfish aquaculture is burdened by annual losses to a myriad of infectious diseases. *Henneguya ictaluri*, the causative agent of proliferative gill disease in channel catfish *Ictalurus punctatus* and female channel catfish x male blue catfish *Ictalurus furcatus* hybrids, is the most commonly diagnosed parasitic disease of catfish in Mississippi. Other myxozoans infect these ictalurid fish, but their impact on catfish production is unknown.

Surveys of actinospores from the oligochaete *Dero digitata* and myxospore stages from fish revealed an unexpected diversity for these production systems. Six genetically distinct actinospores representing four collective groups were observed from *D. digitata*. Herein, two novel *Henneguya* spp. are described from the gills and a novel *Unicauda* sp. is described from the intestinal tract of channel catfish. One *Henneguya* sp. was linked to

its actinospore stage and represents the fourth known life-cycle in the genus. In addition to catfish, smallmouth buffalo *Ictiobus bubalus* polycultured with catfish were examined and two *Myxobolus* spp. were characterized from the gills. Phylogenetic analyses strongly support a clade of ictalurid *Henneguya* spp. and a clade of catostomid *Myxobolus* spp. Although diverse, *H. ictaluri* is the only myxozoan in catfish attributed to significant losses.

With no feasible method of control or treatment, investigations into less susceptible fish were initiated and showed promise. Infectivity trials characterizing *H. ictaluri* development in channel, blue, and hybrid catfish were performed. Channel catfish were suitable hosts with myxospores developing in the gills by six weeks and persisting for at least 14 weeks. In hybrid catfish arrested or limited development was observed with no pseudocysts observed during Trial 1 and only two at 14 weeks during Trial 2. These results may suggest a possible way of decreasing losses attributed to PGD through hybrid catfish monoculture or fish crop rotation to reduce the number of infectious myxospores released into the pond.

DEDICATION

This dissertation is dedicated to Dr. Linda M. Pote and was completed shortly after her passing. The bulk of this work is testament to her ability to instill enthusiasm and love for the field of parasitology in the students she mentored. Her legacy is one that will live on through her students and the people who knew her.

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INTRODUCTION

1.1 Channel catfish aquaculture

Channel catfish (*Ictalurus punctatus*) is the most extensively cultured freshwater fish in the United States and is integral to the economies of several southeastern states (Wellborn 1988; Hargreaves 2002; Stankus 2010; USDA 2012). From the industry's beginnings in the 1960s, extensive growth has occurred in the states occupying the lower flood plain of the Mississippi River, an area more commonly referred to as "the Delta." The culture of channel catfish across Alabama, Arkansas, Louisiana and Mississippi accounts for 95% of the industry's total production, with sales totaling $423 million in 2011 (USDA 2012). Mississippi accounts for approximately 70% of the total industry, with nearly 55,500 water surface acres dedicated to production in 2011 (Robinson and Avery 2000; USDA 2012). Flavorful fillets, dependable breeding, uncomplicated propagation and efficient feed to protein conversion rates make the channel catfish a reliable source of animal protein (Wellborn 1988; Stankus 2010). Recent declines in channel catfish production have been attributed to rising feed costs, influenced primarily by increased prices of corn and soybeans, both critical components of catfish diets (Stankus 2010).

In the 1980s, a growing market for high quality animal protein led to escalated fish demand and necessitated more intensive production strategies. This resulted in

higher stocking rates, larger production ponds, increased feeding rates, and the implement of multi-batch cropping systems. These management practices, designed to increase production, have also created ideal scenarios for the spread of infectious disease (Hargreaves 2002; Tucker and Hargreaves 2004).

1.2 Proliferative gill disease

Proliferative gill disease (PGD) is caused by the myxozoan parasite *Henneguya ictaluri.* Often associated with morbidity and high mortality in fingerling and adult channel catfish, PGD was first reported from catfish aquaculture in the southeastern United States in the early 1980s (Smith and Inslee 1980; Bowser and Conroy 1985; Bowser et al. 1985; Kent et al. 1987). Characterized by swollen gill lamellae, chondrolysis, multifocal interlamellar hyperplasia of the branchial epithelium and anorexia, PGD is the third most commonly diagnosed infectious disease and the most prevalent parasitic disease of channel and hybrid catfish submitted to the Aquatic Research and Diagnostic Laboratory at the Thad Cochran National Warmwater Aquaculture Center, Stoneville, MS (Figure 1.1) (Bowser and Conroy 1985; Bowser et al. 1985; MacMillan et al. 1989; Khoo et al. 2012). The inflamed and severely infected gill lamellae are often clubbed and fused resulting in a loss of surface area compromising respiratory function (Bowser and Conroy 1985; Bowser et al. 1985; Burtle et al. 1991; Kent et al. 1987; MacMillan et al 1989; Styer et al. 1991; Wise et al. 2008). In the catfish aquaculture, PGD is more commonly referred to as “hamburger gill disease” on account of the swollen and mottled appearance of heavily infected gills, which resembles bloody ground hamburger meat (Killian 1994). During an outbreak, PGD is often diagnosed pond-side by farmers, noting the presence of fish piping at the water surface and

swimming listlessly near aerators, both indicators of respiratory distress. Pond-side diagnosis by farmers whose operations endure yearly outbreaks of PGD might result in a lower estimation of actual prevalence of the disease (Wise et al. 2004).

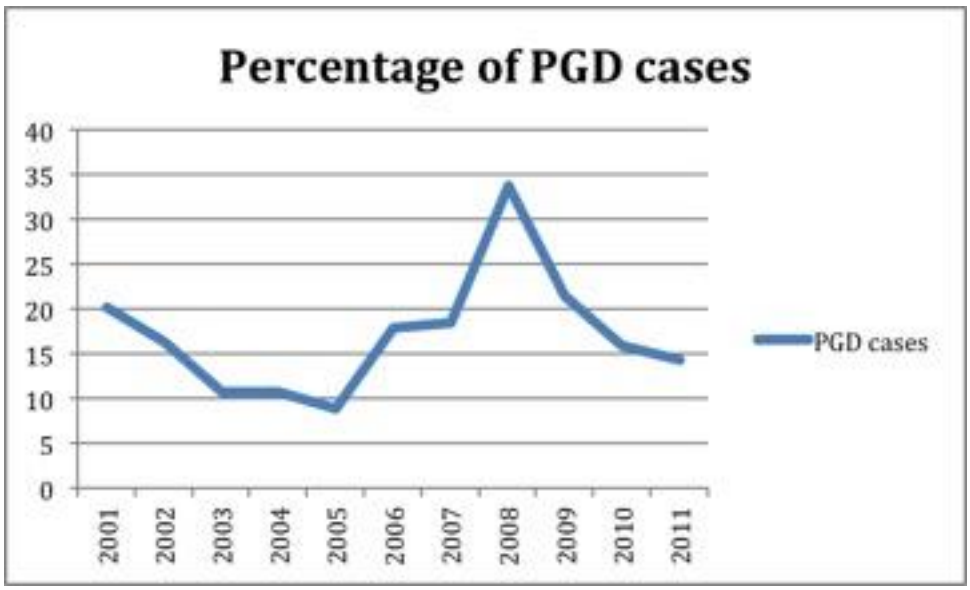

Figure 1.1 Percent of disease case submissions with PGD submitted to the Aquatic Research and Diagnostic Lab at the Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS from 2001-2011.

Most clinical outbreaks of PGD occur in the spring, with a lower incidence in the fall, when the water temperatures are between 15 and 20°C. This seasonal pattern may be associated with ideal temperature ranges for either the development of the parasite or propagation of the oligochaete host, *Dero digitata*, a benthic oligochaete ubiquitous in catfish ponds (Pote et al. 2012; Wise et al. 2004). *Dero digitata*, is the predominant oligochaete observed in benthic samples collected from ponds experiencing epizootics. The population of *D. digitata* in ponds with clinical outbreaks of PGD is significantly

greater than ponds with subclinical outbreaks or no signs of disease (Bellerud et al. 1995). The increased prevalence of *H. ictaluri* in *D. digitata* from ponds with clinical PGD and the static pond environment allows for continuous exposure of the resident fish to the infective actinospore stage. Although actinospores of other myxozoan species have been shown to retain viability at low temperatures, the actinospores of *H. ictaluri* are short lived at normal environmental temperatures and lose viability after 1-2 days (Markiw 1992; Wise et al. 2004; Wise et al. 2008).

Unlike other myxozoans, where mature plasmodia are the root cause of disease (Whitaker and Kent 1991), the catfish host has a limited response to the myxospore stage of *H. ictaluri.* In heavy infections, large numbers of plasmodia can mechanically disrupt gill function, but in general, a majority of the damage associated with *H. ictaluri* stems from the severe inflammatory response associated with the initial penetration and proliferation of the actinospore stage. Prolonged and repeated exposure to high numbers of *H. ictaluri* actinospores results in an increase in clinical severity of PGD in naïve fish. Recent research has demonstrated that naïve fish held in net pens and placed in ponds with active outbreaks develop clinical PGD, sometimes in as little as 24 hours (Wise et al. 2008; Griffin et al. 2010). Similarly, fish held in fiberglass tanks and exposed to infectious pond water over the course of several days developed clinical PGD. However, fish exposed to a single dose of infectious pond water, then removed from the source of infection had fewer chondrolytic lesions and less severe gill inflammation compared to fish maintained in infectious pond water over the same period. In addition, once removed from the source of infection, callous formation was observed at the point of chondrolysis,

suggesting that in the absence of infectious agents, recovery and healing is initiated quickly (Wise et al. 2008).

Under optimal conditions, *D. digitata* populations double every 4 to 7 days (Mischke and Griffin, 2011). In addition, vertical transmission of myxozoans during asexual reproduction of infected oligochaetes has been demonstrated in laboratory studies (Morris and Adams 2006). This phenomenon may further exacerbate actinospore levels in the ponds, as infected oligochaete populations can increase rapidly. These factors all play a role in PGD severity and likely contribute to disease severity of other myxozoan infections in intensively managed aquaculture systems.

1.3 Myxozoa

Myxozoans are a unique group of metazoan parasites of both veterinary and economic importance, especially in commercial aquaculture. The Myxozoa encompass approximately 2,000 species in 62 genera (Lom and Dyková 2006). These spore-forming parasites primarily infect fish (freshwater and marine), with alternate life cycle stages that infect aquatic annelids (oligochaete or polychaete). While uncommon, there are also reports of myxozoans from terrestrial mammals, reptiles and birds are limited (Dyková et al. 2007; Prunescu et al. 2007; Bartholomew et al. 2008; Roberts et al. 2008). The majority of myxozoans that infect fish are innocuous to the fish host, there are several notable exceptions. These exceptions are most often noticed in commercially raised fish species, where intensive management practices such as high stocking densities and multi-batch production systems produce conditions conducive to efficient parasite propagation and transmission (Kent et al. 2001; Lom and Dyková 2006).

To date, more than 50 myxozoan life cycles have been confirmed, linking the actinosporean stage shed by the annelid host with the myxosporean stage in the fish host. *Myxobolus cerebralis* and *Ceratonova* (*Ceratomyxa*) *shasta*, both myxozoan parasites of salmonid fish, have been studied extensively, with molecularly confirmed life cycles linking both stages of the parasites to their respective hosts and working laboratory models facilitating investigations into modes of transmission and developmental studies (Markiw and Wolf 1983; Wolf and Markiw 1984; Meaders and Hendrickson 2009).

Most experimentally and molecularly confirmed myxozoan life cycles in fish are indirect, involving a fish intermediate host and an annelid definitive host with morphologically distinct stages in each host. Fish to fish transmission is rare, and only documented for *Enteromyxum leei* (Eszterbauer et al. 2015).

The actinospores are pelagic, non-motile stages shed from annelid hosts (oligochaetes in freshwater and polychaetes in marine environments) that when released into the water column encounter the fish host and initiate infection via penetration of host tissues (Kent et al. 2001; Køie 2000; Lom and Dyková 2006). Encysted myxospore stages develop within the fish host in a variety of interior and exterior tissue sites. Myxospores are released into the aquatic environment during routine sloughing of the epithelium (exterior plasmodia) or following the death of the animal (interior plasmodia). The released myxospores are ingested during foraging by the annelid hosts and actinospores develop inside a pansporocyst within the epithelial cells lining the annelid's gastrointestinal tract. The life cycle is completed when, upon maturation, actinospores are released into the water column with the annelid's feces and encounter the appropriate

fish host (Wolf and Markiw 1983; Hamilton and Canning 1987; Kent et al. 2001; Gilbert and Granath Jr. 2003).

The taxonomic placement of myxozoans remains in a constant state of flux due to past classification systems. Initially, myxospores (Class: Myxosporea) and actinospores (Class: Actinosporea) were thought to be two separate classes of organisms, which were categorized solely on morphological characteristics of myxospores from fish (Kent et al. 2001). The pioneering work of Markiw and Wolf was the first to recognize that Myxosporea and Actinosporea were not distinct classes of organism, but rather separate components of an indirect life cycle. Their work identified two morphologically distinct stages in the life cycle of *M. cerebralis*, a triactinomyxon actinospore stage in the benthic freshwater oligochaete *Tubifex tubifex* and a myxospore stage in salmonid fish (Wolf and Markiw, 1984). This work was later confirmed with the advent of 18S rDNA sequencing (Wolf et al. 1986). The inclusion of 18S rRNA gene sequences when new species are described has made it possible to link the actinospore and myxospore stages of previously unknown life cycles (Andree et al. 1997; Griffin et al. 2008; Iwanowicz et al. 2008; Work et al. 2008; Griffin et al. 2009a; Griffin et al. 2009b; Camus and Griffin 2010; Walsh et al. 2012). Long considered protists, recent findings have identified myxozoans to be metazoans closely related to the cnidarians, citing ultrastructural similarities between myxozoan polar filaments and cnidarian nematocysts and 18S rDNA gene sequences (Siddall et al. 1995; Andree et al. 1997; Holzer et al. 2004; Whipps et al. 2004; Fiala 2006; Ringuette et al. 2011; Evans et al. 2012). Suppression of the previous classification schemes has been suggested and priority has been given to myxosporean stages when characterizing novel myxozoan species. Meanwhile, collective

morphological groups (aurantiactinomyxon, triactinomyxon, neoactinomyxon, etc.) are used when classifying newly identified actinospore stages (Siddall et al. 1995; Kent et al. 1994; Kent et al. 2001).

1.3.1 *Henneguya ictaluri*

Myxozoans of the genus *Henneguya* Thélohan, 1892 are parasites of predominantly freshwater fish, but some marine forms exist (Minchew 1977; Pote et al. 2000; Eiras 2002; Griffin 2008; Li 2012). Globally, over 200 *Henneguya* spp. parasitize numerous organ systems in a variety of fish hosts. Myxospores of *Henneguya* spp. are ellipsoid or spindle-shaped, possess two elongated polar capsules and are characterized by tapering caudal processes (Pote et al. 2000; Eiras 2002).

Eight species of *Henneguya* have been documented in commercially farmed channel catfish (*Ictalurus punctatus*) based on pseudocyst and myxospore morphology, as well tissue predilection in the fish host (Table 1.1). Of the eight *Henneguya* spp. infecting channel catfish, only *Henneguya exilis* and *H. ictaluri* have molecularly confirmed life histories linking the myxospore stages in the fish host to the actinospore stages in the oligochaete host (*Dero digitata*) (Lin et al. 1999; Pote et al. 2000). The remaining six species have unknown oligochaete hosts and their pathogenicity in fish has not been thorogouhly investigated.

Table 1.1 Overview of *Henneguya* spp. reported from channel catfish.

Henneguya spp.	Fish host(s)	Oligochaete host	Site in fish	Reference:
H. adiposa	*Ictalurus punctatus*	N/A	Adipose fin	Current 1979
H. diversis	*I. punctatus*	N/A	Barbels, pectoral fins, liver and kidneys	Minchew 1977
H. exilis	*I. punctatus,*	*Dero digitata*	Gills	Kudo 1929
H. ictaluri	*I. punctatus*	*D. digitata*	Gills	Pote et al. 2000
H. limatula	*I. punctatus*	N/A	Gall bladder	Minchew 1977
H. longicauda	*I. punctatus*	N/A	Gills	Minchew 1977
H. postexilis	*I. punctatus*	N/A	Gills	Minchew 1977
H. sutherlandi	*I. punctatus*	N/A	Skin	Griffin et al. 2008

Of the multiple *Henneguya* spp. that infect channel catfish, only one has been linked to severe losses. Initially, interlamellar and intralamellar *Henneguya* myxospores were observed in farm-raised catfish, with the interlamellar forming species attributed to more severe losses in fingerlings and respiratory distress in heavily infected market size fish (McCraren et al. 1975). From 1981 through 1983, commercial channel catfish ponds experienced a series of epizootics of unknown cause in fingerlings and market size fish. Histological examination of fish collected from ponds with active disease oubtreaks revealed myxosporean plasmodia located in the gills (Bowser and Conroy 1985). In some instances, mortality rates in ponds reached 100%. Clinical signs included multifocal interlamellar hyperplasia, distortion of gill lamellae, listless swimming and respiratory distress with fish localized near aerators (Bowser and Conroy 1985; Griffin et al. 2008; Kent et al. 1987; Wise et al. 2008).

A myxozoan origin of PGD was initially established by the exposure studies of MacMillan et al. (1989). Specific-pathogen-free (SPF) channel catfish were exposed to sediment collected from the water/benthos interface from ponds with active PGD outbreaks. This resulted in infection with a myxosporean parasite that was detectable by histological processing and microscopic examination of tissues collected over a period of

2 months. Fish exposed to sediments collected from ponds without an active epizootic did not develop PGD (MacMillan et al. 1989).

A severe inflammatory response with marked congestion at the gills, indicative of PGD, was observed as early as 48 hours after exposure to pond sediment. Microscopy displayed a profuse influx of inflammatory cells of differing types, similar to those observed in histological examinations of naturally infected PGD fish. This multifocal inflammatory response contained a mix of lymphocytes, macrophages and neutrophils (Bowser and Conroy 1985; MacMillan et al. 1989; Griffin et al. 2008; Wise et al. 2008). In this study, the number of inflammatory cells and their location varied. Some gill arches possessed small numbers of foci limited to only a few filaments, while in other arches every filament was affected. Closer examination of gill tissues after 48-hour exposure to pond sediments revealed uninucleate cells, 8-10 µm diameter, with a basophilic nucleus and eosinophilic cytoplasm, identified as the primary cell of the myxosporean parasite. Plasmodial stages like those observed by Bowser and Conroy (1985) were observed approximately 3-6 d post exposure and were characterized by numerous secondary cells, 2-4 µm in diameter, surrounding the primary cell (MacMillan et al. 1989). Disease severity was determined to be proportional to the number of developing plasmodia within each filament. Filaments containing 1-4 plasmodia was considered a mild infection, while greater than 4 plasmodia per filament, with 75% of all filaments containing plasmodia, resulting in death (MacMillan et al. 1989).

Surveys of sediment from ponds experiencing PGD epizootics revealed a diverse benthic fauna. The most abundant organism observed in sediment samples from PGD outbreaks was *D. digitata,* a member of the Naididae. Microscopic examination of

squashes of *D. digitata* revealed the presence of actinospore stages of a myxozoan parasite (Burtle et al. 1991; Bellerud et al. 1995; Smith 2001). These actinospores were characterized by the presence of 3 apical polar capsules, a sporoplasm containing approximately 32 nuclei, a triangular spore body, 3 valves and 3 blunt shaped caudal processes (Burtle et al. 1991; Bellerud 1993). Aurantiactinomyxon type actinospores were found in the gut wall of infected *D. digitata* and in the water column of mud samples collected from ponds experiencing a PGD outbreak.

Transmission studies conducted by Styer et al. (1991) established a relationship between *Dero digitata* infected with the unknown species of *Aurantiactinomyxon* and channel catfish. PGD was induced in channel catfish exposed to *D. digitata* isolated from mud dredged from the bottom of a pond during a PGD epizootic. Similarly, PGD was induced in channel catfish exposed to aurantiactinomyxon actinospores collected from squashes of *D. digitata*. Granulomatous inflammation and developing stages indicative of PGD were observed histologically, suggesting a myxozoan life cycle involving the channel catfish and *D. digitata* (Bowser and Conroy 1985; Burtle et al. 1991; Styer et al. 1991; Wise et al. 2008).

Dero digitata is host to a multitude of actinospore types found in commercial catfish ponds. Early work demonstrated that populations of *D. digitata* released a greater variety of myxozoan fauna in ponds with active outbreaks of PGD than ponds without any signs of disease (Bellerud 1993; Bellerud et al. 1995; Burtle et al. 1991). Later, 18S rDNA sequence linked the aurantiactinomyxon type actinospore from *D. digitata* to the myxospore stage of a previously undescribed myxozoan parasite, *Henneguya ictaluri* (Pote et al. 2000). Interlamellar cysts containing mature myxospore stages of *H. ictaluri*

were observed in the gills of an experimentally infected channel catfish 3 months post exposure. Spindle shaped myxospores with split caudal processes were observed on wet mounts with an average spore size of 23.9 µm x 6.0 µm. Two elongate polar capsules of roughly identical length and width (8.1 µm x 2.5 µm) occupied the spore body. Posterior to the spore body, completely split caudal processes extended a length of 63 µm with the split being located immediately posterior to the spore body (Pote et al. 2000).

Investigations into the life cycle of *H. ictaluri* using indirect fluorescent antibody tests suggest multiple portals of entry. Entry points include gills, skin and buccal cavity, with developing organisms detected across multiple organ systems over a period of 4 days. Fluorescent detection of developing organisms was observed in the anterior and poster kidneys, stomach, spleen and liver, with the strongest fluorescence observed in the gills, suggesting the gills are the preferred site of development for early stages in the *H. ictaluri* life cycle (Belem and Pote 2001). Pote and Waterstrat observed a motile amoeboid stage released from the aurantiactinomyxon type actinospores when exposed to gill filaments and mucus of channel catfish (1993). While the gills seem to be the primary site of development of *H. ictaluri* in the fish host, little is known of the precise chemical cues between fish and parasite that initiate infection. Previous studies have found that rainbow trout mucus can be enough to induce the extrusion of polar filaments of actinospore stages of *M. cerebralis.* El Matbouli et al. (1999) used electron microscopy to describe host specific triggering of polar filament extrusion and actinospore attachment during the early stages of *M. cerebralis* infection in susceptible rainbow trout. Triactinomyxons were observed attaching to the epithelium of the fish and initiating infection via penetration of host epithelial surfaces as soon as 1 hour post

exposure. However, exposing triactinomyxons to mucus of non-salmonid fish species failed to induce polar filament extrusion, suggesting some level of host specificity (El-Matbouli et al. 1999).

1.3.2 Diagnosis of PGD

1.3.2.1 Polymerase chain reaction for the detection of *H. ictaluri*

Diagnostic polymerase chain reaction assays for the detection of myxozoans in fish, oligochaetes and the aquatic environement have been developed for numerous myxozoan species (Andree et al. 1997; Andree et al. 1998; Palenzuela et al. 1999; Hanson et al. 2001; Whitaker et al. 2005; Caffara et al. 2009). Hanson et al. (2001) compared 18S rDNA sequences of four different actinospore morphotypes from *D. digitata* to establish a polymerase chain reaction assay for the molecular confirmation of PGD. The PCR primers amplified a 104 base pair product specific to *H. ictaluri* (syn. *Aurantiactinomyxon ictaluri*), targeting a highly variable region within the 18S rRNA gene. The PCR assay is capable of detecting parasite DNA in fish tissue, oligochaetes and environmental pond water samples (Whitaker et al. 2001; 2005). In addition, the PCR assay was more sensitive than conventional diagnostic methods, namely gross examination of gill biopsies and histopathology. Used in conjunction with these other diagnostic methods, the PCR assay provides molecular confirmation of the presence of the parasite (Whitaker et al. 2001; 2005).

While molecular detection of PGD in fish tissues is a valuable confirmatory diagnostic, the ability to detect actinospores in the environment provides more practical information catfish producers can use to determine whether or not it is safe to introduce naïve fish into a pond, or to restock a pond after an outbreak. The detection of the

parasite in the resident oligochaete population is labor intensive and given the patchy distribution of *D. digitata* in the pond can be hit or miss. Alternatively, detecting the parasite in pond water is independent of the oligochaete distribution and can more accurately rank the risk of PGD to naïve fish introduced to the pond. Detecting the infectious stage in the water can identify if an outbreak is likely, or more practically, to assess actinospore levels following an outbreak where losses have occurred.

The PCR assay developed by Hanson et al. (2001) and validated by Whitaker et al. (2001) was used in a survey of 40 commercial catfish ponds. Of the 40 ponds sampled, 32 had *H. ictaluri* positive fish, 35 had *H. ictaluri* positive oligochaetes and 25 had *H. ictaluri* positive water. The PCR test for the water and oligochaetes detected the presence of *H. ictaluri* in all ponds with PGD-positive fish. Of the 40 ponds, only 3 were negative for *H. ictaluri* in the fish, water and oligochaetes, evidence of the widespread nature of *H. ictaluri*. Detecting *H. ictaluri* in pond water offered a more convenient tool than sentinel fish exposures for determining when it is safe to restock ponds following a PGD outbreak (Whitaker et al. 2005).

Conventional endpoint PCR is useful for detecting the presence of target DNA, but yields only qualitative results. Conversely, real-time quantitative PCR (qPCR) allows for the relative enumeration of target DNA. In addition to the ability to quantify target DNA, qPCR provides faster results by negating the need of postreaction processing. As such, qPCR serves as a reliable tool for the detection of pathogens in several areas of study.

Real-time quantitative PCR assays have been employed to detect multiple myxozoan species in host tissue and environmental water samples (Cavender et al. 2004;

Hallet and Bartholomew 2006; Griffin et al. 2009c; True et al. 2009; Jorgensen et al. 2011). Diagnosing and evaluating PGD risk has increased with the development of a real-time PCR assays to detect both the myxospore stage in host tissue and the actinospore stage found in pond water. Previous diagnostic techniques for detecting PGD consist of evaluating wet mounts of 40-80 gill filaments for the presence of chondrolytic lesions indicative of PGD, histological sectioning of gill tissue and amplification of a 104 base pair product of the *H. ictaluri* 18S ribosomal RNA gene (Griffin et al. 2008). While quick to perform, wet mounts are subjective since only a small portion of the gills are examined and infections could be missed if low numbers of parasites are present. Histology is similarly limited. Endpoint PCR has been shown to be a more sensitive diagnostic tool for detecting the presence of *H. ictaluri* in fish tissue, pond water, and the oligochaete host (Hanson et al. 2001; Whitaker et al. 2005). However, the biological significance of qualitative PCR positive results have not been adequately evaluated.

Conversely, research has demonstrated a TaqMan probe based quantitative real-time PCR assay for *H. ictaluri* provides a quantifiable method of detecting the parasite (Griffin et al. 2009c). The assay was sensitive enough to detect a single actinospore, which allows for the detection of early stages of the parasite as soon as 24 hours post exposure. Conversely endpoint PCR, histology, or wet mounts often fail to recognize these early stages.

1.3.2.2 Monitoring program

The use of sentinel fish in evaluating the PGD status of a pond has been well described (Wise et al. 2004; 2008). Briefly, sentinel fish are housed in mesh cages for 7 days, after which gill biopsies (40-80 filaments) are examined for the presence of

chondrolytic lesions indicative of PGD (Wise et al. 2004; 2008). Lesion scores are based on the percentage of filaments with at least one chondrolytic lesion in the filamental cartilage. Scores of 1 to 5% are considered mild, with minimal risk of mortalities. A score of 6 to 15% is moderate, while a lesion score greater than 15% represents a severe infection with a high risk of mortality in naïve fish introduced to the system. In brief, the lesion scoring system gives an indirect estimation of the number of parasites present in the water column.

This scoring system allows producers and fish health professionals to evaluate the risk of losing fish newly stocked into the system or following an outbreak. To gain insight into the progression of the outbreak, a second and sometimes third exposure of sentinel fish is needed. The use of sentinel fish along with the grading scale of severity is an effective diagnostic tool, but is labor intensive, requires at least 1 week for results and can be inconclusive if the fish die before assessment or if the cage system fails (Wise et al. 2004; 2008). Alternatively, the qPCR assay provides a direct, real-time estimate of actinospore levels in the pond, is not as labor intensive, and can provide results in as little as 24 hours (Whitaker et al. 2005; Griffin et al. 2008; Griffin et al. 2009c). However, multiple sampling events for all diagnostic methods allow for observing trends and can be used to better assess outbreak severity and recovery.

1.3.3 Control and treatment of PGD

1.3.3.1 Chemical control

At present, there are no chemotherapeutants or prophylactic treatments for myxozoan infections, as those tested in fish species have shown limited efficacy. Fumagillin is perhaps the most well studied drug used in the treatment of myxozoan

infections in fish (Molnár et al. 1987; Hedrick et al. 1988; El-Matbouli and Hoffmann 1991; Kent and Dawe 1994). Prepared feeds containing fumagilin (dicyclohexylamine) to treat *M. cerebralis* in rainbow trout resulted in decreased infection rates and reduced clinical disease (El-Matbouli and Hoffmann 1991; Wagner 2002). Moreover, fumagillin was successful in treating chinook salmon *Oncorhynchus tshawytscha* experimentally infected with the microsporidian parasite *Loma salmonae*. Daily doses of 10 mg/kg of fish was incorporated into the diet and fed to infected salmon for 30 days. *Loma salmonae* was undetected in all treated fish (Kent and Dawe 1994). The drug was also effective in preventing proliferative kidney disease in experimentally infected chinook salmon at treatment doses of 0.5 and 1.0 g/kg of feed (Hedrick et al. 1988). Lastly, *Sphaerospora renicola* infection of common carp *Cyprinus carpio* has been successfully reduced with fumagillin incorporated into the diet at 0.1% of feed fed (Molnár et al. 1987). Although fumagillin has been shown successful in preventing or reducing myxozoan infections in numerous fish species, the effectiveness has been largely studied when given at the time of initial penetration and proliferation the effectiveness over the course of infection has not been adequately evaluated (El-Matbouli and Hoffmann 1991). In some studies fumagillin has had limited efficacy and mortalities in treated fish has raised concerns of drug related toxicity (Wagner 2002).

Incorporation of nicarbazin into the diets of Atlantic salmon *Salmo salar* was efficacious against *Kudoa thyrsites* with dietary nicarbazin concentrations of at least 2.5 gram per kilogram. When fed at concentrations of 10 and 25 gram per kilogram, dietary nicarbazin was associated with adverse effects in Atlantic salmon, such as anorexia and

mortality, which subsided after returning the fish to a non-medicated diet (Jones et al. 2012).

Quinine and salinomycin appear to be efficacious in the treatment of *Henneguya* sp. when given orally to naturally infected tapir *Gnathonemus petersii* (Dohle et al. 2002). Oral administration of salinomycin, resulted in shrinkage of plasmodia and enlargement of pansporoblastic suture elements in gill trophozoite stages as early as three days post-treatment. Polar capsules and polar filaments were malformed and eventually undetectable. Nine days post-treatment, the pansporoblasts were no longer present. Quinine displayed similar deleterious effects against *Henneguya* sp. in infected tapir fish. Cytoplasmic vacuolization was observed in developing plasmodial stages and destruction of polar capsules was observed 6 days post-treatment. Severing of parasite and host membranous connections was also evident and no parasite stages were detected by microscopy 9 days post-treatment. Moreover, salinomycin and quinine displayed no adverse effects in tapir fish in this short-term study (Dohle et al. 2002).

Proposed control for myxozoan infections in fish often focuses on eliminating the oligochaete host through chemical control, biological control or strategic management strategies. Breaking the life cycle by eradicating or lowering the number of infected oligochaetes could alleviate actinospore exposure levels and may reduce clinical manifestations of disease (Tucker et al. 2004). Chemical treatments of actinospores in the water can provide temporary relief. However, unless the oligochaete host is eliminated, any actinospores eradicated from the water column will be immediately replaced by oligochaetes still present in benthos. As such, in order to be effective, multiple treatments are required. Formalin and potassium permanganate treatment of

water have been found to reduce the number of PGD related lesions in some instances, but there use as a chemical therapeutic is not practical in the production setting due to the high costs of multiple applications (Wise et al. 2004).

Several chemical therapeutics against *D. digitata* have been investigated in laboratory trials in attempts to identify management strategies to eradicate the oligochaete. Cultured *D. digitata* were exposed to Bayluscide® (niclosamide, 70% wettable powder, Bayer Chemical Co., Kansas City, Missouri, USA), Chloramine-T (N-chloro-*p*-toluenesulfonamide, Halamid, H&S Chemical Co., Covington, Kentucky, USA), formalin (37% formaldehyde solution), hydrogen peroxide (H_2O_2), copper sulfate ($CuSO_4$), potassium permanganate ($KMnO_4$), rotenone ($C_{23}H_{22}O_6$) and sodium chloride (NaCl) to test acute toxicity levels at 24 and 48 hours post treatment (Mischke et al. 2001). Though some of these chemical treatments showed promise in controlling *D. digitata*, the application of these compounds in the aquaculture setting is not practical for several reasons. Required dosage of these compounds is often cost prohibitive for catfish producers and doses high enough to penetrate the dense organic matter in the pond bed can be lethal to the resident fish population (Mischke et al. 2001).

1.3.3.2 Biological control

Biological control of *Myxobolus pseudodispar* actinospores through predation by copepods has been shown to lead to a decreased number of triactinomyxon actinospores in laboratory controlled trials (Rácz et al. 2006). Labeling triactinomyxons with fluorescent 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) and microscopically observing the feeding apparatus of copepods confirmed ingestion of actinospores. Stained spores were detected in the alimentary tract of infected copepods

2.5 hours post-exposure and most triactinomyxons exhibited polar filaments and structural damage to caudal processes. To confirm the viability of copepod-ingested actinospores, SPF roach *Rutilus rutilus* fingerlings were fed copepods containing actinospores and necropsied 138 days post-infection to look for *M. pseudodispar* lesions in the musculature. Developing plasmodia were not detected in the roaches challenged with copepods containing myxozoan actinospores, however, infection was observed in positive control fish exposed only to triactinomyxons (Rácz et al. 2006).

Stocking of fathead minnows *Pimephales promelas* in catfish ponds has been proposed to control the *D. digitata* since a large portion of their diet consists of algae and oligochaetes. In order to be effective, large numbers of minnows must be stocked in order to maintain a population large enough to have any measurable impact on oligochaete populations. Unfortunately, predation of minnows by the larger catfish in the system make sustaining a large enough population a difficult task (Burtle 1998).

Similarly, smallmouth buffalo *Ictiobus bubalus* have also been proposed as a method of biological control. Smallmouth buffalo are opportunistic bottom feeders, known to feed on numerous benthic macroinvertebrates. The polyculture of small mouth buffalo with catfish ponds has been suggested to lessen the incidence of PGD in the resident fish population (Steeby et al. 2006). Recent research investigated the use of smallmouth buffalo as a biological control agent in 1-acre research ponds over 2 consecutive production cycles. Researchers stocked ponds (n=9) with 8,000 catfish fingerlings and 300 smallmouth buffalo. Control ponds (n=9) were treated similarly but were not stocked with smallmouth buffalo. Over the course of the 3-year study, there were no differences in the numbers of benthic organisms, actinospore concentrations or

disease severity in sentinel fish. In addition, there were no differences in total feed fed or total weight harvested. Under the conditions used in this study, the presence of smallmouth buffalo did not have a measurable effect on PGD incidence, severity or overall catfish production (Griffin et al. 2014)

Given the limited success in treating PGD with chemotherapeutic drugs, management of the disease is focused on maintaining adequate dissolved oxygen levels in the pond with supplemental aeration, restricting feed during an outbreak and maintaining sufficient chloride levels to minimize osmotic stress. While moving fish into a PGD free pond may reduce mortality, this practice has been considered unfavorable due to risks of introducing myxospores into a pond that has previously been PGD free. However, research has shown PGD to be present at some level in nearly all catfish ponds in the spring of the year. As such, the risk of actually introducing *H. ictaluri* into a naïve pond is likely minimal as truly naïve ponds are rare if nonexistent (Wise et al. 2004).

Current efforts to establish the life cycle of *H. ictaluri* in the laboratory setting have been unsuccessful due to the inability to infect the oligochaete host, *D. digitata*. However recent progress made in propagating large numbers of the oligochaete offers researchers the ability to investigate the biological interactions between the myxospore and oligochaete host (Mischke and Griffin 2011). The experimental maintenance of myxozoan life cycles has been achieved for few species of myxozoans (Wolf et al. 1986; Eszterbauer et al. 2000; Meaders and Hendrickson 2009). Laboratory maintained colonies of *M. cerebralis* and *C. shasta* have provided invaluable information regarding the biology of these myxozoans and their fish hosts (Wolf et al. 1986; Meaders and Hendrickson 2009;). A readily available source of *H. ictaluri* myxospore and actinospore

stages would allow for advancement in the study of PGD in catfish and may provide additional insight into controlling the disease. Establishment and maintenance of *H. ictaluri* in the laboratory warrants further study.

1.3.3.3 Genetic control

The culturing of a less susceptible catfish species has been examined as a method of reducing the incidence of PGD in commercial channel catfish ponds by limiting the number of myxospore stages released back into the system. Blue catfish *Ictalurus punctatus* show resistance to numerous diseases of channel catfish that induce mortalities in aquaculture, such as enteric septicemia, channel catfish virus and PGD, making them an ideal candidate for a culture species (Beecham et al. 2010; Bosworth et al. 2003; Graham 1999; Griffin et al. 2010). However, commercial culture of blue catfish has not been widely adopted due to several limiting production characteristics, including lower dress out, decreased maturation rates and poor spawning in captivity (Graham 1999).

When compared to channel catfish and channel catfish × blue catfish hybrids, blue catfish exhibit a lower incidence of PGD related lesions following 7 day exposures to infectious pond water (Bosworth et al. 2003). Repeated studies combining histopathology with real-time PCR data support the findings of Bosworth et al. (2003) with blue catfish having a significantly lower amount of PGD related lesions than channel catfish and channel catfish × blue catfish hybrids (Griffin et al. 2010). Compounding molecular evidence that blue catfish are refractory to PGD is supported by lower quantities of *H. ictaluri* DNA in gill filaments and blood of blue catfish than channel catfish and channel catfish × blue catfish hybrids (Beecham et al. 2010; Griffin et

al. 2010). Although blue catfish can develop PGD related lesions, it is rare and damage is to a much lesser extent than in concurrently challenged channels and hybrid catfish. Under experimental conditions, *H. ictaluri* DNA was undetectable in blue catfish gills by real-time PCR 14 days post exposure. However comparatively large quantities of *H. ictaluri* DNA was still present in channel and hybrid catfish exposed concurrently. This suggests blue catfish are inherently resistant to PGD and could serve to break the life cycle of *H. ictaluri* in a production system by alternating pond stocks with blue catfish (Bosworth et al. 2003; Beecham et al. 2010; Griffin et al. 2010). However, given the less desirable production characteristics of blue catfish, their application as a rotating aquaculture crop has little chance of adoption. Meanwhile, although hybrids can still become infected with *H. ictaluri* and suffer PGD related mortalities, there is anecdotal evidence that PGD outbreaks occur less often and are less severe in hybrid production ponds compared to traditional channel catfish production. Previous studies have only looked at the early stages of *H. ictaluri* infection in blue and hybrid catfish and investigations into the development of mature myxospores in these fish have not been conducted. Determining if *H. ictaluri* myxospores can develop and mature in hybrid catfish is a critical piece of information in regards to their role as a potential culture species and efforts to reduce the prevalence of *H. ictaluri* in catfish aquaculture.

1.4 References

Andree, K. B., E. MacConnell, and R. P. Hedrick. 1998. A nested polymerase chain reaction for the detection of genomic DNA of *Myxobolus cerebralis* in rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 34:145–154.

Andree, K. B., S. J. Gresoviac, and R. P. Hedrick. 1997. Small subunit ribosomal RNA sequences unite alternate actinosporean and myxosporean stages of *Myxobolus cerebralis* the causative agent of whirling disease in salmonid fish. Journal of Eukaryotic Microbiology 44:208–215.

Bartholomew, J. L., S. D. Atkinson, S. L. Hallett, L. J. Lowenstine, M. M. Garner, C. H. Gardiner, B. A. Rideout, M. K. Keel, and J. D. Brown. 2008. Myxozoan parasitism in waterfowl. International Journal for Parasitology 38:1199–1207.

Beecham, R. V., M. J. Griffin, S. B. LaBarre, D. Wise, M. Mauel, L. M. W. Pote, and C. D. Minchew. 2010. The effects of proliferative gill disease on the blood physiology of channel catfish, blue catfish, and channel catfish × blue catfish hybrid fingerlings. North American Journal of Aquaculture 72:213–218.

Belem, A. M. G., and L. M. Pote. 2001. Portals of entry and systemic localization of proliferative gill disease organisms in channel catfish *Ictalurus punctatus*. Diseases of Aquatic Organisms 48:37–42.

Bellerud, B. L. 1993. Etiological and epidemiological factors effecting outbreaks of proliferative gill disease on Mississippi channel catfish farms. Ph.D. Dissertation. College of Veterinary Medicine, Mississippi State University, Mississippi State, Mississippi, 164 p.

Bellerud, B. L., L. M. Pote, T. L. Lin, M. J. Johnson, and C. R. Boyle. 1995. Etiological and epizootological factors associated with outbreaks of proliferative gill disease in channel catfish. Journal of Aquatic Animal Health 7:124–131.

Bosworth, B. G., D. J. Wise, J. S. Terhune, and W. R. Wolters. 2003. Family and genetic group effects for resistance to proliferative gill disease in channel catfish, blue catfish and channel catfish × blue catfish backcross hybrids. Aquaculture Research 34:569–573.

Bowser, P. R., A. D. Munson, H. H. Jarboe, and F. N. Stiles. 1985. Transmission trials of proliferative gill disease in channel catfish (*Ictalurus punctatus*). Mississippi Agricultural & Forestry Experiment Station Research Report 10: No. 8.

Bowser, P. R., and J. D. Conroy. 1985. Histopathology of gill lesions in channel catfish associated with *Henneguya*. Journal of Wildlife Diseases 21:177–179.

Burtle, G. J. 1998. Control of the oligochaete vector of proliferative gill disease in catfish. 1998 Annual Report Department of Animal and Dairy Science. The University of Georgia, College of Agricultural and Environmental Sciences 22–25.

Burtle, G. J., and L. R. Harrison, E. L. Styer. 1991. Detection of a triactinomyxid myxozoan in an oligochaete from ponds with proliferative gill disease in channel catfish. Journal of Aquatic Animal Health 3:281–287.

Caffara, M., E. Raimondi, D. Florio, F. Marcer, F. Quaglio, and M. L. Fioravanti. 2009. The life cycle of *Myxobolus lentisuturalis* (Myxozoa: Myxobolidae), from goldfish (*Carassius auratus auratus*), involves a raabeia-type actinospore. Folia Parasitologica 56:6–12.

Camus, A. C., and M. J. Griffin. 2010. Molecular characterization and histopathology of *Myxobolus koi* infecting the gills of a koi, *Cyprinus carpio*, with an amended morphological description of the agent. Journal of Parasitology 96:116–124.

Cavender, W. P., J. S. Wood, M. S. Powell, K. Overturf, and K. D. Cain. 2004. Real-time quantitative polymerase chain reaction (QPCR) to identify *Myxobolus cerebralis* in rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 60:205–213.

Current, W. L. 1979. *Henneguya adiposa* Minchew (Myxosporida) in the channel catfish: ultrastructure of the plasmodium wall and sporogenesis. Journal of Protozoology. 26:209–217.

Dohle, A., G. Schmahl, W. Raether, H. Schmidt, and G. Ritter. 2002. Effects of orally administered chemotherapeutics (quinine, salinomycin) against *Henneguya* sp. Thelohán, 1892 (Myxozoa: Myxobolidae), a gill parasite in the tapir fish *Gnathonemus petersii* Günther, 1862 (Teleostei). Parasitology Research 88:861–867.

Dyková, I., T. Tyml, I. Fiala, and J. Lom. New data on *Soricimyxum fegati* (Myxozoa) including analysis of its phylogenetic position inferred from the SSU rRNA gene sequence. Folia Parasitologica 54:272–276.

Eiras, J. C. 2002. Synopsis of the species of the genus *Henneguya* Thélohan, 1892 (Myxozoa: Myxosporea: Myxobolidae). Systematic Parasitology 52:43–54.

El-Matbouli, M., and R. W. Hoffman. 1991. Prevention of experimentally induced whirling disease in rainbow trout *Oncorhynchus mykiss* by fumagillin. Diseases of Aquatic Organisms 10:109–113.

El-Matbouli, M., R. W. Hoffmann, H. Schoel, T. S. McDowell, and R. P. Hedrick. 1999. Whirling disease: host specificity and interaction between the actinosporean stage of *Myxobolus cerebralis* and rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 35:1–12.

Eszterbauer, E., S. Atkinson, A. Diamant, D. Morris, M. El-Matbouli, and H. Hartikainen. 2015. Myxozoan life cycles: Practical approaches and insights. *In* Myxozoan evolution, ecology and development. Okamura, B., A. Gruhl, and J. L. Bartholomew (eds). Springer International Publishing, Switzerland, p.175–198.

Eszterbauer, E., C. Székely, K. Molnár, and F. Baska. 2000. Development of *Myxobolus bramae* (Myxosporea: Myxobolidae) in an oligochaete alternate host, *Tubifex tubifex*. Journal of Fish Diseases 23:19–25.

Evans, N.M., M. T. Holder, M. S. Barbeitos, B. Okamura, and P. Cartwright. 2012. The phylogenetic position of Myxozoa: Exploring conflicting signals in phylogenomic and ribosomal data sets. Molecular Biology and Evolution 27:2733–2746.

Fiala, I. 2006. The phylogeny of Myxosporea (Myxozoa) based on small subunit ribosomal RNA gene analysis. International Journal for Parasitology 36:1521–1534.

Gillbert, M. A., and W. O. Granath, Jr. 2003. Whirling disease of salmonid fish: life cycle, biology, and disease. Journal of Parasitology 89:658–667.

Graham, K. 1999. A review of the biology and management of blue catfish. American Fisheries Society Symposium 24:37–49.

Griffin, M. J., A. C. Camus, D. J. Wise, T. E. Greenway, M. J. Mauel, and L. M. Pote. 2010. Variation in susceptibility to *Henneguya ictaluri* infection by two species of catfish and their hybrid cross. Journal of Aquatic Animal Health 22:21–35.

Griffin, M. J., D. J. Wise, A. C. Camus, M. J. Mauel, T. E. Greenway, and L. M. Pote. 2008. A real-time polymerase chain reaction assay for the detection of the myxozoan parasite *Henneguya ictaluri* in channel catfish. Journal of Veterinary Diagnostic Investigation 20:559–566.

Griffin, M. J., D. J. Wise, and L. M. Pote. 2009a. Morphology and small-subunit ribosomal DNA sequence of *Henneguya adiposa* (Myxosporea) from *Ictalurus punctatus* (Siluriformes). Journal of Parasitology 95:1076–1085.

Griffin, M. J., L. H. Khoo, L. Torrans, B. G. Bosworth, S. M. Quiniou, P. S. Gaunt, and L. M. Pote. 2009b. New data on *Henneguya pellis* (Myxozoa: Myxobolidae), a parasite of blue catfish *Ictalurus furcatus*. Journal of Parasitology 95:1455–1467.

Griffin, M. J., L. M. Pote, A. C. Camus, M. J. Mauel, T. E. Greenway, and D. J. Wise. 2009c. Application of a real-time PCR assay for the detection of *Henneguya ictaluri* in commercial channel catfish ponds. Diseases of Aquatic Organisms 86:223–233.

Griffin, M. J., L. M. Pote, D. J. Wise, T. E. Greenway, M. J. Mauel, and A. C. Camus. 2008. A novel *Henneguya* species from channel catfish described by morphological, histological, and molecular characterization. Journal of Aquatic Health 20:127–135.

Griffin, M. J., C. Mischke, T. Greenway, T. Byars, J. Walker, and D. Wise. 2014. Smallmouth buffalo for control of proliferative gill disease in catfish aquaculture: A three year study. *In* Proceedings of the 39th Eastern Fish Health Workshop. April 28-May 2, 2014. Shepherdstown, WV.

Hallett, S. L., and J. L. Bartholomew. 2006. Application of a real-time PCR assay to detect and quantify the myxozoan parasite *Ceratomyxa shasta* in river water samples. Diseases of Aquatic Organisms 71:109–118.

Hamilton, A. J., and E. U. Canning. 1987. Studies on the proposed role of *Tubifex tubifex* (Müller) as an intermediate host in the life cycle of *Myxosoma cerebralis* (Hofer, 1903). Journal of Fish Diseases 10:145–151.

Hanson, L. A., D. Lin, L. M. W. Pote, and R. Shivaji. 2001. Small subunit rRNA gene comparisons of four actinosporean species to establish a polymerase chain reaction test for the causative agent of proliferative gill diseases in channel catfish. Journal of Aquatic Animal Health 13:117–123.

Hargreaves, J. A. 2002. Channel catfish farming in ponds: Lessons from a maturing industry. Reviews in Fisheries Science 10:499–528.

Hargreaves, J. A., and C. S. Tucker. 2004. Industry development. *In* Bioogy and culture of the channel catfish, ed. Tucker C. S., Hargreaves J. A., 1st ed., pp. 482–488. Elsevier B. V., Amsterdam, The Netherlands.

Hedrick, R. P., J. M. Groff, P. Foley, and T. McDowell. 1988. Oral administration of fumagillin DCH protects chinook salmon *Oncorhynchus tshawytscha* from experimentally-induced proliferative kidney disease. Diseases of Aquatic Organisms 4:165–168.

Holzer, A. S., C. Sommerville, and R. Wootten. 2004. Molecular relationships and phylogeny in a community of myxosporeans and actinosporeans based on their 18S rDNA sequences. International Journal of Parasitology 34:1099–1111.

Iwanowicz, L. R., D. D. Iwanowicz, L. M. Pote, V. S. Blazer, and W. B. Schill. 2008. Morphology and 18S rDNA of *Henneguya gurlei* (Myxosporea) from *Ameiurus nebulosus* (Siluriformes) in North Carolina. Journal of Parasitology 94:46–57.

Jones, S. R. M., I. Forster, X. Liao, and M. G. Ikonomou. 2012. Dietary nicarbazin reduces prevalence and severity of *Kudoa thyrsites* (Myxosporea: Multivalvulida) in Atlantic salmon *Salmo salar* post-smolts. Aquaculture 342–343:1–6.

Jorgensen, A., A. Nylund, V. Nikolaisen, S. Alexandersen, and E. Karlsbakk. 2011. Real-time PCR detection of *Parvicapsula pseudobranchicola* (Myxozoa: Myxosporea) in wild salmonids in Norway. Journal of Fish Diseases 34:365–371.

Kent, M. L., G. E. Duhamel, J. S. Foott, and R. P. Hedrick. 1987. Chronic branchitis (hamburger gill disease) of channel catfish in California and its possible myxosporean etiology. California Fish and Game 73:99–105.

Kent, M. L., K. B. Andree, J. L. Bartholomew, M. El-Matbouli, S. S. Desser, R. H. Devlin, S. W. Feist, R. P. Hedrick, R. W. Hoffmann, J. Khattra, S. L. Hallett, R. J. G. Lester, M. Longshaw, O. Palenzeula, M. E. Siddall, and C. Xiao. 2001. Recent advances in our knowledge of the Myxozoa. The Journal of Eukaryotic Microbiology 48:395–413.

Kent, M. L., L. Margolis, and J. O. Corliss. 1994. The demise of a class of protists: taxonomic and nomenclatural revisions proposed for the protist phylum Myxozoa Grassé, 1970. Canadian Journal of Zoology 72:932–937.

Kent, M. L., and S. C. Dawe. 1994. Efficacy of fumagillin DCH against experimentally induced *Loma salmonae* (Microsporea) infections in chinook salmon *Oncorhynchus tshawytscha*. Diseases of Aquatic Organisms 20:231–233.

Khoo, L. H., P. Gaunt, and M. Mauel. 2012. 2011 Annual Case Summary. Aquatic Research and Diagnostic Laboratory, College of Veterinary Medicine, Mississippi State University. http://tcnwac.msstate.edu//cvm2011.pdf

Killian, H. S. 1994. Proliferative gill disease. University of Arkansas, Division of Agriculture, Cooperative Extension Service, University of Arkansas at Pine Bluff. FSA9073-5M-5-94-S525

Køie, M. 2000. First record of an Actinosporean (Myxozoa) in a marine polychaete annelid. Journal of Parasitology 86:871–872.

Kudo, R. R. 1929. Histozoic Myxosporidia found in freshwater fishes of Illinois, USA. Archiv für Protistenkunde 65:364–378.

Li, Y., H. Sato, Y. Kamata, T. Ohnishi, and Y. Sugita-Konishi. 2012. Three novel myxobolid species of genera *Henneguya* and *Myxobolus* (Myxosporea: Bivalvulida) from marine fish in Japan. Parasitology Research 111:819–826.

Lin, D., L. A. Hanson, and L. M. Pote. 1999. Small subunit ribosomal RNA sequences of *Henneguya exilis* (Class Myxosporea) identifies the actinosporean stage from an oligochaete host. Journal of Eukaryotic Microbiology 46:66–68.

Lom, J., and Dkyová, I. 2006. Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. Folia Parasitologica 53:1–36.

MacMillan, J. R., C. Wilson, and A. Thiyagarajah. 1989. Experimental induction of proliferative gill disease in specific-pathogen-free channel catfish. Journal of Aquatic Animal Health 1:245–254.

Markiw, M. E. 1992. Experimentally induced whirling disease II. Determination of longevity of the infective triactinomyxon stage of *Myxobolus cerebralis* by vital staining. Journal of Aquatic Animal Health 4:44–47.

Markiw, M. E., and K. Wolf. 1983. *Myxosoma cerebralis* (Myxozoa: Myxosporea) etiologic agent of salmonid whirling disease requires tubificid worm (Annelida: Oligochaeta) in its life cycle. Journal of Eukaryotic Microbiology 30:561–564.

McCraren, J. P., M. L. Landolt, G. L. Hoffman, and F. P. Meyer. 1975. Variation in response of channel catfish to *Henneguya* sp. infections (Protozoa: Myxosporidea). Journal of Wildlife Diseases 11:2–7.

Meaders, M. D. and G. L. Hendrickson. 2009. Chronological development of *Ceratomyxa shasta* in the polychaete host *Manayunkia speciosa*. Journal of Parasitology 95:1397–1407.

Minchew, C. D. 1977. Five new specis of *Henneguya* (Protozoa: Myxosporida) from ictalurid fishes. Journal of Protozoology 24:213–220.

Mischke, C. C., J. S. Terhune, and D. J. Wise. 2001. Acute toxicity of several chemicals to the oligochaete *Dero digitata*. Journal of the World Aquaculture Society 32:184–188.

Mischke, C. C., and M. J. Griffin. 2011. Laboratory mass culture of the freshwater oligochaete *Dero digitata*. North American Journal of Aquaculture 73:13–16.

Molnár, K., F. Baska, and C. Székely. 1987. Fumagillin, an efficacious drug against renal sphaerosporosis of the common carp *Cyprinus carpio*. Diseases of Aquatic Organisms 2:187–190.

Morris, D. J., and A. Adams. 2006. Transmission of freshwater myxozoans during the asexual propagation of invertebrate hosts. International Journal for Parasitology 36:371–377.

Palenzuela, O., G. Trobridge, and J. L. Bartholomew. 1999. Development of a polymerase chain reaction diagnostic assay for *Ceratomyxa shasta*, a myxosporean parasite of salmonid fish Diseases of Aquatic Organisms. 36:45–51.

Pote, L. M. W., L. Khoo, and M. Griffin. 2012. *Henneguya ictaluri*. *In* Fish parasites pathobiology and protection, ed. Woo, P.T.K., Buchmann, K., 1st ed., pp.177-192. CAB International, Oxfordshire, United Kingdom.

Pote, L. M., L. A. Hanson, and R. Shivaji. 2000. Small subunit ribosomal RNA sequences link the cause of proliferative gill disease in channel catfish to *Henneguya* n. sp. (Myxozoa: Myxosporea). Journal of Aquatic Animal Health 12:230–240.

Pote, L. M., and P. Waterstrat. 1993. Motile stage of *Aurantiactinomyxon* sp. (Actinosporea: Triactinomyxidae) isolated from *Dero digitata* found in channel catfish ponds during outbreaks of proliferative gill disease. Journal of Aquatic Animal Health 5:213–218.

Prunescu, C., P. Prunescu, Z. Pucek, and J. Lom. 2007. The first finding of myxosporean development from plasmodia to spores in terrestrial mammals: *Soricimyxum fegati* gen. et sp. n. (Myxozoa) from *Sorex araneus* (Soricomorpha). Folia Parasitologica 54:159–164.

Rácz, O., C. S. Székely, and K. Molnár. 2006. The role of copepods (*Cyclops* spp.) in eliminating the actinospore stages of fish-parasitic myxozoans. Acta Veterinaria Hungarica 54:61–70.

Ringuette, M. J., A. Koehler, and S. S. Desser. 2011. Shared antigenicity between the polar filaments of myxosporeans and other Cnidaria. Journal of Parasitology 97:163–166.

Roberts, J. F., C. M. Whipps, J. L. Bartholomew, L. Schneider, and E. R. Jacobson. 2008. *Myxidium scripta* n. sp. identified in urinary and biliary tract of Louisiana-farmed red-eared slider turtles *Trachemys scripta elegans*. Diseases of Aquatic Organisms 80:199–209.

Robinson, E. H., and J. L. Avery. 2000. Farm-raised catfish. Thad Cochran National Warmwater Aquaculture Center Fact Sheet 005.

Siddall, M. E., D. S. Martin, D. Bridge, S. S. Desser, and D. K. Cone. 1995. The demise of a phylum of protists: Phylogeny of Myxozoa and other parasitic cnidaria. Journal of Parasitology 81:961–967.

Smith, D. G. 2001. Pennak's freshwater invertebrates of the United States, 4th ed. Wiley

Smith, C. E., and T. Inslee. 1980. Interlamellar *Henneguya* infestation in adult channel catfish *Ictalurus punctatus* (Rafinesque). Journal of Fish Diseases. 3:257–260.

Stankus, T. 2010. Commodity of the quarter U.S. farm-raised catfish Journal of Agricultural & Food Information 11:176–184.

Steeby, J., D. Wise, T. Byars, and L. Thompson. 2006. Use of smallmouth buffalo (*Ictiobus bubalus*) to reduce the incidence of proliferative gill disease. Thad Cochran National Warmwater Aquaculture Center News 9:3.

Styer, E. L., L. R. Harrison, and G. J. Burtle. 1991. Experimental production of proliferative gill disease in channel catfish exposed to a myxozoan-infected oligochaete, *Dero digitata*. Journal of Aquatic Animal Health 3:288–291.

True, K., M. K. Purcell, and J. S. Foott. 2009. Development and validation of a quantitative PCR to detect *Parvicapsula minibicornis* and comparison to histologically ranked infection of juvenile Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), from the Klamath River, USA. Journal of Fish Diseases 32:183–192.

Tucker, C. S., J. L. Avery, and D. Heikes. 2004. Culture methods. *In* Biology and culture of the channel catfish, ed. Tucker C. S., Hargreaves J. A., Elsevier B.V., Amsterdam, The Netherlands. pp. 166–195

Wagner, E. J. 2002. Whirling disease prevention, control, and management: a review. American Fisheries Society Symposium 29:217–225.

Walsh, H. L., L. R. Iwanowicz, G. W. Glenney, D. D. Iwanowicz, and V. S. Blazer. 2012. Description of two new gill myxozoans from smallmouth (*Micropterus dolomieu*) and largemouth (*Micropterus salmoides*) bass. Journal of Parasitology 98:415–422.

Wellborn, T. L. 1988. Channel catfish life history and biology. Southern Regional Aquaculture Center Publication No. 180.

Whipps, C. M., G. Grossel, R. D. Allard, H. Yokoyama, M. S. Bryant, B. L. Munday, and M. L. Kent. 2004. Phylogeny of the Multivalvulidae (Myxozoa: Myxosporea) based on comparative ribosomal DNA sequence analysis. Journal of Parasitology 90:618–622.

Whitaker D. J., and M. L. Kent. 1991. Myxosporean *Kudoa thyrsites*: a cause of soft flesh in farm-reared Atlantic salmon. Journal of Aquatic Animal Health 3:291–294.

Whitaker J. W., L. M. Pote, and L. A. Hanson. 2005. Assay to detect the actinospore and myxospore stages of proliferative gill disease in oligochaetes and pond water. North American Journal of Aquaculture 67:133–137.

Whitaker J. W., L. M. Pote, L. Khoo, R. Shivaji, and L. A. Hanson. 2001. The use of polymerase chain reaction assay to diagnose proliferative gill disease in channel catfish (*Ictalurus punctatus*). Journal of Veterinary Diagnostic Investigation 13:394–398.

Wise, D. J., A. Camus, T. Schwedler, and J. Terhune. 2004. Health Management. *In* Biology and culture of the channel catfish, ed. Tucker C. S., Hargreaves J. A., pp. 482–488. Elsevier B.V., Amsterdam, The Netherlands.

Wise, D. J., M. J. Griffin, J. S. Terhune, L. M. Pote, and L. H. Khoo. 2008. Induction and evaluation of proliferative gill disease in channel catfish fingerlings. Journal of Aquatic Animal Health 20:236–244.

Wolf, K., and M. E. Markiw. 1984. Biology contravenes taxonomy in the myxozoa: new discoveries show alternation of invertebrate and vertebrate hosts. Science 225:1449–1452.

Wolfe, K., M. E. Markiw, and J. K. Hiltunen. 1986. Salmonid whirling disease: *Tubifex tubifex* (Müller) identified as the essential oligochaete in the protozoan life cycle. Journal of Fish Diseases 9:83–85.

Work, T. M., G. Takata, C. M. Whipps, M. L. Kent. 2008. A new species of *Henneguya* (Myxozoa) in the big-eyed scad (*Selar crumenophthalmus*) from Hawaii. Journal of Parasitology 94:524–529.

CHAPTER II

MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF MYXOZOAN ACTINOSPORE TYPES FROM A COMMERCIAL CATFISH POND IN THE MISSISSIPPI DELTA

2.1 Abstract

The actinospore diversity of infected *Dero digitata* was surveyed (May, 2011) from a channel catfish (*Ictalurus punctatus*) production pond in the Mississippi Delta region for the elucidation of unknown myxozoan life cycles. At present, only 2 myxozoan life cycles have been molecularly confirmed in channel catfish, linking the actinospore stage from an aquatic oligochaete (*D. digitata*) and the myxospore stage from the catfish. In this study *D. digitata* (n=2,592) were isolated from oligochaetes collected from the bottom sediment of a channel catfish production pond. After 1-wk of daily observation, a total of 6 genetically different actinospore types were observed. The collective groups were classified as 2 aurantiactinomyxons, 2 helioactinomyxons, 1 raabeia, and 1 triactinomyxon. Overall prevalence of myxozoan infections in the isolated oligochaetes was 4.4%. Actinospores were photographed and measured for morphological characterization. Four previously undescribed actinospore-types were identified and characterized molecularly and morphologically. Phylogenetic analysis revealed the raabeia and one of the helioactinomyxon (type 1) actinospores were closely related to the group of myxozoans known to parasitize ictalurids in North America. To

date, no myxospores have been linked to the newly sequenced actinospores reported in this survey. The morphological and molecular data generated from this study will assist in the identification of myxospore counterparts for these actinospore stages and aid in the elucidation of unknown myxozoan life cycles in closed production systems.

2.2 Introduction

The Myxozoa are an important group of metazoan spore-forming parasites. Their complex life cycles primarily involve fish (freshwater and marine) and aquatic annelids (oligochaetes and polychaetes) or bryozoans. Some 40 myxozoan species have been described in amphibians, reptiles, waterfowl and more recently mammals, but the most well studied are the >2,000 described myxozoans that cause disease in economically important fish species (Kent et al. 2001; Lom and Dyková 2006; Prunescu et al. 2007; Bartholomew et al. 2008; Roberts et al. 2008). Since the confirmation of the *Myxobolus cerebralis* life cycle (Markiw and Wolf 1983), some 50 myxospore stages in fish have been linked to their corresponding actinospore stage in an annelid, either by experimental infection studies or molecular sequence data (El-Matbouli and Hoffmann 1989; Bartholomew et al. 1997; El-Mansy and Molnár 1997a, 1997b; Yokoyama 1997; Székely et al. 1998; Lin et al. 1999; Székely et al. 1999; Pote et al. 2000; Kallert et al. 2005; Bartholomew et al. 2006; Atkinson and Bartholomew 2009; Caffara et al. 2009).

Surveys of actinospores from aquatic oligochaetes have been conducted in both wild and commercial aquaculture settings (Janiszewska 1955, 1957; Bellerud 1993; McGeorge et al. 1997; El-Mansy et al. 1998a, 1998b; Xiao and Desser 1998a, 1998b, 1998c; Hallett et al. 1999; Negredo and Mulcahy 2001; Oumouna et al. 2003; Székely et al. 2004; Marcucci et al. 2009). Similarly, catfish aquaculture in the southeastern United

States is known to sustain several myxozoan life cycles (Minchew 1977; Current 1979; Bellerud et al. 1995; Griffin et al. 2008). At least 7 *Henneguya* spp. have been described in channel catfish (*Ictalurus punctatus*), the most notable being *Henneguya ictaluri,* the causative agent of proliferative gill disease (PGD) in channel and hybrid catfish (Pote et al. 2000; Bosworth et al. 2003; Griffin et al. 2010). Since it was first reported in 1981, PGD has become the most commonly diagnosed parasitic disease in cultured channel catfish in the southeastern United States (Bowser and Conroy 1985; MacMillan et al. 1989; Burtle et al. 1991; Styer et al. 1991; Wise et al. 2004). True prevalence of this gill pathogen is likely underestimated as the characteristic clinical signs (schooling of fish behind aerators, listless swimming, the hemorrhagic and mottling of the gills resembling raw hamburger) are easily recognized by experienced aquaculturists and the disease can be readily diagnosed pond-side. As a result, infected fish are often not submitted to diagnostic laboratories and many clinical cases go unreported. However, there is evidence that *H. ictaluri* is present in a majority of catfish ponds during the spring of the year, and to a lesser extent in the fall (Wise et al. 2004; Pote et al. 2012).

The oligochaete host in the *H. ictaluri* life cycle is the ubiquitous bottom-dwelling worm *Dero digitata* (Styer et al. 1991; Pote et al. 2000). Common in most catfish production ponds, *D. digitata* is a known host for the aurantiactinomyxon, echinactinomyxon, raabeia, and triactinomyxon collective groups of actinospores, 2 of which have been linked to a myxospore stage in channel catfish (Bellerud 1993; Lin et al. 1999; Pote et al. 2000). Previous surveys of actinospores from catfish aquaculture ponds lack molecular data, but those studies demonstrate *D. digitata* is an important host involved in myxozoan life cycles in these closed production systems (Bellerud 1993;

Bellerud et al. 1995). The objective of this research was to morphologically and molecularly characterize actinospore types released from *D. digitata* in an effort to molecularly confirm unknown myxozoan life cycles within catfish production systems.

2.3 Materials and Methods

2.3.1 Collection of actinospores from *D. digitata*

In May of 2011, benthic sediment samples were collected from a commercial channel catfish pond located in Sunflower County, Mississippi, with a recently confirmed outbreak of proliferative gill disease in the resident catfish population. Sediment samples were dredged from the water/sediment interface of the pond, transported to the laboratory in 5 gallon buckets, covered with pond water and left to settle overnight. Following established protocols (Bellerud 1993; Pote et al. 1994), the mud was washed with reverse osmosis water and initially screened through a 2 mm aperture brass screen, followed by a second wash through a 300 µm aperture brass screen. The filtrate was then washed into a white plastic tray for visualization of oligochaetes. *Dero digitata,* identified by their characteristic serpentine swimming motion and morphology, were isolated, rinsed thoroughly and placed individually into the wells of 96-well plates. Oligochaetes were observed every 24 hr for 1 wk for the release of actinospores (Bellerud 1993; Pote et al. 1994). Once actinospore stages were observed, infected *D. digitata* were placed into sterile 1.5 ml microcentrifuge tubes with 1 ml of nuclease free water and allowed to shed actinospores for 24 hr. A 20 µl suspension of actinospores was placed on a microscope slide, cover slipped, and viewed using an Olympus BX-50 microscope (Olympus, Center Valley, Pennsylvania). Images of actinospores from infected oligochaetes were captured using a Spot Insight QE digital camera and morphological measurements were made

using Spot Basic 3.1 image analysis software (Diagnostic Instruments, Sterling Heights, Michigan). Identification and morphological characterization of actinospores was followed using the guidelines of Lom et al. (1997).

2.3.2 Actinospore DNA extraction

Infected *D. digitata* were removed from the 1.5 ml microcentrifuge tubes and genomic DNA was extracted from the remaining actinospores following the suggested protocol of the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, California). Genomic DNA was re-suspended in 100 µl of PowerSoil® DNA Isolation Kit Solution C6 before being stored at -80ºC.

2.3.3 Amplification of the 18S SSU rRNA gene

Initial amplification of the 18S SSU rRNA gene was performed using universal eukaryotic primers ERIB1 and ERIB10 and subsequent nested polymerase chain reactions (PCR) were carried out using primers designed to amplify the myxozoan 18S rRNA gene for each actinospore isolate. Primers used in the amplification of the 18S rRNA gene were the H2 and H9 primers developed by Hanson et al. (2001), MyxospecF and MyxospecR primers described by Fiala (2006), and combinations of the Genmyxo3, Genmyxo4, and Genmyxo5 primers designed by Griffin et al. (2008). The 25-µl PCR reaction mixtures contained 20 pmol of each primer (Table 2.1) using EconoTaq® Plus Green 2X Master Mix (Lucigen, Madison, Wisconsin). Amplification was carried out using an MJ Research PTC-200 thermal cycler (GMI, Ramsey, Minnesota) with a denaturation step of 95ºC for 10 min, followed by 35 cycles of 95ºC for 1 min, 48ºC for 1 min, 72ºC for 2 min, and the final extension step was at 72ºC for 10 min. For the nested

PCR reactions, 1 µl of PCR product from the initial amplification using the ERIB1 and ERIB10 primers was used with the primer combinations according to Griffin et al. (2008). In the nested PCR reactions all reaction mixtures remained the same, but the annealing temperature was 52ºC. Amplicons were run through a 1.2% agarose gel containing 0.1µg/ml ethidium bromide and visualized under ultraviolet light to ensure the presence of a single appropriate sized band, estimated by direct comparison to a concurrently run molecular weight marker (Hyperladder II, Bioline, London, U.K.).

Table 2.1 Primers used in 18S rRNA genetic analysis of actinospores

Primer	Sequence (5'-3')	Reference
ERIB1	ACCTGGTTGATCCTGCCAG	Barta et al. (1997)
ERIB10	CCTCCGCAGGTTCACCTACGG	Barta et al. (1997)
H2	CGACTTTTACTTCCTCGAAATTGC	Hanson et al. (2001)
H9	TTACCTGGTCCGGACATCAA	Hanson et al. (2001)
Myxospec F	TTCTGCCCTATCAACTWGTTG	Fiala (2006)
Myxospec R	GGTTTCNCDGRGGGMCCAAC	Fiala (2006)
Genmyxo3	TGATTAAGAGGAGCGGTTGG	Griffin et al. (2008)
Genmyxo4	GGATGTTGGTTCCGTATTGG	Griffin et al. (2008)
Genmyxo5	TAAGCGCAGCAACTTTGAGA	Griffin et al. (2008)

2.3.4 Sequencing of the 18S rRNA gene

All PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, California) and primers used for PCR were also used for sequencing. Purified products were sequenced directly or cloned using a plasmid vector (pCR®4Blunt-TOPO®; Zero Blunt® TOPO® PCR cloning kit (Invitrogen, Carlsbad, California) and sequenced according to the manufacturer's protocol. Purified PCR

products were sequenced in both directions and sequencing reactions were carried out using ABI BigDye™ chemistry (Applied Biosystems, Foster City, California), and run on an ABI Prism 3730™ automated sequencer (Applied Biosystems). Contiguous sequences were assembled using the corresponding chromatograms and the SeqMan™ utility of the Lasergene software package (DNAStar, Madison, Wisconsin) and submitted to the NCBI nucleotide database (Accession numbers: KF263537, KF263538, KF263539, KF263540).

2.3.5 Phylogenetic analysis

The obtained DNA sequences were compared with similar published myxozoan sequences deposited in the National Center for Biotechnology Information non-redundant nucleotide database using the blastn suite optimized for highly similar sequences (Altschul et al. 1990). For the phylogenetic analysis, the 20 most closely related published sequences generated by the blastn search of each actinospore sequences of this study were obtained and aligned using the Clustal W application of the MEGA5 (Molecular Evolutionary Genetics Analysis, 5.0) program (Tamura et al. 2011). *Tetracapsuloides bryosalmonae* was chosen as an outlying organism for the phylogenetic analyses.

Phylogenetic analyses performed on the 18S rRNA sequences of the newly sequenced actinospores were conducted using the MEGA5 software (Tamura et al. 2011). Maximum parsimony analysis was performed using the close-neighbor-interchange search level 3, with the random addition of 100 trees (Nei and Kumar 2000). The bootstrap consensus tree for both analyses was inferred from 1,000 replicates (Felsenstein 1985). Minimum evolution analysis was performed using a close-neighbor interchange

search level 3 with the initial tree obtained by the neighbor-joining algorithm using the pairwise deletion option for gaps/missing data (Rzhetsky and Nei 1992; Nei and Kumar 2000).

2.4 Results

A total of 2,592 *D. digitata* were isolated from sediments collected from the bottom mud of a commercial channel catfish pond harboring an outbreak of PGD in the resident population. Actively shedding *D. digitata* (*N*=114; 4.4% overall prevalence) released 6 morphotypes belonging to 4 collective groups of actinospores (Figure 2.1). Two aurantiactinomyxon types, 2 helioactinomyxon types, 1 raabeia type and 1 triactinomyxon type were observed in this survey.

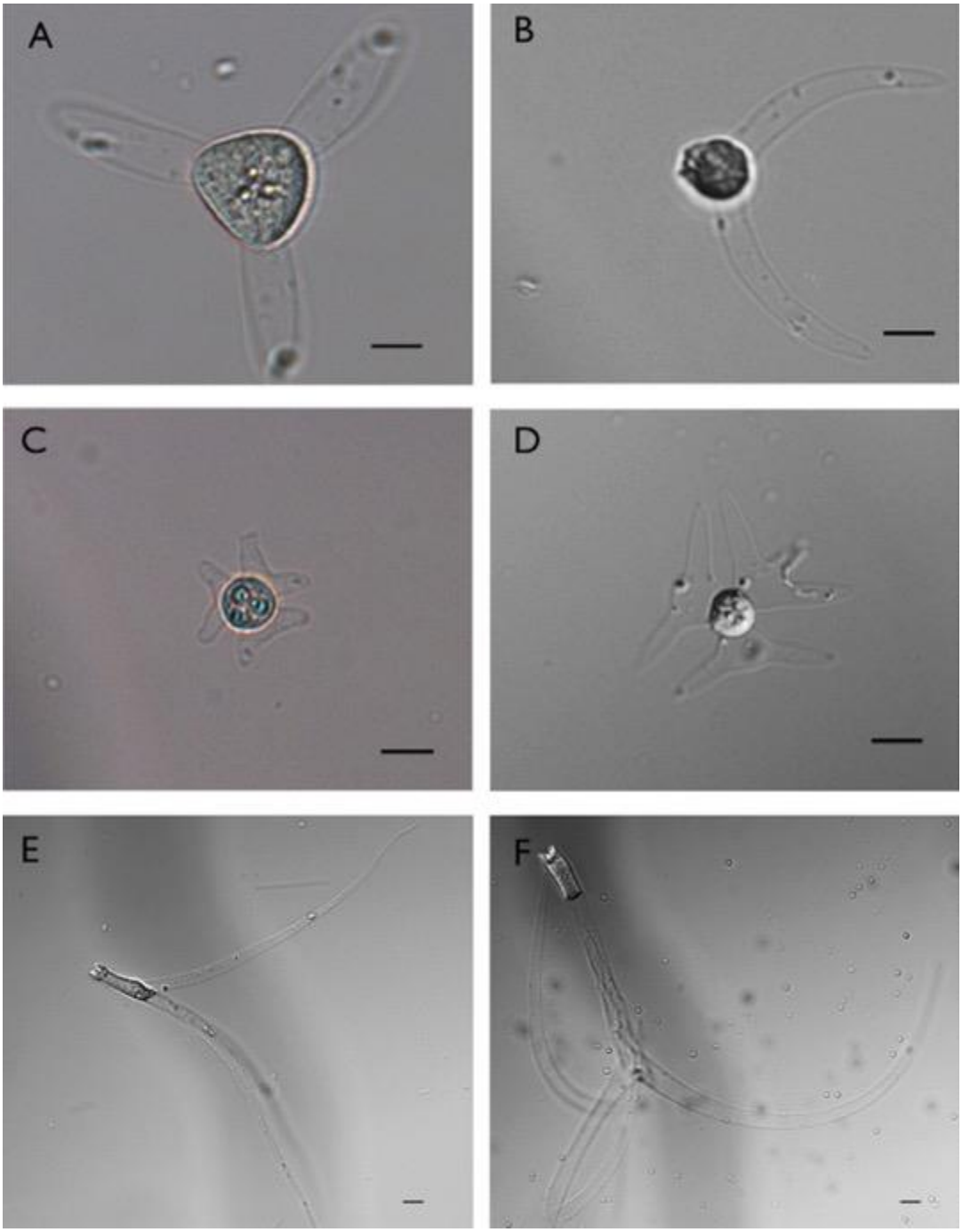

Figure 2.1 Photomicrographs of myxozoan actinospore types from *Dero digitata*

(A) Aurantiactinomyxon type actinospore of *Henneguya ictaluri*. (B) Aurantiactinomyxon type actinospore of *Henneguya exilis*. (C) Helioactinomyxon type 1 actinospore. (D) Helioactinomyxon type 2 actinospore. (E) Raabeia type actinospore. (F) Triactinomyxon type actinospore. Scale bars for A-D represent 10 µm in length. Scale bars for E-F represents 25 µm in length.

2.4.1 Actinospore descriptions

2.4.1.1 Aurantiactinomyxon type 1

Spore body spherical to triangular, diameter 20.87 ± 0.60 µm (range, 20.1–22.3 µm) (Figure 2.1A). Three polar capsules, spherical at apex of spore body. Three caudal processes, length 27.69 ± 0.71 µm (range, 26.3–28.9 µm) and width 10.02 ± 0.40 µm (range, 9.4–10.9 µm). Measurements were obtained from 25 actinospores.

2.4.1.1.1 Taxonomic summary

Host: Dero digitata Mueller, 1773.

Locality: Commercial catfish pond, Sunflower County, Mississippi.

Prevalence of infection: Thirty-five of 2,592 worms (1.35%).

2.4.1.1.1.1 Remarks

The aurantiactinomyxon types were similar to the aurantiactinomyxon type description of Lom and Dyková (2006). Aurantiactinomyxons are characterized by 3 stout, leaf-like caudal processes that are sometimes curved downward, a spore body spherical in shape, and protruding polar capsules (Lom et al. 1997; Lom and Dyková 2006). Partial sequencing of the 18S rRNA gene revealed Aurantiactinomyxon type 1 was a 100% match to *Henneguya ictaluri*, the myxozoan that causes proliferative gill disease, or "hamburger gill" in channel and hybrid catfish.

2.4.1.2 Aurantiactinomyxon type 2

Spore body spherical, diameter 11.74 ± 0.86 µm (range, 10.2–13.3 µm) (Figure 2.1B). Three polar capsules, spherical at apex of spore body. Three caudal processes, elongate and extending in downward curve from spore body, length 42.47 ± 2.47 µm

(range, 37.6–46.2 μm) and width 6.54 ± 0.88 μm (range, 5.2–8.5 μm). Measurements were obtained from 25 actinospores.

2.4.1.2.1 Taxonomic summary

Host: Dero digitata Mueller, 1773.

Locality: Commercial catfish pond, Sunflower County, Mississippi.

Prevalence of infection: Sixty-one of 2,592 worms (2.35%).

2.4.1.2.1.1 Remarks

Actinospores of aurantiactinomyxon type 2 were morphologically similar to those of *Henneguya exilis* reported by Bellerud (1993), but the average caudal process length of the actinospores observed in this study were shorter than previously reported (42.5 versus 52.0). However, the range of both descriptions overlapped across all measured features (Table 2.2). The longer caudal processes of *H. exilis* are uncommon when compared to other aurantiactinomyxon type actinospores reported in the literature (El-Mansy et al. 1998b; Székely et al. 2000; Oumouna et al. 2003; Székely et al. 2003, 2004).

Table 2.2 Comparison of aurantiactinomyxon type actinospores.

Species/type	Host	Caudal Process Length	Caudal Process Width	Caudal Process Span	Spore Body Diameter	Reference
Aurantiactinomyxon type 1 or *Henneguya ictaluri*	*Dero digitata*	28.4	9.9	NA	21.8	Bellerud et al. (1993), Pote et al. (2000)
Aurantiactinomyxon type 2 or *Henneguya exilis*	*Dero digitata*	52.0 (42.5)	5.9 (6.5)	NA (65.5)	11.8 (11.7)	Bellerud (1993), Lin et al. (1999), (This study)
Aurantiactinomyxon type 1	*Branchiura sowerbyi*	51.3	9.5	103.2	18.8	El-Mansy et al. (1998a)
Aurantiactinomyxon type 2	*Limnodrilus*	22.6	11.7	52.2	21.1	El-Mansy et al. (1998a)

Table 2.2 (continued)

Aurantiactinomyxon type 3	*Branchiura sowerbyi*	17.2	3.9	39.5	9.9	El-Mansy et al. (1998a)
Aurantiactinomyxon type 1	*Tubifex tubifex*	17.5	9.9	45.4	18.3	El-Mansy et al. (1998b)
Aurantiactinomyxon type 2	*Branchiura sowerbyi*	65.7	10.5	142.5	22.8	El-Mansy et al. (1998b)
Aurantiactinomyxon type 3	*Branchiura sowerbyi*	70.3	8.0	149.3	22.8	El-Mansy et al. (1998b)
Aurantiactinomyxon type 4	*Branchiura sowerbyi*	55.7	11.2	122	19.4	El-Mansy et al. (1998b)
Aurantiactinomyxon type 5	*Branchiura sowerbyi*	17.2	3.9	39.5	9.9	El-Mansy et al. (1998b)
Aurantiactinomyxon type 6	*Limnodrilus* sp.	24.2	11.2	55.6	19.7	El-Mansy et al. (1998b)
Aurantiactinomyxon type 7	Unidentified	24.4	9.5	58.4	18.9	El-Mansy et al. (1998b)
Aurantiactinomyxon type 8	*Limnodrilus* sp.	12.2	9.0	39.8	22.6	El-Mansy et al. (1998b)
Aurantiactinomyxon type 9	*Branchiura sowerbyi*	51.3	9.5	103.2	18.8	El-Mansy et al. (1998b)
Aurantiactinomyxon type 10	*Branchiura sowerbyi*	16.7	8.8	39.5	15.5	El-Mansy et al. (1998b)
Aurantiactinomyxon type 11	Unidentified	31.9	3.7	46.5	8.5	El-Mansy et al. (1998b)
Aurantiactinomyxon type 12	*Branchiura sowerbyi*	26.5	8.7	59.2	12.1	El-Mansy et al. (1998b)
Aurantiactinomyxon of *Myxobolus intimus*	*Limnodrilus hoffmeisteri*	20.1	10.4	43.6	13.8	Hallett et al. (2006)
Aurantiactinomyxon type 1	Unidentified	26.6	10.1	49.9	12	Hallett et al. (2006)
Aurantiactinomyxon type	Lumbriculid	17.4	7.7	NA	9.7	Marcucci et al. (2009)
Aurantiactinomyxon type	Tubificid	25.6	12	NA	13.7	McGeorge et al. (1997)
Aurantiactinomyxon type 1	*Tubifex ignotus*	21.1	16.1	NA	14.4	Negredo and Mulcahy (2001)
Aurantiactinomyxon type 2	*Limnodrilus hoffmeisteri*	31	10.6	NA	14.1	Negredo and Mulcahy (2001)
Aurantiactinomyxon type 3	*Tubifex ignotus*	20.8	10.4	NA	9.1	Negredo and Mulcahy (2001)
Aurantiactinomyxon of *Aurantiactinomyxon pavinsis*	*Tubifex* sp.	12	NA	NA	10	Oumouna et al. (2003)
Aurantiactinomyxon type 1	Unidentified	76	NA	NA	16	Oumouna et al. (2003)

Table 2.2 (continued)

Aurantiactinomyxon type 1	*Tubifex tubifex*	32	NA	NA	14.4	Özer et al. (2002)
Aurantiactinomyxon type 2	*Tubifex tubifex*	24.7	NA	NA	14.9	Özer et al. (2002)
Aurantiactinomyxon type 3	*Tubifex tubifex*	114.5	NA	NA	21.8	Özer et al. (2002)
Aurantiactinomyxon type 4	*Tubifex tubifex*	28.3	NA	NA	11.9	Özer et al. (2002)
Aurantiactinomyxon of *Thelohanellus nikolskii*	*Tubifex tubifex*	13.4	9.0	40.5	21.1	Székely et al. (1998)
Aurantiactinomyxon type	*Branchiura sowerbyi*	6.1	5.6	17.1	8.1	Székely et al. (2000)
Aurantiactinomyxon type	*Tubfiex tubifex*	12.4	12.4	26.8	13.5	Székely et al. (2003)
Aurantiactinomyxon type 1	*Branchiura sowerbyi*	10.4	15.0	36	19.5	Székely et al. (2004)
Aurantiactinomyxon type 2	*Branchiura sowerbyi*	10.5	15.2	35.8	19.6	Székely et al. (2004)
Aurantiactinomyxon type of *Hoferellus carassii*	*Nais* sp.	48.8	11.7	NA	23.5	Trouillier et al. (1996)
Aurantiactinomyxon type	*Branchiura sowerbyi*	170.8	12.9	NA	19.7	Xi et al. (2013)
Aurantiactinomyxon	*Limnodrilus hoffmeisteri*	24	13-16	NA	12	Xiao & Desser (1998c)
Aurantiactinomyxon of *Thelohanellus hovorkai*	*Branchiura sowerbyi*	29	9.2	65.2	18.6	Yokyama (1997), Székely et al. (1998)

Partial sequencing of the 18S rRNA gene revealed this type shared 100% identity (100% coverage) to *Henneguya exilis* (AF021881) a gill parasite of channel catfish (Lin et al. 1999).

2.4.1.3 Helioactinomyxon type 1

Spore body spherical, diameter 10.07 ± 0.39 µm (range, 9.7–10.7 µm). Three polar capsules spherical, at apex of spore (Figure 2.1C). Caudal processes, 3, joined at side of spore body, bi-lobed, narrows at base, length 6.34 ± 0.58 µm (range, 5.6–8.5 µm).

Span between caudal processes 11.47±1.33 µm (range, 10.0–13.9 µm). Measurements were obtained from 7 actinospores.

2.4.1.3.1 Taxonomic summary

Host: Dero digitata Mueller, 1773.

Locality: Commercial catfish pond, Sunflower County, Mississippi.

Prevalence of infection: Two of 2,592 worms (0.08%).

2.4.1.3.1.1 Remarks

Bellerud (1993) proposed a novel type of actinospore designated as *Helioactinomyxon minutus* isolated from *D. digitata* collected from channel catfish production ponds. The helioactinomyxon actinospore bears similarities to the aurantiactinomyxon type, but is much smaller than other actinospore types described from *D. digitata* in channel catfish ponds. The caudal processes extend from the base of the spore body and are bifurcated at about half their length as compared to the non-bifurcated caudal processes exhibited by aurantiactinomyxon types (Bellerud 1993; Lom and Dyková 2006). No other actinospore types described in the literature fit the helioactinomyxon type described by Bellerud and the types described in this paper, suggesting the inclusion of helioactinomyxon as a new collective group (Janiszewska 1955, 1957; Bellerud 1993; Lom and Dyková 2006).

The contiguous sequence of the 18S rRNA gene obtained for the helioactinomyxon type 1 actinospore was 2,015 bp in length and was not a complete match to any sequence in the NCBI nucleotide database. The helioactinomyxon type 1 actinospore shared a 98% identity (100% coverage) with *Aurantiactinomyxon*

mississippiensis (AF021878), an actinospore released by *D. digitata*, but was not observed in this study, 98% identity (100% coverage) with *H. ictaluri* (AF195510), the myxozoan that causes proliferative gill disease in channel and hybrid catfish, 94% identity (100% coverage) with *H. adiposa* (EU492929) which forms epidermal plasmodia on the adipose fin of channel catfish (Minchew 1977; Griffin et al. 2009b), 94% identity (100% coverage) with *H. exilis* (AF021881), a gill myxozoan of channel catfish (Lin et al. 1999), and 91% identities (98–100% coverage) with *Henneguya pellis* (FJ468488) and *Henneguya sutherlandi* (EF191200), which form epidermal plasmodia in blue (*Ictalurus furcatus*) and channel catfish, respectively (Griffin et al. 2008, 2009a). Minimum evolution and maximum parsimony analysis were in agreement with the placement of helioactinomyxon type 1 within the clade of *Henneguya* species known to parasitize ictalurid fish and was well supported by bootstrap values (100% bootstrap support for both maximum parsimony and minimum evolution (Figure 2.2 and Figure 2.3).

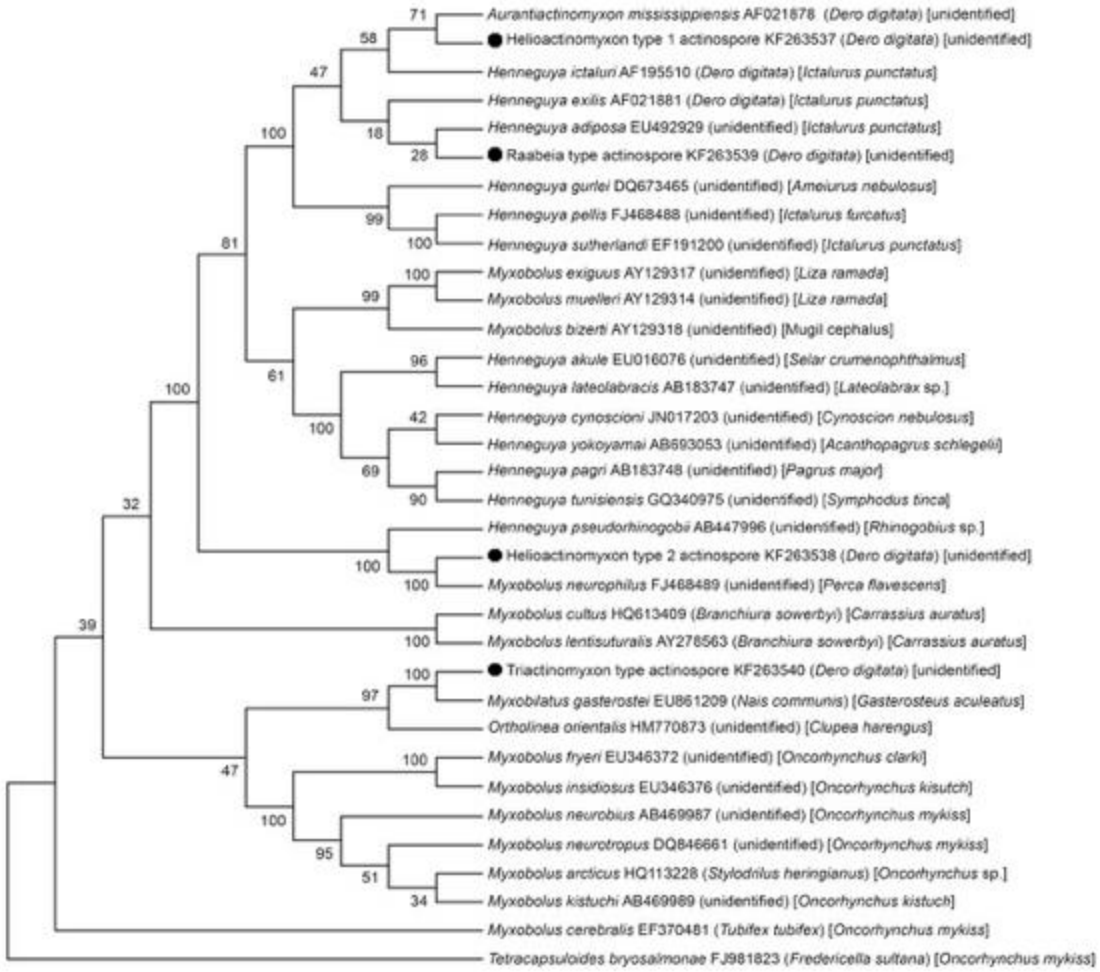

Figure 2.2 Maximum parsimony analysis of the actinospore 18S SSU rRNA gene sequences.

Maximum parsimony tree of the actinospore 18S SSU rRNA gene sequences and the 30 most relevant myxozoan sequences (oligochaete host) [fish host] obtained by a blast search of the NCBI non-redundant nucleotide (nr/nt) database and rooted at *Tetracapsuloides bryosalmonae*. Numbers at the nodes represent bootstrap confidence values (n=1,000 replicates). Circles indicate the undescribed myxozoans in this paper.

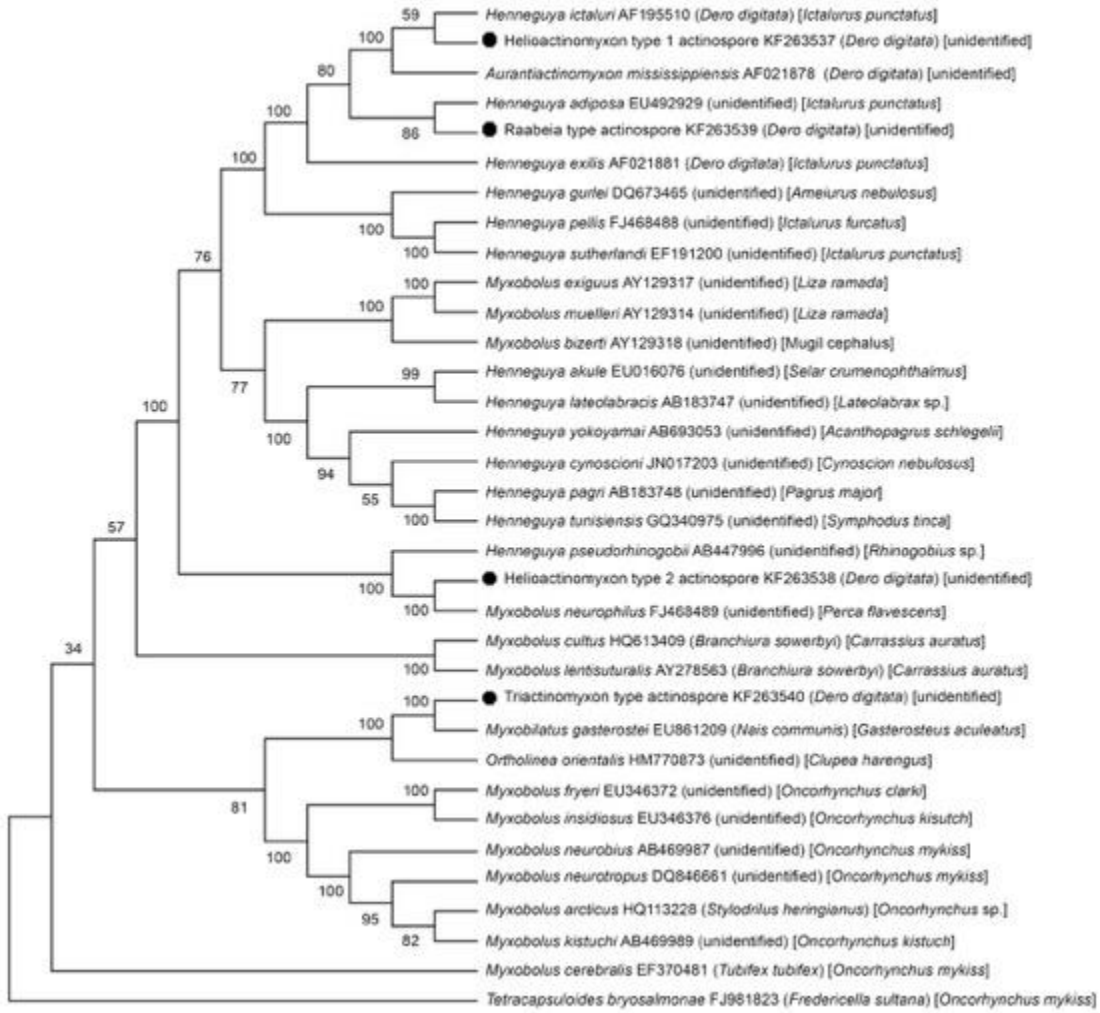

Figure 2.3 Minimum evolution analysis of the actinospore 18S SSU rRNA gene sequences.

Minimum evolution tree of the actinospore 18S SSU rRNA gene sequences and the 30 most relevant myxozoan sequences (oligochaete host) [fish host] obtained by a blast search of the NCBI nonredundant nucleotide (nr/nt) database and rooted at *Tetracapsuloides bryosalmonae*. Numbers at the nodes represent bootstrap confidence values (n=1,000 replicates). Circles indicate the undescribed myxozoans in this paper.

2.4.1.4 Helioactinomyxon type 2

Spore body spherical, diameter 8.93 ± 0.64 µm (range, 7.4–10.0 µm) (Figure 2.1D). Polar capsules, 3, pyriform at apex of spore body. Three caudal processes, broad,

bilobed, extending from base of spore body, length 22.43 ± 1.69 µm (range, 19.3–24.7 µm). Span between caudal processes 14.34 ± 1.91 µm (range, 10.3–17.7 µm). Measurements were obtained from 15 actinospores.

2.4.1.4.1 Taxonomic summary

Host: Dero digitata Mueller, 1773.

Locality: Commercial catfish pond, Sunflower County, Mississippi.

Prevalence of infection: Eight of 2,592 worms (0.31%).

2.4.1.4.1.1 Remarks

The contiguous sequence of the 18S rRNA gene obtained for the helioactinomyxon type 2 actinospore was 2,101 bp in length. The helioactinomyxon type 2 actinospore had a 91% identity (79% coverage) with *Myxobolus neurophilus* from the brain of yellow perch *Perca flavescens* from the United States (FJ468489), 94% identity (82% coverage) with 3 isolates of *Henneguya pseudorhinogobii* (AB447994, AB447995, AB447996) a gill myxozoan of freshwater goby *Rhinogobius* sp. from Japan (Kageyama et al. 2009), 89% identity (89% coverage) with *Henneguya tunisiensis* (GQ340975) found in the gill-arches of East Atlantic peacock wrasse *Symphodus tinca* off the Kerkennah Islands, Tunisia (Bahri et al. 2010) and *Henneguya cynoscioni* (JN017203) that is associated with lesions in the bulbus arteriosus of spotted seatrout *Cynoscion nebulosus* (Dyková et al. 2011). Phylogenetic placement of the helioactinomyxon type 2 actinospore within clade containing *Myxobolus neurophilus* and *Henneguya pseudorhinogobii* was well supported (100% bootstrap value for both maximum parsimony analysis and minimum evolution analysis).

2.4.1.5 Raabeia type

Spore body elongate, cylindrical, length 28.2 ± 3.13 µm (24.1–33.7 µm) and width 6.44 ± 0.52 µm (range, 5.7–7.4 µm) (Figure 2.1E). Three polar capsules, pyriform, protruding from apex of spore body. Caudal processes, 3, joined at base of spore body and downward curving before curving upward to tapered ends, length 150.65 ± 19.46 µm (range, 117.0–171.2 µm) and width 7.3 ± 0.83 µm (6.1–8.9 µm). Measurements were obtained from 9 actinospores.

2.4.1.5.1 Taxonomic summary

Host: Dero digitata Mueller, 1773.

Locality: Commercial catfish pond, Sunflower County, Mississippi.

Prevalence of infection: Four of 2,592 worms (0.15%).

2.4.1.5.1.1 Remarks

Raabeia type actinospores are described as having an elliptical spore body usually containing 3 protruding polar capsules at the apical portion. Caudal processes are jointed at the base of the spore body and are often long, tapered, and curved in an upward direction. Raabeia actinospores are differentiated from triactinomyxon type actinospores based on the absence of a style (Janiszewska 1957; Lom and Dyková 2006).

The raabeia type actinospore observed in this study was not consistent with the morphology of the raabeia type (*Raabeia noxubeensis*) reported by Bellerud (1993) from *Amphichaeta* sp. isolated from channel catfish ponds (Table 2.3). This raabeia type possessed longer caudal processes (150.7 versus 53.9) and the spore body width was narrower (6.4 versus 11.8). The closest morphological match to the raabeia type

observed in this study is the raabeia type 4 of Özer et al. (2002) from *Tubifex tubifex* isolated from a salmon farm in Northern Scotland. They share similar caudal process lengths (150.7 and 142.7) and spore body lengths (28.2 and 29.6), but the spore body width of the type described in this paper is much narrower (6.44 versus 16.5). The raabeia type actinospore belonging to the myxozoan parasite *Myxobolus cultus*, as reported by Xi et al. (2013) from a freshwater pond in China, also has overlapping morphological features with the raabeia type of this study, but the caudal process length of *M. cultus* is greatly extended (150.7 versus 250.8).

Table 2.3 Comparison of raabeia type actinospores.

Species/type	Host	Caudal Process Length	Caudal Process Width	Caudal Process Span	Spore Body Length	Spore Body Width	Reference
Raabeia type	*Dero digitata*	150.7	7.3	248.5	28.2	6.44	This study
Raabiea type of *Raabeia noxubeensis*	*Amphicheta* sp.	53.9	9.1	NA	27.5	11.8	Bellerud (1993)
Raabeia type of *Myxobolus lentisuturalis*	*Branchiura sowerbyi*	196	NA	NA	22.1	10.8	Caffara et al. (2009)
Raabeia type 1	*Limnodrilus* sp.	202.8	8.2	NA	14.1	12.4	El-Mansy et al. (1998a)
Raabeia type 2	*Tubifex* sp.	209.4	6.6	NA	21.7	7.7	El-Mansy et al. (1998a)
Raabeia type 1	*Branchiura* sp. and *Tubifex* sp.	294	9	NA	25.9	11.8	El-Mansy et al. (1998b)
Raabeia type 2	*Branchiura* sp.	202.8	8.2	NA	14.1	12.4	El-Mansy et al. (1998b)
Raabeia type 3	*Tubifex* sp.	183.6	10.6	NA	28.2	14.1	El-Mansy et al. (1998b)
Raabeia type 4	Unidentified	209.4	6.6	NA	21.7	7.7	El-Mansy et al. (1998b)
Raabeia type 1	Unidentified	213.2	11.2	NA	27.2	16.8	Hallett et al. (2006)
Raabeia type 2	Unidentified	120.7	7.7	NA	22	14.2	Hallett et al. (2006)

Table 2.3 (continued)

Raabeia type	*Uncinais uncinata*	34.6	NA	NA	11.2	4.9	Koprivnikar and Desser (2002)
Raabeia type	Unidentified	219	10	NA	18.2	12.8	McGeorge et al. (1997)
Raabeia type 1	*Tubfiex* sp.	245	NA	NA	35	12	Oumouna et al. (2003)
Raabeia type 2	Unidentified	80	NA	NA	18	15	Oumouna et al. (2003)
Raabeia type 1	Unidentified	94.5	10	NA	18.1	15.7	Özer et al. (2002)
Raabeia type 2	*Lumbriculus variegatus*	85.6	NA	NA	18.1	16.1	Özer et al. (2002)
Raabeia type 3	*Tubifex tubifex*	228.3	NA	NA	33.9	12.8	Özer et al. (2002)
Raabeia type 4	*Tubifex tubifex*	142.7	NA	NA	29.6	16.5	Özer et al. (2002)
Raabeia type 5	*Lumbriculus variegatus*	133.3	NA	NA	23.7	20.2	Özer et al. (2002)
Raabeia type 6	*Tubifex tubifex*	164.8	NA	NA	29.8	17.4	Özer et al. (2002)
Raabeia type of *Myxobolus cultus*	*Branchiura sowerbyi*	250.8	6.7	NA	24.8	8.2	Xi et al. (2013)
Raabeia 'A'	*Limnodrilus hoffmeisteri*	145	8-9	NA	16	10	Xiao & Desser (1998b)
Raabeia 'B'	*Limnodrilus hoffmeisteri*	230	14	NA	25.5	9	Xiao & Desser (1998b)
Raabeia 'C'	*Limnodrilus hoffmeisteri*	210	10-12	NA	16.5	9	Xiao & Desser (1998b)
Raabeia 'D'	*Tubifex tubifex*	290	11-13	NA	21.5	9.5	Xiao & Desser (1998b)
Raabeia 'E'	*Tubifex tubifex*	215	9-11	NA	24	11	Xiao & Desser (1998b)
Raabeia 'F'	*Limnodrilus hoffmeisteri*	145	6-7	NA	16.5	8.5	Xiao & Desser (1998b)

The contiguous sequence of the 18S rRNA gene obtained for the raabeia type actinospore was 2,081 bp in length and was not a complete match to any sequence in the NCBI nucleotide database. The raabeia type actinospore showed 96% identity (100% coverage) with *H. ictaluri* (AF195510) from the gills of experimentally infected channel

catfish (Pote et al. 2000), 95% identity (100% coverage) with *A. mississippiensis* (AF021878), an aurantiactinomyxon type actinospore that was not observed in this study, but is a known parasite of *D. digitata* (Hanson et al. 2001), 96% identity (97% coverage) with *H. adiposa* (EU492929) from the adipose fin of channel catfish, 95% identity (100% coverage) with *H. exilis* (AF021881) a gill myxozoan of channel catfish, and 92% identities (97% coverage) with *H. pellis* (FJ468488) and *H. sutherlandi* (EF191200), skin myxozoans of blue (*Ictalurus furcatus*) and channel catfish, respectively. Minimum evolution and maximum parsimony analysis placed the raabeia type actinospore among the group of *Henneguya* species that infect ictalurid fish and was well supported (100% bootstrap support for both minimum evolution and maximum parsimony).

2.4.1.6 Triactinomyxon type

Spore body elongate, length 19.85 ± 2.32 µm (range, 16.4–22.9 µm) and width 9.66 ± 0.92 µm (range, 9.6–10.8 µm) (Figure 2.1F). Three polar capsules protruding from apex of spore body. Style length 84.55 ± 4.38 µm (range, 78.2–88.9 µm) and width 8.98 ± 0.87 µm (range, 7.6–10.1 µm) with irregular placement of valve cell nuclei. Three caudal processes, 3, (see previous query) curving upward and tapered at ends, length 201.93 ± 3.96 (range, 196.0–206.7 µm). Measurements were obtained from 6 actinospores.

2.4.1.6.1 Taxonomic summary

Host: Dero digitata Mueller, 1773.

Locality: Commercial catfish pond, Sunflower County, Mississippi.

Prevalence of infection: Four of 2,592 worms (0.15%).

2.4.1.6.1.1 Remarks

Triactinomyxon type actinospores are commonly characterized by an elongated spore body that forms the style which splits into 3 slightly upward curving caudal processes that taper to sharp tips (Janiszewska 1955, 1957; Lom and Dyková 2006). Similar to the type described by Székely et al. (2007), the valve cell nuclei of the style are irregularly positioned.

The triactinomyxon type actinospore observed in this study was morphologically distinct across multiple features from the 2 triactinomyxon types from *D. digitata* described by Bellerud (1993) (Table 2.4). The closest morphological match to the triactinomyxon of this study was the triactinomyxon actinospore of *Myxobolus hungaricus*, a gill parasite of common bream *Abramis brama* (El-Mansy and Molnár 1997a). The actinospore of *M. hungaricus* is released from 2 oligochaetes, *Tubifex tubifex* and *Limnodrilus hoffmeisteri*. The triactinomyxon actinospore described in this paper has a shorter spore body than the actinospore of *M. hungaricus* (19.9 versus 38.9).

Table 2.4 Comparison of triactinomyxon type actinospores

Species/type	Host	Caudal Process Length	Caudal Process Width	Spore Body Length	Spore Body Width	Style Length	Style Width	Reference
Triactinomyxon type	*Dero digitata*	201.93	NA	19.85	9.66	84.55	8.98	This study
Triactinomyxon of *Triactinomyxon brevis*	*Dero digitata*	127.3	8.6	33	10	NA	NA	Bellerud (1993)
Triactinomyxon of *Triactinomyxon* marquesi	*Amphicheta* sp.	274.1	14.3	28.9	10.3	126.08	NA	Bellerud (1993)
Triactinomyxon of *Triactinomyxon funiformes*	*Dero digitata*	343.3	20.3	49.3	19	66.3	NA	Bellerud (1993)
Triactinomyxon of *Myxobolus hungaricus*	*Tubifex tubifex* and *Limnodrilus hoffmeisteri*	196.7	NA	38.9	9.5	80.8	6	El-Mansy & Molnár (1997a)

Table 2.4 (continued)

Triactinomyxon of *Myxobolus drjagini*	*Tubifex tubifex*	126	10	32	10.5	66	10	El-Mansy and Molnár (1997b)
Triactinomyxon type 1	*Tubifex* or *Limnodrilus*	230	18.8	50.6	12.9	123.6	21.2	El-Mansy et al. (1998a)
Triactinomyxon type 2	*Tubifex* or *Limnodrilus*	152.2	17.6	25.4	10.6	117.7	15.3	El-Mansy et al. (1998a)
Triactinomyxon type 3	*Tubifex*	224.6	17.5	44.7	11.8	87.1	20	El-Mansy et al. (1998a)
Triactinomyxon type 4	*Limnodrilus*	281.7	20.8	45	12.9	149	23.5	El-Mansy et al. (1998a)
Triactinomyxon type 5	*Tubifex or Limnodrilus*	*249*	16.1	37.7	13.5	90.6	12.9	El-Mansy et al. (1998a)
Triactinomyxon type 1	*Stylaria* sp. and *Tubifex* sp.	128	10.6	36.6	10.6	102	16.5	El-Mansy et al. (1998b)
Triactinomyxon type 2	Unidentified	NA	NA	101.2	14.1	ND	ND	El-Mansy et al. (1998b)
Triactinomyxon type 3	*Nais* sp. and *Tubifex* sp.	127.5	14.5	47.1	10.6	150	10.6	El-Mansy et al. (1998b)
Triactinomyxon type 4	*Limnodrilus* sp.	173.4	14.3	41.2	8.8	137.7	20.0	El-Mansy et al. (1998b)
Triactinomxyon	*Tubifex tubifex*	2 longer: 193.1 1 shorter: 115.4	2 longer: 12.7 1 shorter: 12.7	25.6	10	112	19.2	Hallett et al. (2004)
Triactinomyxon of *Henneguya nuesslini*	*Tubifex tubifex*	227.5	12.9	34.6	11.1	138.4	17.24	Kallert et al. (2005)
Triactinomyxon type 1	Tubificid	96	NA	24	13	41	10	Lowers & Bartholomew (2003)
Triactinomyxon type 2	Tubificid	188	NA	29	13	162	16	Lowers & Bartholomew (2003)
Triactinomyxon type 3	Tubificid	270	NA	36	12	192	16	Lowers & Bartholomew (2003)
Triactinomyxon type 4	Tubificid	221	NA	56	9	103	15	Lowers & Bartholomew (2003)
Triactinomyxon type 5	Tubificid	123	NA	19	8	94	11	Lowers & Bartholomew (2003)
Triactinomyxon type 6	Tubificid	183	NA	29	15	92	14	Lowers & Bartholomew (2003)
Triactinomyxon type 7	Tubificid	200	NA	35	13	129	16	Lowers & Bartholomew (2003)
Triactinomyxon type	Unidentified	129	NA	52	12.4	130	25	McGeorge et al. (1997)
Triactinomyxon type 1	*Tubifex* sp.	160	NA	NA	NA	170	NA	Oumouna et al. (2003)
Triactinomyxon type 2	*Tubifex* sp.	75	NA	NA	NA	67	NA	Oumouna et al. (2003)

Table 2.4 (continued)

Triactinomyxon type 3	Unidentified	175	NA	NA	NA	80	NA	Oumouna et al. (2003)
Triactinomyxon type 4	*Tubifex* sp.	202	NA	NA	NA	155	NA	Oumouna et al. (2003)
Triactinomyxon type	Tubificid	161.1	NA	47.6	15.2	136.5	NA	Özer et al. (2002)
Triactinomyxon of *Myxobolus pseudodispar*	*Tubifex tubifex*	2 longer: 196.6 1 shorter: 127.2	13.6	50.4	15.8	157.3	15.8	Székely et al. (1999)
Triactinomyxon type 1	*Rhyacodrilus komarovi*	178	10	35	12.5	125	NA	Székely et al. (2002)
Triactinomyxon type 2	*Rhyacodrilus komarovi*	187	13.8	62	9.1	125	NA	Székely et al. (2002)
Triactinomyxon Syrian type	*Psammoryctides albicola*	120	NA	30	NA	130	NA	Székely et al. (2007)
Triactinomyxon 'A'	*Limnodrilus hoffmeisteri*	370	21	55	11-13	NA	NA	Xiao & Desser (1998b)
Triactinomyxon 'B'	*Limnodrilus hoffmeisteri*	205	20	23	18	NA	NA	Xiao & Desser (1998b)
Triactinomyxon 'C'	*Limnodrilus hoffmeisteri*	290	25	18	10-13	NA	NA	Xiao & Desser (1998b)
Triactinomyxon 'D'	*Limnodrilus hoffmeisteri*	110	NA	NA	NA	NA	NA	Xiao & Desser (1998b)
Triactinomyxon 'E'	*Limnodrilus hoffmeisteri* and *Tubifex tubifex*	285	32	50	16	NA	NA	Xiao & Desser (1998b)
Triactinomyxon 'F'	*Limnodrilus hoffmeisteri* and *Rhyacodrilus coccineus*	180	10-11	50	6.4	NA	NA	Xiao & Desser (1998b)
Triactinomyxon dubium	*Tubifex tubifex*	260	27	31	19	NA	NA	Xiao & Desser (1998b)
Triactinomyxon ignotum	*Tubifex* sp.	110	11	22	14	NA	NA	Xiao & Desser (1998b)

The contiguous sequence of the 18S rRNA gene obtained for the triactinomyxon type actinospore was 2,073 bp in length and was not a complete match to any sequence in NCBI nucleotide database. The triactinomyxon type actinospore shared greater than 97% identity (60—92% coverage) with 4 isolates of *Myxobilatus gasterostei* (EU861209,

EU861210, AY495703, AJ582063) found in the urinary system of three-spined sticklebacks *Gasterosteus aculeatus* (Atkinson and Bartholomew 2009) and 95% identity (82% coverage) with 5 isolates of *Ortholinea orientalis* (HM770873, HM770872, HM770871, HM770875, HM770874) in the ureters of herring *Clupea harengus* and sprat *Sprattus sprattus* (Karlsbakk and Køie 2011). The phylogenetic placement of the triactinomyxon type actinospore was within the clade containing the renal myxozoan parasites *Myxobilatus gasterostei* and *Ortholinea orientlis* (97% bootstrap support for maximum parsimony; 100% bootstrap support for minimum evolution).

2.5 Discussion

This current study demonstrated *D. digitata* to be host to at least 6 genetically distinct myxozoan parasites, consisting of the following collective groups: aurantiactinomyxon, helioactinomyxon, raabeia, and triactinomyxon. Two of the actinospores observed in this study, aurantiactinomyxon type 1 and aurantiactinomyxon type 2, have corresponding myxospore stages that have been described from channel catfish *I. punctatus* (*H. ictaluri* and *H. exilis,* respectively). The 4 remaining actinospore types observed here have not been linked with any corresponding myxospore stage in a fish. As such, little is known of their pathology in the fish host or impacts on production.

The actinospore prevalence in oligochaetes sampled from intensively managed aquaculture systems appears to be higher than in natural waters, which can be expected. In catfish production systems, there is a close association of the oligochaete and fish hosts. In addition, catfish production ponds are often managed as multi-batch systems, are rarely clean harvested between production cycles, and often harbor contaminating wild fish species. As a result, holdover fish from the previous production cycle can serve

as a reservoir of infection for the oligochaete populations in these earthen ponds. This, along with the close proximity in which the fish and oligochaete hosts are held in these closed systems, provides an optimal environment for the propagation of myxozoan life cycles (Wise et al. 2004; Pote et al. 2012). In this current study, collected *D. digitata* were only observed for a 1 wk period. It is likely that several oligochaetes that were found negative were actually infected, but not yet releasing actinospores. In addition, oligochaetes were only sampled over a 1 mo period. It is likely other actinospore stages may be observed if sampling continued over the course of an entire year, although previous research has found the presence of actively shedding oligochaetes in catfish ponds to be highest in the spring (Bellerud 1993; Bellerud et al. 1995).

The overall infection prevalence of *D. digitata* actively releasing actinospores over the 1 wk observational period was 4.4%. This was in accordance with values observed in other surveys of actinosporean-infected oligochaetes. In a previous account of the *D. digitata* populations of 8 channel catfish farms in Mississippi, the prevalence of actinospore releasing *D. digitata* varied between 0.6–21.7% (Bellerud 1993). Comparatively, prevalence rates of the actinospore types from oligochaetes isolated from an inflow brook of a salmon hatchery system in Japan varied among the different types of actinospores released and were between 0.7–7% (Székely et al. 2002). Other farm-based surveys report prevalence rates of less than 1%, but these rates may be underestimated in studies where oligochaetes are collected at a single time point, rather than year round or seasonally (McGeorge et al. 1997; Oumouna et al. 2003; Eszterbauer et al. 2006). In surveys of oligochaetes from natural aquatic systems the prevalence levels are often low (<1%) (Xiao and Desser 1998b). Conversely, El-Mansy et al. (1998a) reported an

infection prevalence of greater than 30% for a triactinomyxon from *Tubifex tubifex* and attributed their higher prevalence as a result of the extended length of time the same population of oligochaetes were sampled.

Historically, actinospore surveys have focused on morphology and host species, but descriptions including 18S rRNA gene sequences allow a more rapid identification of the corresponding myxospores stage (Székely et al. 2005; Marcucci et al. 2009). Approximately 200 different types of actinospores have been reported in the literature worldwide, but many surveys report only morphological characteristics and most are deficient in molecular data and phylogenetic inferences that would provide a more reliable identification (Janiszewska 1955, 1957; Bellerud 1993; McGeorge et al. 1997; El-Mansy et al. 1998a, 1998b; Xiao and Desser 1998a, 1998b, 1998c; Hallett et al. 1999; Negredo and Mulcahy 2001; Oumouna et al. 2003; Székely et al. 2004; Lom and Dyková 2006; Atkinson and Bartholomew 2009; Marcucci et al. 2009). As a result, numerous researchers have suggested morphological descriptions of actinospore or myxospore isolates now be accompanied by 18S rRNA gene sequence data, especially in situations where actinospores or myxospores of a single type have overlapping or similar morphological features. Hallett et al. (2002) first reported 2 aurantiactinomyxon type actinospores that were genetically identical, but differed phenotypically in spore shape and process length. Additionally, others have reported similar findings of a single actinospore or myxospore genotype having multiple phenotypes, suggesting morphological characterization alone could result in taxonomic redundancy and any new description or redescription of either actinospore or myxospore stage should include 18S

rRNA sequences (Hallett et al. 2004; Eszterbauer et al. 2006; Atkinson and Bartholomew 2009; Urawa et al. 2011).

The now common use of DNA sequencing of the 18S rRNA gene in identifying actinospore and myxospore types has not only provided a more precise means of identification, but also allows researchers to compare and infer relationships between and among this diverse group of organisms (Hallett et al. 2002, 2004; Holzer et al. 2004; Eszterbauer et al. 2006; Fiala 2006; Karlsbakk and Køie 2011; Urawa et al. 2011). Additionally, elucidation of numerous myxozoan life cycles has been greatly aided by the comparison of 18S rRNA gene sequences of the actinospore and myxospore stage in their respective host (Lin et al. 1999; Pote et al. 2000; Fiala 2006). Four of the 18S rRNA sequences generated in this study were novel sequences and have been submitted to NCBI nucleotide database in hopes that identification of their corresponding myxospore stages may be identified through 18S rRNA sequence comparisons.

In a detailed phylogenetic study of novel *Myxobolus* and *Henneguya* myxosporeans, Carriero et al. (2013) demonstrated the evolutionary placement of myxozoans was largely based on fish host. The phylogenetic placement of both the helioactinomyxon type 1 and raabeia type actinospores within the group of *Henneguya* known to infect ictalurid fish in North America suggests these 2 actinospore types likely infect an ictalurid host, although this has yet to be experimentally confirmed (Fig 1 and Fig 2). To date no myxospore stage in an ictalurid fish has been molecularly linked to the helioactinomyxon type 1 or raabeia type actinospore described in this paper.

Interestingly, both helioactinomyxon type 2 and triactinomyxon type actinospores are placed outside the group of myxozoans that infect ictalurid fish. Triactinomyxon type

actinospore grouped well within the group of myxozoans that make up the urinary bladder clade described by Fiala (2006). The urinary bladder clade is a relatively small group within the larger freshwater *Myxobolus* clade. The closest genetic match to the triactinomyxon type actinospore was *Myxobilatus gasterostei,* which parasitizes the urinary system of the three-spined sticklebacks *Gasterosteus aculeatus* and has been experimentally demonstrated to have a triactinomyxon actinospore involved in its life cycle (Atkinson and Bartholomew 2009). As the corresponding myxospore stage associated with the triactinomyxon type actinospore is unknown, it is unclear if this organism parasitizes the urinary system of its corresponding vertebrate host. Phylogenetic grouping of myxozoans by site of infection has also been documented (Andree et al. 1999; Eszterbauer 2004; Holzer et al. 2004; Fiala 2006). In the description of *Ortholinea orientalis*, a myxozoan that parasitizes the ureters of *Clupea harengus* and *Sprattus sprattus* from Denmark, Karlsbakk and Køie (2011) observed the grouping of *O. orientalis* among the myxozoans that constitute the urinary bladder clade.

Both maximum parsimony analysis and minimum evolution analysis placed the helioactinomyxon type 2 actinospore with *Myxobolus neurophilus* found in the brain of the yellow perch *Perca flavescens* (Khoo et al. 2010). At this time the helioactinomyxon type 2 actinospore has not yet been linked to any myxospore stage in a fish host.

The helioactinomyxon type actinospores described in this survey match no descriptions of any previously identified actinospore types (Lom et al. 1997; Lom and Dyková 2006). Bellerud (1993) first described *Helioactinomyxon minutus* as an actinospore released from *D. digitata* collected from commercial channel catfish ponds in Mississippi. The actinospores of both the helioactinomyxon type 1 (*H. minutus*) and

helioactinomyxon type 2 in this study both resemble aurantiactinomyxon types, but are considerably smaller and possess bilobed caudal processes, a feature that has not been reported among the described actinospore types (Janiszewska 1955, 1957; Lom et al. 1997; Lom and Dyková 2006). Hexactinomyxon type actinospores, as described by Hallett et al. (2003) bare similar bifurcations, but these are actually 6 caudal processes that arise from the division of the 3 valve cells that form the style, which is lacking in the helioactinomyxon types described here and by Bellerud (1993). Based on these findings, we propose the adoption of helioactinomyxon as a novel actinospore morphotype.

It is important to consider other fish species that are found in commercial channel catfish ponds as a potential intermediate host in the life cycle of these myxozoans. Although given the close association with these parasites and the target production species, several other fish species are often present in catfish production and are just as likely to serve as a fish host in these life cycles as the cultured ictalurids. A wide array of fish species are often associated with catfish production, including, but not limited to, mosquitofish (*Gambusia affinis*), grass carp (*Ctenopharyngodon idella*), silver carp (*Hypothalmichthys molotrix*), bighead carp *(Aristichthys nobilis*), bigmouth buffalo *(Ictiobus cyprinellus*), smallmouth buffalo *(Ictiobus bubalus*), threadfin shad *(Dorosoma petenense*), and fathead minnows *(Pimephales promelas*) (Tucker et al. 2004; Mischke et al. 2012, 2013). It is possible several of the actinospore stages identified in this study do not cycle through channel or hybrid catfish, but utilize one of the many incidental species present in catfish ponds, which are considerably understudied. Future research will focus on these alternative fish species, as well as other vertebrate species that may play a role in the propagation of myxozoan life cycles in catfish aquaculture.

This survey is the first account of actinospores from *D. digitata* collected from a channel catfish aquaculture system using both morphology and sequencing of the 18S rRNA gene. Other than *H. ictaluri*, the impact these actinospore types may have on catfish production remain unclear. Investigations of other resident fish populations routinely found in catfish ponds are necessary to determine if any unidentified myxospore stages could be associated with the actinospores reported in this survey and the potential impact this could have on catfish health and production.

2.6 References

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403–410.

Andree, K. B., C. Székely, K. Molnár, S. J. Gresoviac, and R. P. Hedrick. 1999. Relationships among members of the genus *Myxobolus* (Myxozoa: Bivalvulidae) based on small subunit ribosomal DNA sequences. Journal of Parasitology 85:68–74.

Atkinson, S. D., and J. L. Bartholomew. 2009. Alternate spore stages of *Myxobilatus gasterostei*, a myxosporean parasite of three-spined sticklebacks (*Gasterosteus aculeatus*) and oligochaetes (*Nais communis*). Parasitology Research 104:1173–1181.

Bahri, S., S. Marton, A. Marques, and E. Eszterbauer. 2010. *Henneguya tunisiensis* n. sp. (Myxosporea: Bivalvulida), a new gill parasite of *Symphodus tinca* (L.) (Teleostei: Labridae) off Tunisia. Systematic Parasitology 76:93–101.

Barta, J. R., D. S. Marin, P. A. Liberator, M. Dashkevicz, J. W. Anderson, S. D. Feigner, A. Elbrecht, A. Perkins-Barrow, M. C. Jenkins, H. D. Danforth, M. D. Ruff, and H. Profous-Juchelka. 1997. Phylogenetic relationships among eight *Eimeria* species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. Journal of Parasitology 83:262–271.

Bartholomew, J. L., S. D. Atkinson, and S. L. Hallett. 2006. Involvement of *Manayunkia speciosa* (Annelida: Polychaeta: Sabellidae) in the life cycle of *Parvicapsula minibicornis*, a myxozoan parasite of pacific salmon. Journal of Parasitology 92:742–748.

Bartholomew, J. L., S. D. Atkinson, L. J. Lowenstine, M. M. Garner, C. H. Gardiner, B. A. Rideout, M. K. Keel, and J. D. Brown. 2008. Myxozoan parasitism in waterfowl. International Journal for Parasitology 38:199–1207.

Bartholomew, J. L., M. J. Whipple, D. G. Stevens, and J. L. Fryer. 1997. The life cycle of *Ceratomyxa shasta*, a myxosporean parasite of salmonids, requires a freshwater polychaetes as an alternate host. Journal of Parasitology 83:859–868.

Bellerud, B. L. 1993. Etiological and epidemiological factors effecting outbreaks of proliferative gill disease on Mississippi channel catfish farms. Ph.D. Dissertation. College of Veterinary Medicine, Mississippi State University, Mississippi State, Mississippi, 164 p.

Bellerud, B. L., L. M. Pote, T. L. Lin, M. J. Johnson, and C. R. Boyle. 1995. Etiological and epizootological factors associated with outbreaks of proliferative gill disease in channel catfish. Journal of Aquatic Animal Health 7:124–131.

Bosworth, B. G., D. J. Wise, J. S. Terhune, and W. R. Wolters. 2003. Family and genetic group effects for resistance to proliferative gill disease in channel catfish, blue catfish and channel catfish x blue catfish backcross hybrids. Aquaculture Research 34:569–573.

Bowser, P. R., and J. D. Conroy. 1985. Histopathology of gill lesions in channel catfish associated with *Henneguya*. Journal of Wildlife Diseases 21:177–179.

Burtle, G. J., L. R. Harrison, and E. L. Styer. 1991. Detection of a triactinomyxid myxozoan in an oligochaete from ponds with proliferative gill disease in channel catfish. Journal of Aquatic Animal Health 3:281–287.

Caffara, M., E. Raimondi, D. Florio, F. Marcer, F. Quaglio, and M. L. Fioravanti. 2009. The life cycle of *Myxobolus lentisuturalis* (Myxozoa: Myxobolidae), from gold fish (*Carassius auratus auratus*), involves a raabeia-type actinospore. Folia Parasitologica 56:6–12.

Carriero, M. M., E. A. Adriano, M. R. M. Silva, P. S. Ceccarelli, and A. A. M. Maia. 2013. Molecular phylogeny of the *Myxobolus* and *Henneguya* genera with several new South American species. PLoS One 8**:**e73713.

Current, W. L. 1979. *Henneguya adipose* Minchew (Myxosporida) in the channel catfish: ultrastructure of the plasmodium wall and sporogenesis. Journal of Protozoology 26:209–217.

Dyková, I., I. Deburon, W. A. Roumillat, and I. Fiala. 2011. *Henneguya cynoscioni* sp. n. (Myxosporea: Bivalvulida), an agent of severe cardiac lesions in the spotted seatrout, *Cynoscion nebulosus* (Teleostei: Sciaenidae). Folia Parasitologica 58:169–177.

El-Mansy, A., and K. Molnár. 1997a. Development of *Myxobolus hungaricus* (Myxosporea: Myxobolidae) in oligochaete alternate hosts. Diseases of Aquatic Organisms 31:227–232.

El-Mansy A., and K. Molnár. 1997b. Extrapiscine development of *Myxobolus drjagini* Akhmerov, 1954 (Myxosporea: Myxobolidae) in oligochaete alternative hosts. Acta Veterinaria Hungarica 45:427–438.

El-Mansy, A., C. S. Székely, and K. Molnár. 1998a. Studies on the occurrence of actinosporean stages of myxosporeans in Lake Balaton, Hungary, with the description of triactinomyxon, raabeia and aurantiactinomyxon types. Acta Veterinaria Hungarica 46:437–450.

El-Mansy, A., C. S. Székely, and K. Molnár. 1998b. Studies on the occurrence of actinosporean stages of fish myxosporeans in a fish farm of Hungary, with the description of triactinomyxon, raabeia, aurantiactinomyxon and neoactinomyxon types. Acta Veterinaria Hungarica 46:259–284.

El-Matbouli, M., and R. Hoffmann. 1989. Experimental transmission of two *Myxobolus* spp. developing bisporogeny via tubificid worms. Parasitology Research 75:461–464.

Eszterbauer, E. 2004. Genetic relationship among gill-infecting *Myxobolus* species (Myxosporea) of cyprinids: Molecular evidence of importance of tissue-specificity. Diseases of Aquatic Organisms 58:35–40.

Eszterbauer, E., S. Marton, O. Z. Rácz, M. Letenyei, and K. Molnár. 2006. Morphological and genetic differences among actinosporean stages of fish-parasitic myxosporeans (Myxozoa): difficulties of species identification. Systematic Parasitology 65:97–114.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the boostrap. Evolution 39:783–791.

Fiala, I. 2006. The phylogeny of Myxosporea (Myxozoa) based on small subunit ribosomal RNA gene analysis. International Journal of Parasitology 36:1521–1534.

Griffin, M. J., A. C. Camus, D. J. Wise, T. E. Greenway, M. J. Mauel, and L. M. Pote. 2010. Variation in susceptibility to *Henneguya ictaluri* infection by two species of catfish and their hybrid cross. Journal of Aquatic Animal Health 22:21–35.

Griffin, M. J., L. H. Khoo, L. Torrans, B. G. Bosworth, S. M. Quiniou, P. S. Gaunt, and L. M. Pote. 2009a. New data on *Henneguya pellis* Myxozoa: Myxobolidae), a parasite of blue catfish *Ictalurus furcatus*. Journal of Parasitology 95:1455–1467.

Griffin, M. J., L. M. Pote, D. J. Wise, T. E. Greenway, M. J. Mauel, and A. C. Camus. 2008. A novel *Henneguya* species from channel catfish described by morphological, histological and molecular characterization. Journal of Aquatic Animal Health 20:127–135.

Griffin, M. J., D. J. Wise, and L. M. Pote. 2009b. Morphology and small-subunit ribosomal DNA sequence of *Henneguya adiposa* (Myxosporea) from *Ictalurus punctatus* (Siluriformes). Journal of Parasitology 95:1076–1085.

Hallett, S. L., S. D. Atkinson, and M. El-Matbouli. 2002. Molecular characterization of two aurantiactinomyxon (Myxozoa) phenotypes reveals one genotype. Journal of Fish Diseases 25:627–631.

Hallett, S. L., S. D. Atkinson, C. Erséus, and M. El-Matbouli. 2004. Molecular methods clarify morphometric variation in triactinomyxon spores (Myxozoa) released from different oligochaete hosts. Systematic Parasitology 57:1–14.

Hallett, S. L., S. D. Atkinson, C. Erséus, and M. El-Matbouli. 2006. Myxozoan parasites disseminated via oligochaete worms as live food for aquarium fishes: descriptions of aurantiactinomyxon and raabeia actinospore types. Diseases of Aquatic Organisms 69:213–225.

Hallett, S. L., C. Erséus, and R. J. G. Lester. 1999. Actinosporeans (Myxozoa) from marine oligochaetes of the Great Barrier Reef. Systematic Parasitology 44:49–57.

Hallett, S. L., H. Schöl, and M. El-Matbouli. 2003. Characterisation of two novel types of hexactinomyxon spores (Myxozoa) with subsidiary protrusions on their caudal processes. Diseases of Aquatic Organisms 55:45–57.

Hanson, L. A., D. Lin, L. M. W. Pote, and R. Shivaji. 2001. Small subunit rRNA gene comparisons of four actinosporean species to establish a polymerase chain reaction test for the causative agent of proliferative gill disease in channel catfish. Journal of Aquatic Animal Health 13:117–123.

Holzer, A. S., C. Sommerville, and R. Wootten. 2004. Molecular relationships and phylogeny in a community of myxosporeans and actinosporeans based on their 18S rDNA sequences. International Journal for Parasitology 34:1099–1111.

Janiszewska, J. 1955. Actinomyxidia: morphology, ecology history of investigations, systematics, development. Acta Parasitologica Polonica 2:405–453.

Janiszewska, J. 1957. Actinomyxidia II. New systematics, sexual cycle, description of new genera and species. Zoologica Poloniae 8:3–34.

Kageyama, T., T. Yanagida, K. Ohara, and H. Yokoyama. 2009. *Henneguya pseudorhinogobii* n. sp. (Myxozoa: Myxosporea) parasitizing the gills of the freshwater goby *Rhinogobius* sp. OR from the Nagara River and redescription of *Henneguya rhinogobii*. Fisheries Science 75:657–663.

Kallert, D. M., E. Eszterbauer, M. El-Matbouli, C. Erséus, and W. Haas. 2005. The life cycle of *Henneguya neusslini* Schuber & Schröder, 1905 (Myxozoa) involves a triactinomyxon-type actinospore. Journal of Fish Diseases 28:71–79.

Karlsbakk, E., and M. Køie. 2011. Morphology and SSU rDNA sequences of *Ortholinea orientalis* (Shul'man and Shul'man-Albova, 1953) (Myxozoa, Ortholineidae) from *Clupea harengus* and *Sprattus sprattus* (Clupeidae) from Denmark. Parasitology Research 109:139–145.

Kent, M. L., K. B. Andree, J. L. Bartholomew, M. El-Matbouli, S. S. Desser, R. H. Devlin, S. W. Feist, R. P. Hedrick, R. W. Hoffmann, J. Khattra, S. L. Hallett, R. J. G. Lester, M. Longshaw, O. Palenzeula, M. E. Siddall, and C. Xiao. 2001. Recent advances in our knowledge of the Myxozoa. Journal of Eukaryotic Micriobiology 48:395–413.

Khoo, L., F. A. Rommel, S. A. Smith, M. J. Griffin, and L. M. Pote. 2010. *Myxobolus neurophilus*: morphologic, histopathologic and molecular characterization. Diseases of Aquatic Organisms 89:51–61.

Koprivnikar, J., and S. S. Desser. 2002. A new form of raabeia-type actinosporean (Myxozoa) from the oligochaete *Uncinais uncinata*. Folia Parasitologica 49:89–92.

Lin, D., L. A. Hanson, and L. M. Pote. 1999. Small subunit ribosomal RNA sequence of *Henneguya exilis* (class Myxosporea) identifies the actinosporean stage from an oligochaete host. Journal of Eukaryotic Microbiology 46:66–68.

Lom, J. and I. Dyková. 2006. Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. Folia Parasitologica 53:1–36.

Lom, J., J. Mcgeorge, S. W. Feist, D. Morris, and A. Adams. 1997. Guidelines for the uniform characterisation of the actinosporean stages of parasites of the phylum Myxozoa. Diseases of Aquatic Organisms 30:1–9.

Lowers, J. M., and J. L. Bartholomew. 2003. Detection of myxozoan parasites in oligochaetes imported as food for ornamental fish. Journal of Parasitology 89:84–91.

MacMillan, J. R., C. Wilson, and A. Thiyagarajah. 1989. Experimental induction of proliferative gill disease in specific-pathogen-free channel catfish. Journal of Aquatic Animal Health 1:245–254.

Marcucci, C., M. Caffara, and E. Goretti. 2009. Occurrence of actinosporean stages (Myxozoa) in the Nera River system (Umbria, central Italy). Parasitology Research 105:1517–1530.

Markiw, M. E., and K. Wolf. 1983. *Myxosoma cerebralis* (Myxozoa: Myxosporea) etiologic agent of salmonid whirling disease requires Tubificid worm (Annelida: Oligochaeta) in its life cycle. Journal of Protozoology 30:561–564.

Mcgeorge, J., C. Sommerville, and R. Wootten. 1997. Studies of actinosporean myxozoan stages parasitic in oligochaetes from the sediments of a hatchery where Atlantic salmon harbor *Sphaerospora truttae* infection. Diseases of Aquatic Organisms 30:107–119.

Minchew, C. D. 1977. Five new species of *Henneguya* (Protozoa: Myxosporida) from ictalurid fishes. Journal of Protozoology 24:213–220.

Mischke, C. C., M. J. Griffin, T. E. Greenway, and D. J. Wise. 2013. Effects of mosquitofish, *Gambusia affinis*, on channel catfish, *Ictalurus punctatus*, production ponds. Journal of World Aquaculture Society 44:288–292.

Mischke, C. C. S. Tucker, And M. H. Li. 2012. Channel catfish polyculture with fathead minnows or threadfin shad: effects on pond plankton communities and catfish fillet flavor, color, and fatty acid composition. Journal of World Aquaculture Society 43:208–217.

Negredo, C., and M. F. Mulcahy. 2001. Actinosporean infections in oligochaetes in a river system in southwest Ireland with descriptions of three new forms. Diseases of Aquatic Organisms 46:67–77.

Nei, M., and S. Kumar. 2000. Molecular evolution and phylogenetics. Oxford University press, New York, New York, 333 p.

Oumouna, M., S. L. Hallett, R. W. Hoffmann, and M. El-Matbouli. 2003. Seasonal occurrence of actinosporeans (Myxozoa) and oligochaetes (Annelida) at a trout hatchery in Bavaria, Germany. Parasitology Research 89:170–184.

Özer, A., R. Wootten, and A. P. Shinn. 2002. Survey of actinosporean types (Myxozoa) belonging to seven collective groups found in a freshwater salmon farm in Northern Scotland. Folia Parasitologica 49:189–210.

Pote, L. M., B. Bellerud, D. L. Lin, and E. F. Chenney. 1994. The isolation and propagation of *Dero digitata* infected with *Aurantiactinomyxon* sp. Journal of the World Aquaculture Society 25:303–307.

Pote, L., L. A. Hanson, and R. Shivaji. 2000. Small subunit ribosomal RNA sequences link the cause of proliferative gill disease in channel catfish to *Henneguya* n. sp. (Myxozoa: Myxosporea). Journal of Aquatic Animal Health 12:230–240.

Pote, L., L. Khoo, and M. Griffin. 2012. *Henneguya ictaluri*. *In* Fish parasites pathobiology and protection, P. T. K. Woo and K. Buchmann (eds.). CAB International, Oxfordshire, U. K., p. 177–192.

Prunescu, C., P. Prunescu, Z. Pucek, and J. Lom. 2007. The first finding of myxosporean development from plasmodia to spores in terrestrial mammals: *Soricimyxum fegati* gen. et sp. n. (Myxozoa) from *Sorex araneus* (Soricomorpha). Folia Parasitologica 54:159–164.

Roberts, J. F., C. M. Whipps, J. L. Bartholomew, L. Schneider, and E. R. Jacobson. 2008. *Myxidium scripta* n. sp. identified in urinary and biliary tract of Louisiana-farmed red-eared slider turtles *Trachemys scripta elegans*. Diseases of Aquatic Organisms 80:199–209.

Rzhetsky, A., and M. Nei. 1992. A simple method for estimating and testing minimum evolution trees. Molecular Biology and Evolution 9:945–967.

Styer, E. L., L. R. Harrison, and G. J. Burtle. 1991. Experimental production of proliferative gill disease in channel catfish exposed to a myxozoan-infected oligochaete, *Dero digitata*. Journal of Aquatic Animal Health 3:288–291.

Székely, C., A. Avenant-Oldewage, and K. Molnár. 2004. Description of a new actinosporean type from South African freshwaters. Diseases of Aquatic Organisms 61:95–102.

Székely, C., J. C. Eiras, and E. Eszterbauer. 2005. Description of a new synactinomyxon type from the River Sousa, Portugal. Diseases of Aquatic Organisms 66:9–14.

Székely, C., A. El-Mansy, K. Molnár, and F. Baska. 1998. Development of *Thelohanellus hovorkai* and *Thelohanellus nikolskii* (Myxosporea: Myxozoa) in oligochaete alternate hosts. Fish Pathology 33:107–114.

Székely, C., S. L. Hallett, A. Al-Samman, and A. Dayoub. 2007. First description of myxozoans from Syria: Novel records of hexactinomyxon, triactinomyxon and endocapsa actinospore types. Diseases of Aquatic Organisms 74:127–137.

Székely, C., K. Molnár, E. Eszterbauer, and F. Baska. 1999. Experimental detection of the actinospores of *Myxobolus pseudodispar* (Myxoporea: Myxobolidae) in oligochaete alternate hosts. Diseases of Aquatic Organisms 38:219–224.

Székely, C., A. Sitjà-Bobadilla, and P. Alvarez-Pellitero. 2000. First report on the occurrence of an actinosporean stage (Myxozoa) in oligochaetes from Spanish freshwaters. Acta Veterinaria Hungarica 48:433–441.

Székely, C., S. Urawa, and H. Yokoyama. 2002. Occurrence of actinosporean stages of myxosporeans in an inflow brook of a salmon hatchery in the Mena River System, Hokkaido, Japan. Diseases of Aquatic Organisms 49:153–160.

Székely, C., H. Yokoyama, S. Urawa, T. Timm, and K. Ogawa. 2003. Description of two new actinosporean types from a brook of Fuji Mountain, Honshu, and fromo Chitose River, Hokkaido, Japan. Diseases of Aquatic Organisms 53:127–132.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology & Evolution 28:2731–2739.

Trouillier, A., M. El-Matbouli, and R. W. Hoffmann. 1996. A new look at the life-cycle of *Hoferellus carassii* in the goldfish (*Carassius auratus auratus*) and its relation to "kidney enlargement disease" (KED). Folia Parasitologica 43:173–187.

Tucker, C. S., J. L. Avery, and D. Heikes. 2004. Culture Methods. *In* Biology and culture of the channel catfish, C. S. Tucker and J. A. Hargreaves (eds.). Elsevier B. V., Amsterdam, The Netherlands, p. 166–195.

Urawa, S., M. A. Freeman, S. C. Johnson, S. R. M. Jones, and H. Yokoyama. 2011. Geographical variation in spore morphology, gene sequences, and host specificity of *Myxobolus arcticus* (Myxozoa) infecting salmonid nerve tissues. Diseases of Aquatic Organisms 96:229–237.

Wise, D. J., A. Camus, T. Schwedler, and J. Terhune. 2004. Health Management. *In* Biology and culture of the channel catfish, C. S. Tucker and J. A. Hargreaves (eds.). Elsevier B. V., Amersterdam, The Netherlands, p. 444–502.

Xi, B., J. Zhang, J. Xie, L. Pan, P. Xu, and X. Ge. 2013. Three actinosporean types (Myxozoa) from the oligochaete *Branchiura sowerbyi* in China. Parasitology Research 112:1575–1582.

Xiao, C., and S. S. Desser. 1998a. The oligochaetes and their actinosporean parasites in Lake Sasajewun, Algonquin Park, Ontario. Journal of Parasitology 84:1020–1026.

Xiao, C., and S. S. Desser. 1998b. Actinosporean stages of myxozoan parasites of oligochaetes from Lake Sasajewun, Algonquin Park, Ontario: New forms of triactinomyxon and raabeia. Journal of Parasitology 84:998–1009.

Xiao, C. And S. S. Desser. 1998c. Actinosporean stages of myxozoan parasites of oligochaetes from Lake Sasajewun, Algonquin Park, Ontario: New forms of echinactinomyxon, neoactinomyxum, aurantiactinomyxon, guyenotia, synactinomyon and antonactinomyxon. Journal of Parasitology 84:1010–1019.

Yokoyama, H. 1997. Transmission of *Thelohanellus hovorkai* Achmerov, 1960 (Myxosporea: Myxozoa) to common carp *Cyprinus carpio* through the alternate oligochaete host. Systematic Parasitology 36:79–84.

CHAPTER III

18S rRNA GENE SEQUENCING IDENTIFIES A NOVEL SPECIES OF *HENNEGUYA* PARASITIZING THE GILLS OF THE CHANNEL CATFISH (ICTALURIDAE)

3.1 Abstract

In the southeastern United States, the channel catfish *Ictalurus punctatus* is a host to at least eight different species of myxozoan parasites belonging to the genus *Henneguya*, four of which have been characterized molecularly by small subunit ribosomal RNA gene (SSU rRNA) sequencing. However, only two of these have molecularly confirmed life cycles that involve the oligochaete *Dero digitata* as the definitive host. During a health screening of farm-raised channel catfish, several fish presented with deformed primary lamellae. Lamellae harbored large, nodular, white pseudocysts 1.25 mm in diameter and upon rupturing, these pseudocysts released *Henneguya* myxospores, with a typical lanceolate shaped spore body, measuring 17.1 ± 1.0 µm (mean ± SD; range = 15.0-19.3 µm) in length and 4.8 ± 0.4 µm (3.7-5.6 µm) in width. Pyriform shaped polar capsules were 5.8 ± 0.3 µm in length (5.1-6.4 µm) and 1.7 ± 0.1 µm (1.4-1.9 µm) in width. The two caudal processes were 40.0 ± 5.1 µm in length (29.5-50.0 µm) with a spore length of 57.2 ± 4.7 (46.8-66.8 µm). The contiguous SSU rRNA gene sequence obtained from myxospores of five excised cysts did not match any *Henneguya* sp. in Genbank. The greatest sequence homology (91% over 1900 bp) was with *Henneguya pellis*, associated with blister-like lesions on the skin of blue catfish

Ictalurus furcatus. Based on the unique combination of pseudocyst and myxospore morphology, tissue location, host and SSU rRNA gene sequence data, we report this isolate to be a previously unreported species, *Henneguya bulbosus* sp. nov.

3.2 Introduction

The genus *Henneguya* Thélohan, 1892, contains approximately 200 species of freshwater and marine parasites of fish, described primarily by host records and morphological descriptions of the myxospore stage in the fish host. However, with the advent of molecular sequencing more precise identification and descriptions of novel species can be made, supplementing morphological descriptions, tissue predilection and host records with molecular characterization of the 18S rRNA gene (Lom and Dyková 2006; Eiras and Adriano 2012).

In the southeastern United States, with its closed earthen ponds, intensive management strategies, and multibatch production systems (Wise et al. 2004), catfish aquaculture provides an optimal environment for the maintenance and propagation of myxozoan life cycles. *Henneguya ictaluri,* the myxozoan parasite responsible for proliferative gill disease in channel and hybrid catfish, has been persistent in catfish aquaculture since the early 1980s (Bowser and Conroy 1985; Pote et al. 2000; Bosworth et al. 2003; Wise et al. 2004; Griffin et al. 2010). In addition to *H. ictaluri*, eight other species of *Henneguya* are described from the channel catfish (Minchew 1977; Eiras and Adriano et al. 2002; 2012) but most are often incidental findings on routine diagnostic screenings. Of these eight species, four have been molecularly characterized by sequencing of the 18S rRNA gene (Lin et al. 1999; Pote et al. 2000; Griffin et al. 2008; Griffin et al. 2009a; Griffin et al. 2009b). In this paper, we present detailed

morphological, histological and molecular sequencing data describing a previously unidentified species of *Henneguya* from the gills of farm-raised channel catfish.

3.3 Materials and methods

Four fingerling and one stocker channel catfish from a local commercial aquaculture operation were submitted for routine diagnostic screening to the Aquatic Research & Diagnostic Laboratory at the Thad Cochran National Warmwater Aquaculture Center, Stoneville, MS. Four of the fish displayed several nodular myxosporean pseudocysts (~1 mm in diameter) that presented as a bulbous protrusion of the primary lamellae. Numerous lanceolate *Henneguya* myxospores were released when the pseudocysts were mechanically ruptured. Gill tissue containing pseudocysts was pooled and preserved in 70% ethanol for future morphological and molecular characterization.

Pseudocysts (n=7) were individually excised from gill tissue by sharp dissection and placed onto a clean glass microscope slide with a drop of physiological saline. A glass coverslip was placed over each excised pseudocyst, which was photographed prior to mechanical rupture to release the myxospores. Tissues and myxospores were viewed with a BX-50 Olympus microscope (Olympus Optical Co Ltd, Tokyo, Japan) and representative images captured with an Olympus DP72 camera and DP-2-Twain/cellSens software (Olympus Optical Co Ltd, Tokyo, Japan).

A single primary lamella containing a pseudocyst was excised from the gill arch of one fish. The tissue was trimmed, processed, and embedded in paraffin wax, and sectioned at 5 µm. Slides were originally stained with hematoxylin and eosin (H & E). However, due to the limited amount of tissue left in the paraffin block and the need to

better demonstrate the presence and morphology of myxospores, Giemsa stain was then applied to the H & E stained slides.

Myxospores from each excised pseudocyst were collected into individual 1.5 ml microcentrifuge tubes and centrifuged at 15,000 x *g* for 10 min. The supernatant was removed and myxospores were washed with 1 mL of nuclease free water before repeating the centrifugation step as previously described. After removal of the supernatant, genomic DNA was extracted from myxospores using the QIAGEN DNeasy Tissue Kit (QIAGEN Inc., Valencia, California) according to manufacturer protocol. Primers (Table 3.1) designed to amplify the 18S rRNA gene of myxozoans were used in the molecular characterization of the myxospores (Barta et al. 1997; Hanson et al. 2001; Fiala 2006; Griffin et al. 2008). The first amplification was performed using the general eukaryotic primers ERIB1 and ERIB10 (Barta et al 1997) and the remaining primers were used in nested PCR reactions according to Griffin et al. (2008). All 25 µl reaction mixtures contained 20 pmol of each primer using EconoTaq® Plus Green 2X Master Mix (Lucigen, Madison, Wisconsin). The initial amplification was 95°C for 10 min, followed by 35 cycles of 95°C for 1 min, 48°C for 1 min, 72°C for 2 min, and a final extension step of 72°C for 10 min. The nested PCR reactions were carried out using 1 µl of PCR product from the initial amplification by the ERIB1 and ERIB10 primer set. The cycling parameters were the same, but the annealing temperature was 52°C. Amplification of the 18S rRNA gene was carried out using an MJ Research PTC-200 thermal cycler (GMI, Ramsey, Minnesota). A 1.2% agarose gel stained with ethidium bromide (0.1 µg/ml) was used to visualize amplicons after exposure to ultraviolet light. All products were

compared to an appropriate molecular weight marker (Hyperladder II, Bioline, London, United Kingdom) to confirm the amplification of an appropriately sized product.

Table 3.1 Primers used in the 18S rRNA genetic characterization of *H. bulbosus* n. sp.

Primer	Sequence (5'-3')	Reference
ERIB1	ACCTGGTTGATCCTGCCAG	Barta et al. (1997)
ERIB10	CCTCCGCAGGTTCACCTACGG	Barta et al. (1997)
H2	CGACTTTTACTTCCTCGAAATTGC	Hanson et al. (2001)
H9	TTACCTGGTCCGGACATCAA	Hanson et al. (2001)
Myxospec F	TTCTGCCCTATCAACTWGTTG	Fiala (2006)
Myxospec R	GGTTTCNCDGRGGGMCCAAC	Fiala (2006)
Genmyxo3	TGATTAAGAGGAGCGGTTGG	Griffin et al. (2008)
Genmyxo4	GGATGTTGGTTCCGTATTGG	Griffin et al. (2008)
Genmyxo5	TAAGCGCAGCAACTTTGAGA	Griffin et al. (2008)

Amplicons were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, California) and the primers used in amplification of the 18S rRNA gene were also used for sequencing. Prior to sequencing, purified products were quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, Delaware). Products were sequenced directly in both directions using ABI BigDye™ chemistry (Applied Biosystems, Foster City, California), and run on an ABI Prism 3730™ automated sequencer (Applied Biosystems). All contiguous sequences were assembled using the SeqMan™ program of the Lasergene software package (DNAStar, Madison, Wisconsin). The consensus sequence generated was used in further phylogenetic analysis and submitted to GenBank (Accession number: KM000055).

The myxozoan sequences presented in Table 3.2 were downloaded from the National Center for Biotechnology Information's Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and used in the creation of phylogenetic trees:

Table 3.2 Myxozoan sequences used in phylogenetic analyses

Species	Accession #
Henneguya adiposa	EU492929
Henneguya akule	EU016076
Henneguya corruscans	JQ654971
Henneguya cynoscioni	JN017203
Henneguya doori	U37549
Henneguya exilis	AF021881
Henneguya gurlei	DQ673465
Henneguya ictaluri	AF195510
Henneguya lateolabracis	AB183747
Henneguya lesteri	AF306794
Henneguya mauritaniensis	JQ687060
Henneguya ogawai	AB693051
Henneguya pellis	FJ468488
Henneguya pseudorhinogobii	AB447995
Henneguya rhinogobii	AB447992
Henneguya sutherlandi	EF191200
Henneguya yokoyamai	AB693053
Myxobolus bibullatus	AF378336
Myxobolus cerebralis	EF370481
Myxobolus episquamalis	AY129312
Myxobolus exiguus	AY129317
Myxobolus hakyi	FJ816269
Myxobolus ichkeulensis	AF378337
Myxobolus inornatus	JN896706
Myxobolus koi	FJ841887
Myxobolus lentisuturalis	AY278563
Myxobolus machidai	AB693054
Myxobolus oliveirai	HM754633
Myxobolus osburni	AF378338
Myxobolus pangasii	FJ816270
Tetracapsuloides bryosalmonae	FJ981823

Further molecular and phylogenetic analyses of the 18S rRNA gene obtained from the myxospores were conducted using the Molecular Evolutionary Genetic Analysis 5.0 (MEGA5) software (Tamura et al. 2011). Maximum parsimony and minimum evolutionary analyses were performed using the close-neighbor interchange search level

3. Both bootstrap consensus trees were inferred from 1000 replicates. For minimum evolution analysis, the initial tree was obtained by the neighbor-joining algorithm using the pairwise deletion option for gaps/missing data (Rzhetsky and Nei 1992; Nei and Kumar 2000).

3.4 Results

3.4.1 Gross morphology and histology

Pseudocysts presented as grossly visible (~1.25 mm diameter) white nodules in the primary lamellae (Figure 3.1). Mechanical rupturing of the pseudocysts on wet mount observation revealed numerous *Henneguya* myxospores.

Figure 3.1 Light microscopic image of a *Henneguya bulbosus* n. sp. pseudocyst on gill wet mount.

Pseudocyst previously fixed in 70% ethanol.

Within the expanded branchial tissue was a large (~ 620 X 350 µm) , well circumscribed oval, non-epithelial lined cyst-like structure containing granular eosinophilic material that fractured during processing and blue staining central portion consisting of asynchronous maturing myxospores (Figures 3.2 and 3.3). The more mature spores were located towards the center of the structure (Figure 3.4). The displaced epithelial tissue immediately surrounding the pseudocyst had a loose infiltrate of mononuclear inflammatory cells (mainly lymphocytes) and the outermost portion of

the tissue covered by an almost contiguous layer of mucous cells especially along one leading edge (Figure 3.3).

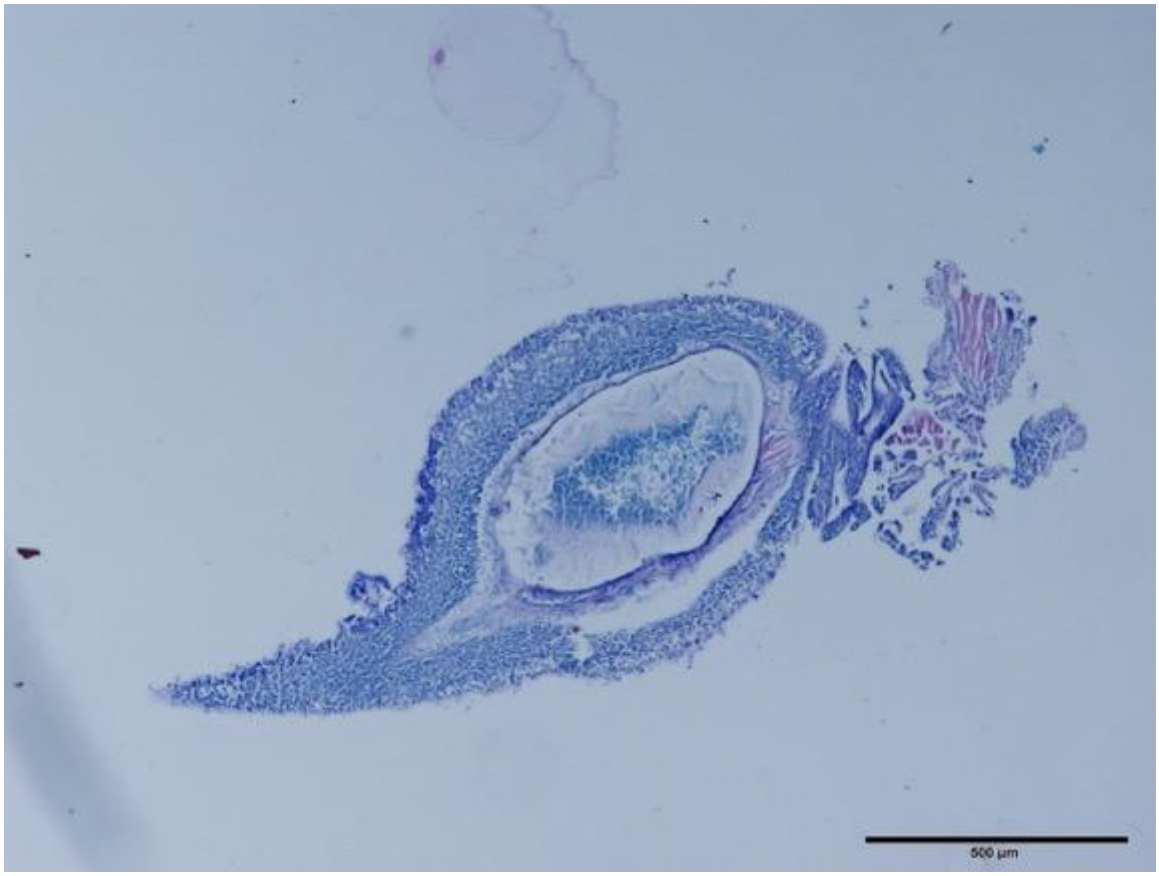

Figure 3.2 Low magnification view of the affected secondary lamella with the pseudocyst.

H & E and Giemsa stained.

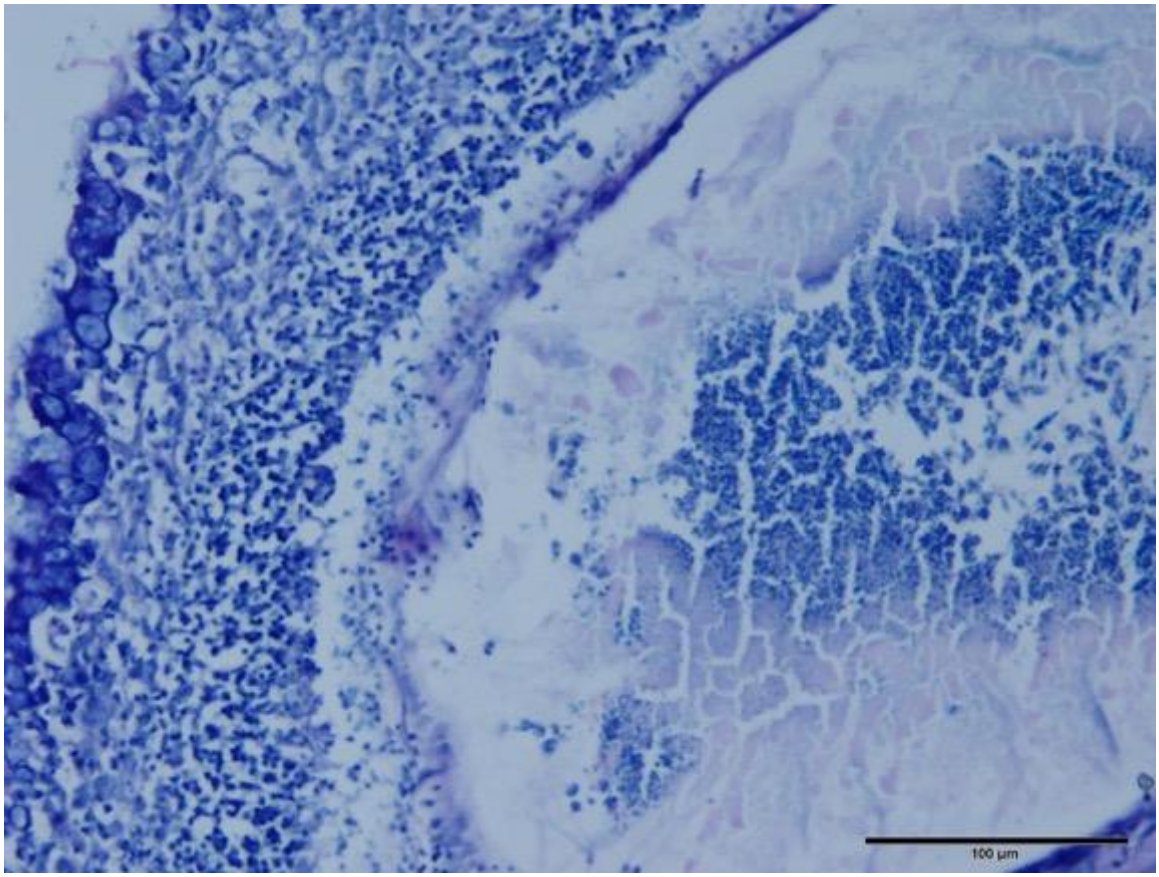

Figure 3.3 Higher magnification view of the pseudocyst of *H. bulbosus* n. sp.

Note the blue staining mucus cells on the edge, the mononuclear inflammatory infiltrate and the granular central core consisting of asynchronously maturing myxospores. H & E and Giemsa stain.

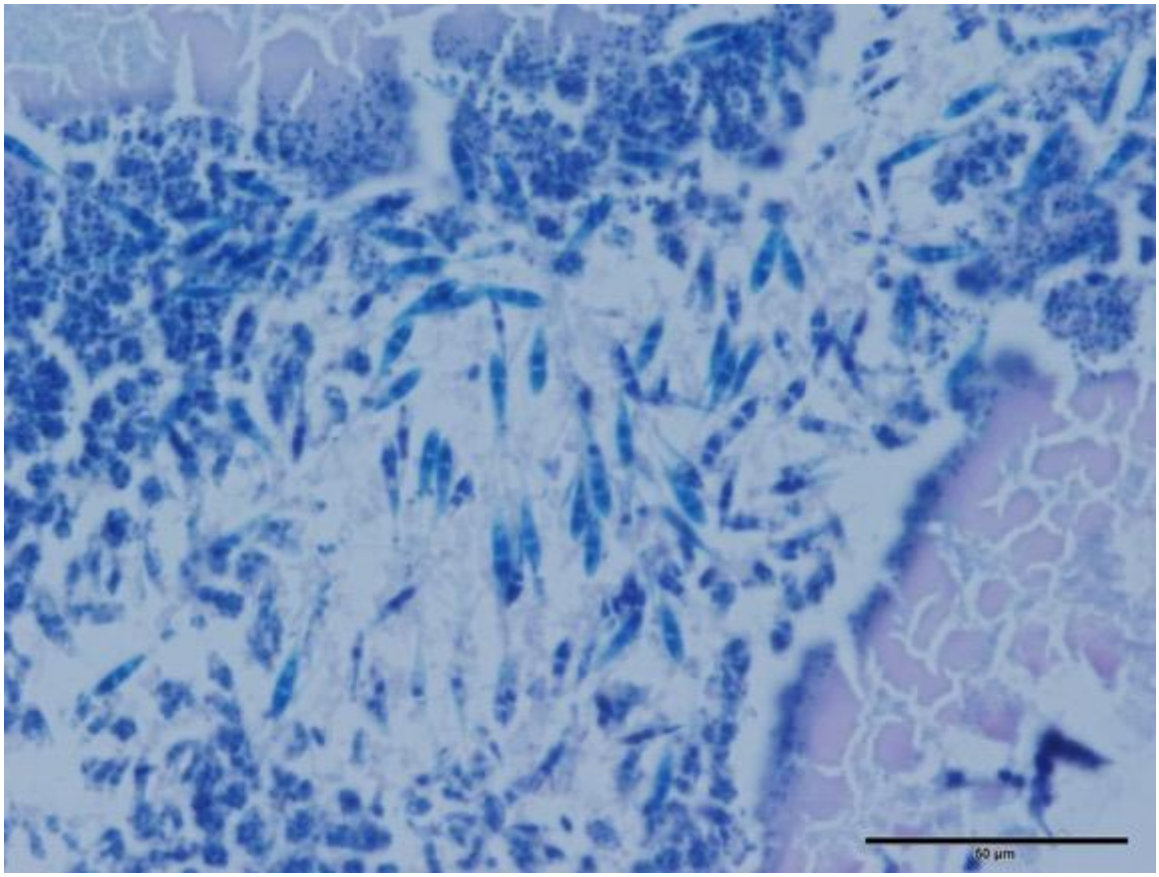

Figure 3.4 Higher magnification to illustrate the asynchronous maturation and morphology of myxospores of *H. bulbosus* n. sp.

H & E and Giemsa stain.

3.4.2 Myxospore morphology

Myxospores (Figures 3.5 and 3.6) were consistent with the description of the genus *Henneguya* and were characterized by a lanceolate shaped spore body 17.1 ± 0.1 µm (mean ± SD; range = 15.0-19.3 µm) in length and 4.8 ± 0.4 µm (3.7-5.6 µm) in width. Pyriform shaped polar capsules were 5.8 ± 0.3 µm in length (5.1-6.4 µm) and 1.7 ± 0.1 µm (1.4-1.9 µm) in width. The two caudal processes were 40.0 ± 5.1 µm in length (29.5 - 50.0 µm) with a total spore length of 57.2 ± 4.7 (46.8-66.8 µm). Measurements obtained from 25 myxospores.

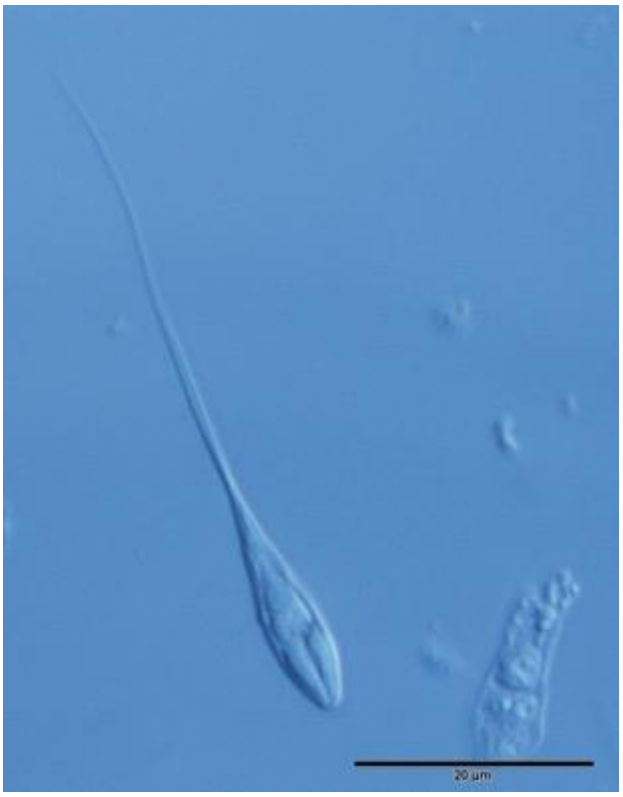

Figure 3.5 Wet mount of a representative mature myxospore of *H. bulbosus* n. sp. released from the mechanically ruptured pseudocyst.

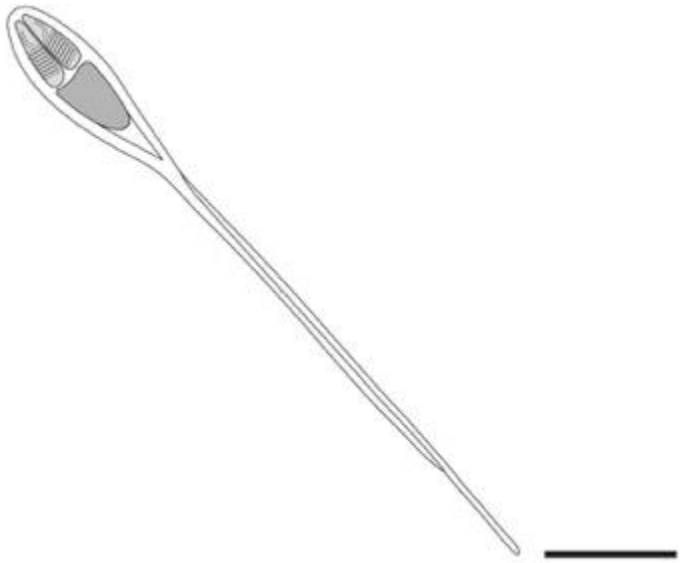

Figure 3.6 Line drawing of a representative myxospore of *H. bulbosus* n. sp. Scale bar is 10 µm.

Morphologically the myxospores had similar features that overlapped with numerous *Henneguya* species described from ictalurid fish in North America, but were not an exact match to any previously described species from ictalurid fish (Table 3.3). While morphology and host species can be useful tools in the identification of myxozoan species, they alone should not be relied upon as criteria to describe novel species. Descriptive morphological reports supplemented with 18S rRNA gene sequence data is currently the most widely accepted means for describing novel myxozoan species (Fiala 2006).

Table 3.3 Morphological measurements of *Henneguya bulbosus* n. sp. myxospores and other *Henneguya* spp. from North American ictalurid fish

Species	Total Spore Length	Spore Body Length	Spore Body Width	Polar Capsule Length	Polar Capsule Width	Caudal Process Length	Reference
H. bulbosus	57.2 (46.8-66.8)	17.1 (15.0-19.3)	4.8 (3.7-5.6)	5.8 (5.1-6.4)	1.7 (1.4-1.9)	40.0 (29.5-50.0)	This paper
H. adiposa	55.6 (40.7-65.8)	17.1 (14.7-20.5)	4.1 (3.4-4.6)	7.2 (5.8-8.3)	1.3 (0.9-1.9)	38.0 (23.2-48.8)	Griffin et al. 2009
H. ameiurensis	NA	23.3	4.1	5.4	1.6	15.0-41.5	Nigrelli and Smith 1940
H. diversis	49.5 (40.0-62.0)	14.8 (13.0-16.5)	4.0 (3.2-5.0)	6.5 (6.0-7.5)	1.5 (1.0-2.0)	34.6 (25.0-47.0)	Minchew 1977
H. exilis	69.9 (60.0-90.0)	17.6 (16.0-19.0)	4.9 (4.0-5.0)	8.5 (7.0-9.0)	1.5-2	52.3 (41.0-73.0)	Kudo 1929
H. ictaluri	NA	23.9 (20.8-26.1)	6.0 (4.5-6.4)	8.1 (7.6-9.6)	2.5 (2.0-3.2)	63.0 (48.1-80.2)	Pote et al. 2000
H. limatula	NA	13.7	5.0-6.0	6.5-8.0	1.5-2.0	27.0-37.0	Meglitsch 1937
H. longicauda	108.3 (91.0-127.0)	16.2 (14.0-17.5)	4.0 (3.5-4.5)	7.7 (7.0-8.5)	1.8 (1.5-2.0)	90.5 (75.0-110.0)	Minchew 1977
H. pellis	100.4 (79.0-124.0)	13.0 (11.0-14.5)	5.0 (4.5-5.2)	6.9 (5.5-8.5)	1.8 (1.5-2.0)	87.8 (66.0-112.0)	Minchew 1977
H. postexilis	52.0 (42.0-62.0)	15.0 (13.5-17.)	3.4 (3.5-4.0)	6.6 (5.9-7.2); 7.0 (6.0-8.0)	1.5 (1.0-2.0)	37.0 (28.0-49.0)	Minchew 1977
H. sutherlandi	65.9 (48.2-90.0)	15.4 (12.2-19.3)	5.5 (4.5-6.8)	6.1 (4.0-7.9)	1.7 (1.0-2.2)	50.5 (34.8-71.4)	Griffin et al. 2008

3.4.3 Molecular analyses

The 2073 bp 18S rRNA gene sequence did not completely match any sequences deposited in NCBI nr/nt database after performing a BLASTn search for somewhat similar sequences and has been deposited in GenBank (Accession number KM000055). Comparison to the most homologous sequences revealed that the myxospores were most similar to *Henneguya pellis* (97.1% sequence homology) and *Henneguya sutherlandi* (95.7% sequence homology) both of which form epidermal pseudocysts in blue and channel catfish, respectively (Minchew 1977; Griffin et al. 2008; Griffin et al. 2009a). Although the myxospores in this paper share overlapping morphological characteristics and significant sequence homology (>90%) to *Henneguya* spp. parasitizing North American ictalurid fish, the discrete pseudocyst morphology and location, coupled with distinct morphological features suggest this to be an undescribed species of *Henneguya*.

3.4.4 Taxonomic summary

Species: *Henneguya bulbosus* n. sp. (Myxozoa: Myxosporea)

Type host: *Ictalurus punctatus* (Rafinesque, 1818) (Siluriformes, Ictaluridae)

Site of infection: Gills (Intralamellar type)

Type locality: Commercial catfish pond, Washington County, Mississippi, USA

Materials deposited: Holotype USNM 1251670, Smithsonian Institution, National Museum of Natural History, Washington DC, United States of America

3.5 Discussion

Henneguya bulbosus n. sp. myxospore shares overlapping morphological characteristics with those of the eight species of *Henneguya* identified from the channel

catfish (Table 3.3). These similar morphological characteristics make proper identification by routine microscopy difficult and emphasize the importance of noting other identifiers such as pseudocyst morphology and location, host species, geographic location, associated pathology, and molecular analysis of at least the 18S rRNA gene.

Phylogenetic analysis of the 18S rRNA gene of *H. bulbosus* n. sp. supports its placement among the *Henneguya* species infecting North American ictalurids (Figures 3.6 and 3.7). The ictalurid infecting *Henneguya* species, based on 18S rRNA gene analysis (sharing >90% sequence homology), are genetically similar and appear to group based on spore morphology, host family (Ictaluridae) and to a lesser extent tissue site. *H. bulbosus* n. sp., a myxozoan parasite of the gills of the channel catfish, groups most closely with the epidermal pseudocyst forming species *H. pellis* and *H. sutherlandi* from the blue catfish and channel catfish, respectively (Griffin et al. 2008, Griffin et al. 2009a). The pseudocysts of *H. bulbosus* n. sp. form grossly visible spherical 1 mm pseudocysts similar in size to those of *H. pellis* and *H. sutherlandi*, but differ in tissue location.

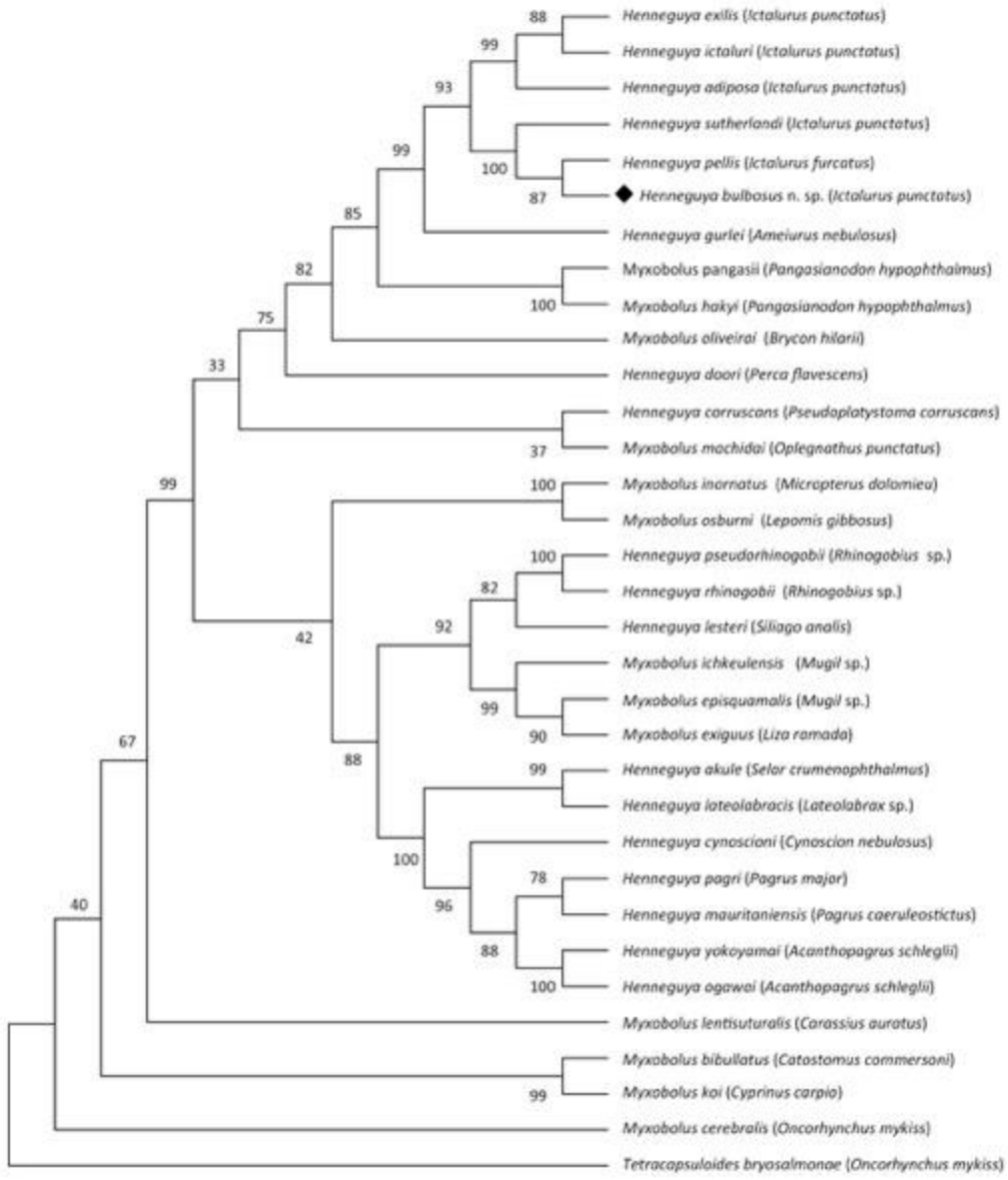

Figure 3.7 Minimum evolution analysis of *H. bulbosus* 18S SSU rRNA gene sequence.

Fish hosts are indicated in parentheses. Numbers at each node represent bootstrap confidence values (n=1,000 replicates). Diamond indicates the novel species *H. bulbosus*.

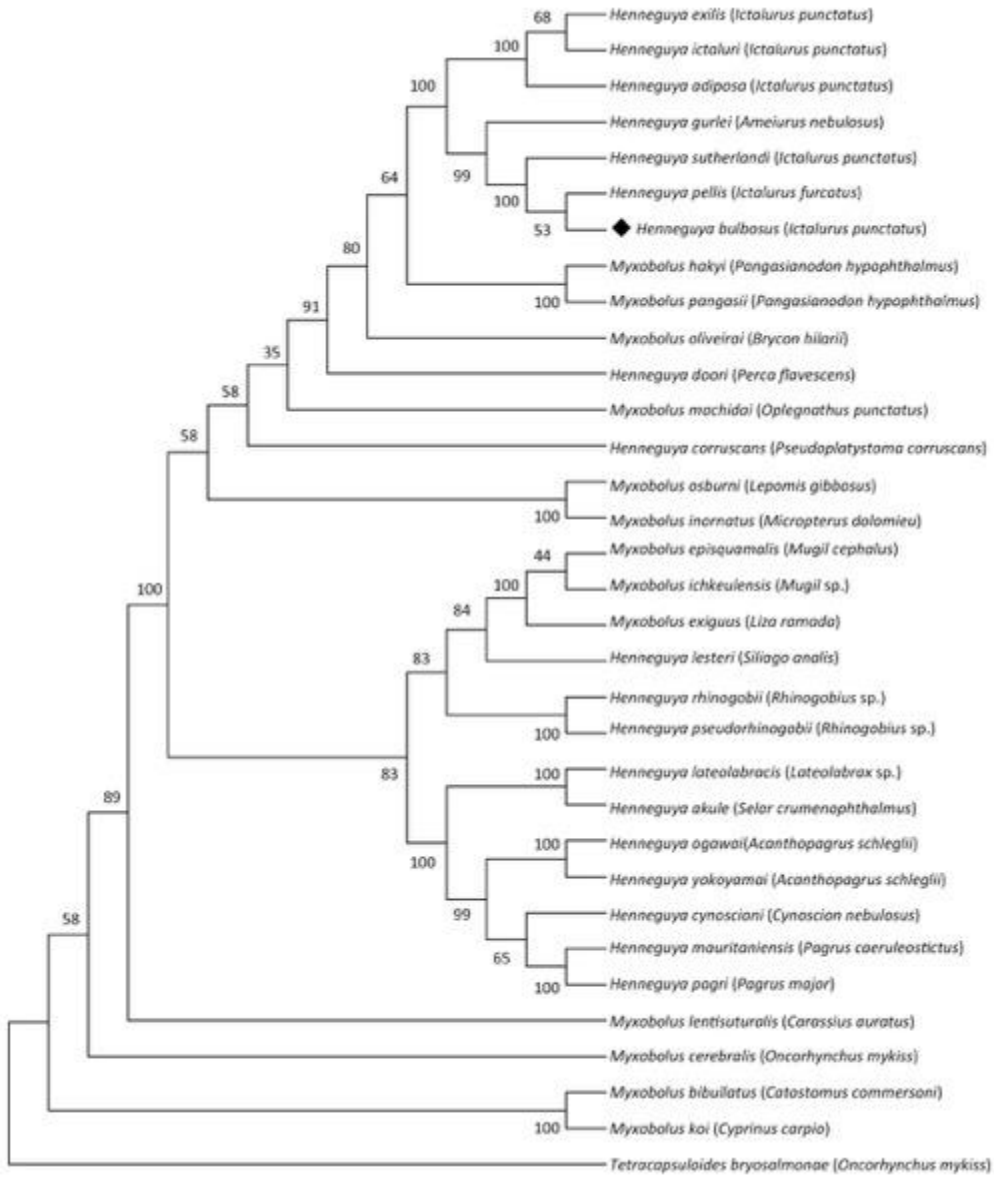

Figure 3.8 Maximum parsimony analysis of *H. bulbosus* 18S SSU rRNA gene sequence.

Fish hosts are indicated in parentheses. Numbers at each node represent bootstrap confidence values (n=1,000 replicates). Diamond indicates the novel species *H. bulbosus*.

In the past, spore morphology and site of infection were heavily relied upon to determine myxosporean species among closely related fish (Lom and Arthur 1989). The

validity of the host record of the gill parasite *Myxobolus rotundus* of the common bream *Abramis brama* was evaluated using 18S rRNA gene sequencing to clarify previous records that were speculated to be misidentifications. In their analyses, Molnár et al. (2009) noted that previous accounts of *M. rotundus* extended to hosts and tissue locations previously undescribed from the type species and type locality. Sequencing of the 18S rRNA gene concluded these were indeed misidentified species of *Myxobolus* that were erroneously reported as *M. rotundus* and reinforces the importance of using multiple identifying characters such as spore and cyst morphology, type host, type locality and 18S rRNA gene sequencing when characterizing a new species. Further examination of the genetic relatedness of gill infecting species of *Myxobolus* based on 18S rRNA gene analyses revealed close phylogenetic clustering of morphological similar *Myxobolus* species infecting the gills of cyprinids in Hungary, seperate from the muscle infecting *Myxobolus* species. This further supports the value of including detailed descriptions of host species and tissue tropism in new species designations (Eszterbauer 2004). In the past, little detail is given on the precise location within gill tissue of myxosporean pseudocyst development. In an effort to provide more definite species descriptions, Molnar (2002) provides detailed descriptions of various gill tissue sites that pseudocysts of *Henneguya*, *Myxobolus* and *Thelohanellus* form. The pseudocysts described here of *H. bulbosus* n. sp. displayed an intralamellar type pseudocyst, similar to those described by Molnar (2002). In African catfish *Clarias garipenus* and tilapia *Oreochromis niloticus* in Egypt, *H. suprabranchiae* develops in the suprabranchial organ and gill tissue, respectively, and has similar presentation as *H. bulbosus* n. sp. (i.e. distortion of

host tissue at infection sites and deformation of gill filaments) (Abdel-Ghaffar et al. 2008, Morsy et al. 2012).

Histologically, the pseudocyst appears similar to other *Henneguya* species affecting the gills except for the very large bullous-like structure, which displaces the branchial epithelium. The apparent inflammatory infiltrate may be the immune response to the pseudocyst but more likely is a result of the location of the pseudocyst which is close to the lamellar tip, which often houses larger numbers of lymphocytes. Both of these explanations may also be applicable for the increased presence of mucus cells along one of the edges of the affected portion of the lamella.

At this time the potential impact of this parasite on the health of cultured channel catfish is uncertain and the complete life cycle of this myxozoan remains unknown. The 18S rRNA gene sequence deposited in GenBank will facilitate molecular confirmation of the life cycle once the corresponding actinospore stage is identified and sequenced. To date, the definitive oligochaete host of *H. bulbosus* n. sp. has yet to be isolated, but previous studies have demonstrated that the benthic oligochaete *Dero digitata* is a host to numerous species of myxozoan parasites in the catfish industry. Through a survey of over 2,000 *D. digitata* collected from a commercial channel catfish pond during the spring, Rosser et al. (2014; Chapter II) observed six genetically distinct actinospore types released from infected *D. digitata*. None of the 18S rRNA gene sequences of the actinospore types described from commercial catfish ponds were a match to *H. bulbosus* n. sp. It is important to note that other species of oligochaetes other than *D. digitata* also inhabit the benthic strata of these ponds and have been found to be releasing actinospore stages of myxozoan life cycles (Bellerud 1993). Further research into these other species

of benthic oligochaetes is necessary to determine their roles as hosts in unknown myxozoan life cycles in catfish production ponds.

Currently, in the commercial catfish industry, the only myxozoan species of economic concern is the pathogenic *H. ictaluri*, the etiological agent of proliferative gill disease (Pote et al. 2000). To date no other species of *Henneguya* from catfish production ponds have been associated with fish mortalities, although *Henneguya* spp. cysts are often observed in the gills during routine diagnostic screenings. The distinct morphology of the pseudocyst and myxospore stage and molecular sequencing of the 18S rRNA gene of *Henneguya bulbosus* n. sp. confirms this to be a previously undescribed *Henneguya* species infecting the gill tissue of the channel catfish.

3.6 References

Abdel-Ghaffar, F., A. S. Abdel-Baki, E. M. Bayoumy, A. R. Bashtar, S. Al-Quraishy, K. S. Morsy, A. Alghamdy, H. Mehlohrn. 2008. Light and electron microscopic study on *Henneguya suprabranchiae* Landsberg, 1987 (Myxozoa: Myxosporea) infecting *Oreochromis niloticus*, a new host record. Parasitology Research 103:609–617.

Barta, J. R., D. S. Marin, P. A. Liberator, M. Dshkevicz, J. W. Anderson, S. D. Feigner, A. Elbrecht, A. Perkins-Barrow, M. C. Jenkins, H. D. Danforth, M. D. Ruff, and H. Profous-Juchelka. 1997. Phylogenetic relationships among eight *Eimeria* species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. Journal of Parasitology 83:262–271.

Bellerud, B. L. 1993. Etiological and epidemiological factors effecting outbreaks of proliferative gill disease on Mississippi channel catfish farms. Dissertation, College of Veterinary Medicine, Mississippi State University

Bosworth, B. G., D. J. Wise, J. S. Terhune, and W. R. Wolters. 2003. Family and genetic group effects for resistance to proliferative gill disease in channel catfish, blue catfish and channel catfish x blue catfish backcross hybrids. Aquaculture Research 34: 569–573.

Bowser, P. R., and J. D. Conroy. 1985. Histopathology of gill lesions in channel catfish associated with *Henneguya*. Journal Wildlife Disease 21:177–179.

Eiras, J. C., and E. A. Adriano. 2012. A checklist of new species of *Henneguya* Thélohan, 1892 (Myxozoa: Myxosporea, Myxobolidae) described between 2002 and 2012. Systematic Parasitology 83:95–104.

Eiras, J. C. 2002. Synopsis of the species of the genus *Henneguya* Thélohan, 1892 (Myxozoa: Myxosporea: Myxobolidae). Systematic Parasitology 52:43–54.

Eszterbauer, E. 2004. Genetic relationship among gill-infecting *Myxobolus* species (Myxosporea) of cyprinids: molecular evidence of importance of tissue-specificity. Diseases Aquatic Organisms 58:35–40.

Fiala, J. 2006. The phylogeny of Myxosporea (Myxozoa) based on small subunit ribosomal RNA gene analysis. International Journal for Parasitology 36:1521–1534.

Griffin, M. J., L. H. Khoo, L. Torrans, B. G. Bosworth, S. M. Quiniou, P. S. Gaunt, and L. M. Pote. 2009a. New data on *Henneguya pellis* Myxozoa: Myxobolidae), a parasite of blue catfish *Ictalurus furcatus*. Journal of Parasitology 95:1455–1467.

Griffin, M. J., L. H. Khoo, D. J. Wise, T. E. Greenway, M. J. Mauel, A. C. Camus. 2008. A novel *Henneguya* species from channel catfish described by morphological, histological and molecular characterization. Journal of Aquatic Animal Health 20:127–135.

Griffin, M. J., D. J. Wise, and L. M. Pote. 2009b. Morphology and small-subunit ribosomal DNA sequence of *Henneguya adiposa* (Myxosporea) from *Ictalurus punctatus* (Siluriformes). Journal of Parasitology 95:1076–1085.

Griffin, M. J., A. C. Camus, D. J. Wise, T. E. Greenway, M. J. Mauel, and L. M. Pote. 2010. Variation in susceptibility to *Henenguya ictaluri* infection by two species of catfish and their hybrid cross. Journal of Aquatic Animal Health 22:21-35.

Hanson, L. A., D. Lin, L. M. W. Pote, and R. Shivaji. 2001. Small subunit rRNA gene comparisons of four actinosporean species to establish a polymerase chain reaction test for the causative agent of proliferative gill disease in channel catfish. Journal of Aquatic Animal Health 13:117–123.

Kudo, R. R. 1920. Studies on Myxosporidia: A synopsis of genera and species of Myxosporidia. Illinois Biological Monographs 5:1–265.

Lin, D, L. A. Hanson, and L. M. Pote. 1999. Small subunit ribosomal RNA sequences of *Henneguya exilis* (Class Myxosporea) identifies the actinosporean stage from an oligochaete host. Journal of Eukaryotic Microbiology 46:66–68.

Lom, J., and J. R. Arthur. 1989. A guideline for the preparation of species descriptions in Myxosporea. Journal of Fish Diseases 12:151–156.

Lom, J., and I. Dyková. 2006. Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. Folia Parasitologica 53:1–36.

Meglitsch, P. A. 1937. On some new and known Myxosporidia of the fishes of Illinois. Journal of Parasitology 23:467–477.

Minchew, C. D. 1977. Five new species of *Henneguya* (Protozoa: Myxosporida) from ictalurid fishes. Journal of Protozoology 24:213–220.

Molnár, K. 2002. Site preference of fish myxosporeans in the gill. Disease of Aquatic Organisms 48:197–207.

Molnár, K., C. Székely, S. L. Hallett, and S. D. Atkinson. 2009. Some remarks on the occurrence, host-specificity and validity of *Myxobolus rotundus* Nemeczek, 1911 (Myxozoa: Myxosporea). Systematic Parasitology 72:71–79.

Morsy, K., F. Abdel-Ghaffar, A. R. Bashtar, H. Mehlhorn, S. Al-Quraishy, and R. Abdel-Gaber. 2012. Morphology and small subunit ribosomal DNA sequence of *Henneguya suprabranchiae* (Myxozoa), a parasite of the catfish *Clarias gariepinus* (Clariidae) from the River Nile, Egypt. Parasitology Research 111:1423–1435.

Nei, M., and S. Kumar. 2000. Molecular evolution and phylogenetics. Oxford University Press, New York, New York

Nigrelli, R. F., and G. M. Smith. 1940. A papillary cystic disease affecting the barbells of *Ameiurus nebulosus* (la Sueur), caused by the myxosporidian *Henneguya ameiurus* sp. nov. Zoologica 25:89–94.

Pote, L. M., L. A. Hanson, and R. Shivaji. 2000. Small subunit ribosomal RNA sequences link the cause of proliferative gill disease in channel catfish to *Henneguya* n. sp. (Myxozoa: Myxosporea). Journal of Aquatic Animal Health 12:230–240.

Rosser, T. G., M. J. Griffin, S. M. Quiniou, T. E. Greenway, L. H. Khoo, D. J. Wise, and L. M. Pote. 2014. Molecular and morphological characterization of myxozoan actinospore types from a commercial catfish pond in the Mississippi Delta. Journal of Parasitology 100:828–839.

Rzhetsky, A., and M. Nei. 1992. A simple method for estimating and testing minimum evolution trees. Molecular Biology and Evolution 9:945–967.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28:2731–2739.

Wise, D. J., A. Camus, T. Schwedler, and J. Terhune. 2004. Health Management. *In*: Tucker CS, Hargreaves JA (ed) Biology and culture of the channel catfish, Elsevier BV, Amsterdam, The Netherlands, pp 482-488.

CHAPTER IV

SMALL SUBUNIT RIBOSOMAL RNA SEQUENCE LINKS THE MYXOSPORE STAGE OF *HENNEGUYA MISSISSIPPIENSIS* N. SP. FROM CHANNEL CATFISH *ICTALURUS PUNCTATUS* TO AN ACTINOSPORE RELEASED BY THE BENTHIC OLIGOCHAETE *DERO DIGITATA*

4.1 Abstract

There are more than 200 species of *Henneguya* described from fish. Of these, only three life cycles have been determined, identifying the actinospore and myxospore stages from their respective hosts. Two of these life cycles involve the channel catfish (*Ictalurus punctatus*) and the freshwater oligochaete *Dero digitata*. Herein we molecularly confirm the life cycle of a previously undescribed *Henneguya* sp. by matching 18S rRNA gene sequence of the myxospore stage from channel catfish with the previously described actinospore stage (*Aurantiactinomyxon mississippiensis*) from *D. digitata*. Gill tissue from naturally infected channel catfish contained pseudocysts restricted to the apical end of the primary lamellae. Myxospores were morphologically consistent with *Henneguya* spp. from ictalurid fishes in North America. The spores measured 48.8 ± 4.8 µm (range = 40.7-61.6 µm) in total spore length. The lanceolate spore body was 17.1 ± 1.0 µm (14.4-19.3 µm) in length and 5.0 ± 0.3 µm (4.5-5.5 µm) in width. The two polar capsules were 6.2 ± 0.4 µm (5.8-7.0 µm) long and 5.0 ± 0.3 µm

(4.5-5.5 µm) wide. Polar capsule contained 8-9 coils in the polar filament. The two caudal processes were of equal length, measuring 31.0 ± 4.1 µm (22.9-40.6 µm). The 1980 bp 18S rRNA gene sequence obtained from two excised cysts shared 99.4% similarity (100% coverage) to the published sequence of *A. mississippiensis*, an actinospore previously described from *D. digitata*. The sequence similarity between the myxospore from channel catfish and actinospore from *D. digitata* suggests they are conspecific, representing alternate life stages of *H. mississippiensis* n. sp.

4.2 Introduction

There are over 2,000 described species of myxozoan parasites; most of which have only been characterized by the myxospore stage (Kent et al. 2001; Lom and Dyková 2006). New descriptions of myxozoan species occur at a fairly rapid rate, however relative few studies have linked the myxospore to an actinospore stage (Kent et al. 2001; Lom and Dyková 2006). Those known myxozoan life cycles adhere to the findings of Markiw and Wolf (1983), involving an actinospore stage released from an aquatic oligochaete and a myxospore stage that develops in the fish host. In the literature, there is a paucity of actinospore descriptions from oligochaetes when compared to the myriad of myxospore stages described thus far. Presently, at least 200 actinospores have been described, with limited molecular sequence data available for comparisons to myxospore stages (Kent et al. 2001; Lom and Dyková 2006). Myxozoan life cycles have been elucidated through experimental infections of naïve hosts, as well as molecular comparisons of 18S rRNA gene sequences (Andree et al. 1997; El-Mansy and Molnár 1997a; El-Mansy and Molnár 1997b; Yokoyama 1997; El-Mansy et al. 1998; Székely et al. 1998; Lin et al. 1999; Székely et al. 1999; Molnár et al. 1999; Eszterbauer et al. 2000;

Pote et al. 2000; Székely et al. 2001; Székely et al. 2002; Holzer et al. 2004; Kallert et al. 2005; Bartholomew et al. 2006; Holzer et al. 2006; Køie et al. 2008; Atkinson and Bartholomew 2009; Caffara et al. 2009; Székely et al. 2009). Although the genus *Henneguya* consists of more than 200 described species only 3 life cycles are known (Lom and Dyková 2006; Eiras and Adriano 2012). The life cycles of *H. exilis* (Lin et al. 1999) and *H. ictaluri* (Pote et al. 2000) involve the channel catfish *Ictalurus punctatus* as the fish host and the oligochaete *Dero digitata* as the alternate host, while *Henneguya nuesslini* myxospores from brown trout *Salmo trutta* and brook trout *Salvelinus fontinalis* have been linked to an actinospore stage from *Tubifex tubifex* (Kallert et al. 2005).

Presently, there are 9 species of *Henneguya* known to parasitize the channel catfish, of which only 2 have been linked to actinospore stages (Kudo 1929; Minchew 1977; Lin et al. 1999; Pote et al. 2000; Griffin et al. 2008; Rosser et al. 2014b). Similarly, the oligochaete fauna associated with channel catfish production ponds is parasitized by a diversity of actinospore stages, many of which have unknown myxospore counterparts (Bellerud 1993, Rosser et al. 2014a). Bellerud (1993) first described the aurantiactinomyxon actinospore *Aurantiactinomyxon mississippiensis* from *Dero digitata* collected in commercial catfish ponds. The 18S rRNA gene of *A. mississippiensis* was later sequenced by Hanson et al. (2001). This current study links the actinospore stage of *A. mississippiensis* with a previously undescribed *Henneguya* sp. from the gills of the channel catfish using 18S rRNA gene sequences. According to conventional classifications of myxozoan parasites (Kent et al. 1994), the actinospore nomenclature is suppressed and the new taxon *Henneguya mississippiensis* n. sp. is proposed.

4.3 Materials and Methods

4.3.1 Myxospore isolation and characterization

Channel catfish gill tissue collected from a routine diagnostic screening harbored myxozoan pseudocysts restricted to the apical portion of the primary lamellae. When ruptured these pseudocysts released myxospore stages consistent with those of the genus *Henneguya*. Gill tissue was pooled and preserved in 70% molecular biology grade ethanol for future analysis. Individual filaments (n=2) containing pseudocysts were excised and placed on glass slides with a drop of nuclease-free water and covered with a glass coverslip. Pseudocysts were mechanically ruptured to release the myxospore stages for morphological characterization. Images of pseudocysts and myxospores were captured using a BX-50 Olympus microscope (Olympus Optical Co Ltd, Tokyo, Japan) with an attached Olympus DP72 camera and the accompanying DP-2-Twain/cellSens software (Olympus Optical Co Ltd, Tokyo, Japan). Aliquots of ethanol fixed myxospores were air dried on clean glass slides, fixed in methanol, stained using DiffQuik®, sealed with Permount® (Fisher Scientific, Fair Lawn, New Jersey) and deposited in Smithsonian Institution, National Museum of Natural History, Washington DC, United States of America (Accession number: Holotype USNM 1270623). Line drawings of representative myxospores were made from micrographs using Adobe Illustrator CC 2014 (Adobe, San Jose, California).

4.3.2 DNA isolation from myxospores

Aliquots of myxospores were washed into 1.5 ml microcentrifuge tubes and suspended in 1 ml of sterile nuclease free water before being centrifuged at 15,000 x *g* for 10 minutes. The supernatant was removed from the pelleted myxospores and DNA was

extracted using the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN Inc., Valencia, California) following the manufacturer recommendations.

4.3.3 Sequencing of the 18S rRNA gene

Amplification of the 18S rRNA gene was carried out using general eukaryotic and myxozoan specific primer sets according to Griffin et al. (2008). Briefly, the general eukaryotic primers of Barta et al. (1997) were used in the first polymerase chain reaction and the remaining primers were used in nested PCR reactions as previously described (Griffin et al. 2008; Rosser et al. 2014a,b). Amplicons were visualized under ultraviolet light on 1.2% agarose gels stained with 0.1 μg/ml ethidium bromide and compared to a concurrently run molecular weight marker (HyperLadder™ 50bp, Bioline, London, United Kingdom) to confirm the presence of appropriate sized bands. Amplicons were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, California), quantified spectrophotometrically (NanoDrop Technologies, Inc., Wilmington, Delaware), and sequenced directly from both strands. Sequencing was performed using ABI BigDye™ chemistry (Applied Biosystems, Foster City, California) and read on an ABI Prism 3730™ automated sequencer. All sequencing reads were edited manually and aligned using SeqMan™ (DNAStar, Madison, Wisconsin). The contiguous sequence was submitted to GenBank (Accession number: KP404438).

4.3.4 Phylogenetic analyses

The consensus sequence was analyzed against other published myxozoan sequences available in the National Center for Biotechnology Information (NCBI) non-redundant nucleotide database using the Blastn suite for highly similar sequences

(Altschul et al. 1990). The most closely related published sequences greater than 1500 bp in length (n=40) were downloaded and used in the construction of phylogenetic trees using Molecular Evolutionary Genetic Analysis 6.0 (MEGA6) (Tamura et al. 2013). The 40 sequences were aligned using the Clustal W utility and trimmed to equal lengths. The final dataset consisted of 1155 positions. Phylogenetic placement of the newly sequenced myxospore isolate was inferred by the maximum likelihood method using the Tamura-Nei model and the complete deletion option (Tamura and Nei 1993). The final tree was constructed from 1000 bootstrap replicates with the initial tree being obtained by maximum parsimony (Felsenstein 1985).

4.4 Results

4.4.1 Myxospore morphology

Pseudocysts spherical, located at the apical portion of the primary lamellae and approximately 0.3 – 0.5 mm in diameter (Figure 4.1). Intralamellar pseudocyst type according to Molnár (2002). Myxospores characteristic of the genus *Henneguya* (Lom and Dyková 2006). Spore body, lanceolate, 17.1 ± 1.0 µm (mean ± standard deviation; range = 14.4-19.3 µm) in length and 5.0 ± 0.3 µm (4.5-5.5 µm) in width. Polar capsules, 2, 6.2 ± 0.4 µm (5.8-7.0 µm) in length and 1.7 ± 0.2 µm (1.4-1.9 µm) in width. Caudal processes, 2, 31.0 ± 4.1 µm (22.9-40.6 µm) in length. Total spore length 48.8 ± 4.8 µm (40.7-61.6 µm). Coils in polar filament, 8-9 when observable. Measurements derived from 22 myxospores (Figures 4.2 and 4.3).

Figure 4.1 Light microscopic image of apical pseudocyst of *Henneguya mississippiensis* n. sp.

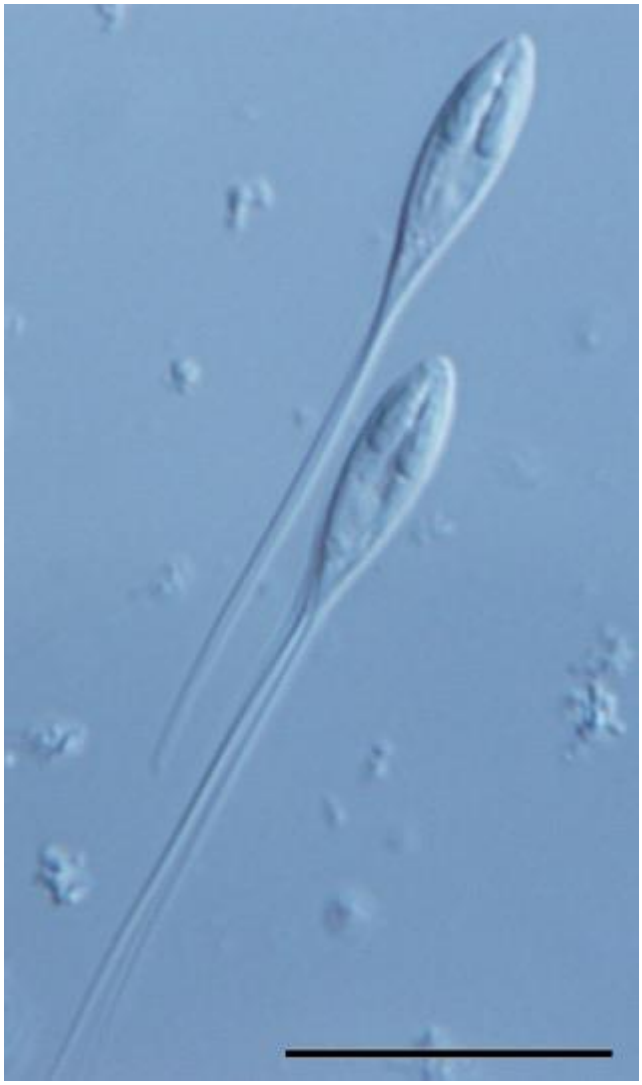

Figure 4.2 Wet mount preparation of myxospores of *Henneguya mississippiensis* n. sp. Scale bar represents 20 µm.

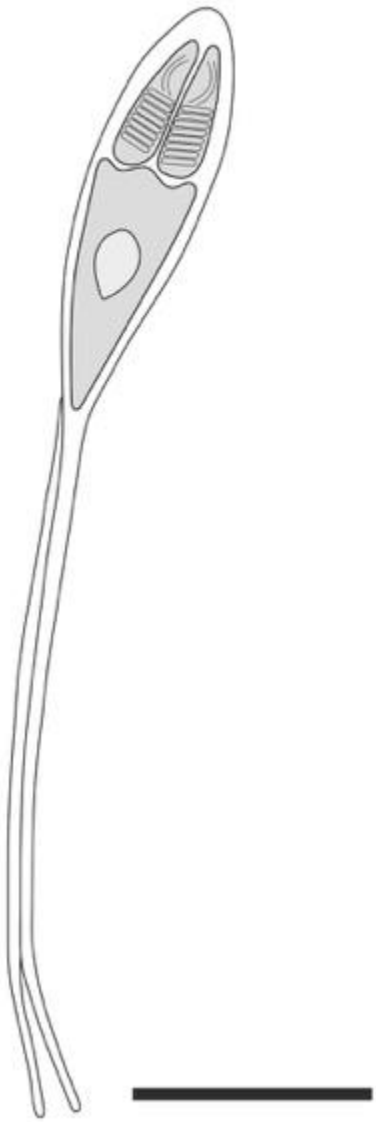

Figure 4.3 Line drawing of *Henneguya mississippiensis* n. sp. myxospore. Scale bar represents 10 µm.

The myxospores share overlapping characteristics with other *Henneguya* species from North American ictalurid fish (Table 4.1). *H. mississippiensis* n. sp. was morphologically most similar to *H. diversis*, reported both internally (liver and kidney) and externally as tumor-like growths in the channel catfish (Minchew 1977). However,

the spore body of *H. mississippiensis* n. sp. was longer (17.1 µm versus 14.8 µm) and wider (5.0 µm versus 4.0 µm) and the caudal processes of *H. mississippiensis* n. sp. were slightly shorter (31.0 µm versus 34.6 µm).

Table 4.1 Morphological data of *Henneguya mississippiensis* n. sp. myxospores and other *Henneguya* spp. from North American ictalurid fish.

Species	Total Spore Length	Spore Body Length	Spore Body Width	Polar Capsule Length	Polar Capsule Width	Caudal Process Length	Reference
H. mississippiensis n. sp.	48.8 (40.7-61.6)	17.1 (14.4-19.3)	5.0 (4.5-5.5)	6.2 (5.8-7.0)	1.7 (1.4-1.9)	31.0 (22.9-40.6)	This paper
H. adiposa	55.6 (40.7-65.8)	17.1 (14.7-20.5)	4.1 (3.4-4.6)	7.2 (5.8-8.3)	1.3 (0.9-1.9)	38.0 (23.2-48.8)	Griffin et al. 2009
H. ameiurensis	NA	23.3	4.1	5.4	1.6	15.0-41.5	Nigrelli and Smith 1940
H. bulbosus	57.2 (46.8-66.8)	17.1 (15.0-19.3)	4.8 (3.7-5.6)	5.8 (5.1-6.4)	1.7 (1.4-1.9)	40.0 (29.5-50.0)	Rosser et al. 2014b
H. diversis	49.5 (40.0-62.0)	14.8 (13.0-16.5)	4.0 (3.2-5.0)	6.5 (6.0-7.5)	1.5 (1.0-2.0)	34.6 (25.0-47.0)	Minchew 1977
H. exilis	69.9 (60.0-90.0)	17.6 (16.0-19.0)	4.9 (4.0-5.0)	8.5 (7.0-9.0)	1.5-2	52.3 (41.0-73.0)	Kudo 1920
H. gurlei	60.9 (48.7-68.5)	18.2 (15.7-20.3)	5.4 (3.8-6.1)	5.9 (4.8-7.1)	1.2 (1.0-1.5)	41.1 (34.0-49.9)	Iwanowicz et al. 2008
H. ictaluri	NA	23.9 (20.8-26.1)	6.0 (4.5-6.4)	8.1 (7.6-9.6)	2.5 (2.0-3.2)	63.0 (48.1-80.2)	Pote et al. 2000
H. limatula	NA	13.7	5.0-6.0	6.5-8.0	1.5-2.0	27.0-37.0	Meglitsch 1937
H. longicauda	108.3 (91.0-127.0)	16.2 (14.0-17.5)	4.0 (3.5-4.5)	7.7 (7.0-8.5)	1.8 (1.5-2.0)	90.5 (75.0-110.0)	Minchew 1977
H. pellis	100.4 (79.0-124.0)	13.0 (11.0-14.5)	5.0 (4.5-5.2)	6.9 (5.5-8.5)	1.8 (1.5-2.0)	87.8 (66.0-112.0)	Minchew 1977
H. postexilis	52.0 (42.0-62.0)	15.0 (13.5-17.)	3.4 (3.5-4.0)	6.6 (5.9-7.2); 7.0 (6.0-8.0)	1.5 (1.0-2.0)	37.0 (28.0-49.0)	Minchew 1977
H. sutherlandi	65.9 (48.2-90.0)	15.4 (12.2-19.3)	5.5 (4.5-6.8)	6.1 (4.0-7.9)	1.7 (1.0-2.2)	50.5 (34.8-71.4)	Griffin et al. 2008

4.4.2 Molecular analyses of myxospores

The 1980 bp 18S rRNA gene sequence was used in a BLASTn search for highly similar sequences in the NCBI nr/nt database (Altschul et al. 1990). The closest match (99.4% (1970/1982 bp) sequence similarity; 100% coverage) was the aurantiactinomyxon actinospore of *A. mississippiensis* (AF021878). The second closest matches were the myxospores of *H. adiposa* (95% similarity and 100% coverage; EU492929), which forms pseudocysts on the adipose fin of the channel catfish (Griffin et al. 2009) and *H. ictaluri* (95% similarity and 100% coverage; AF195510), the causative agent of proliferative gill disease in channel catfish (Pote et al. 2000). Phylogenetic analysis using maximum likelihood clustered the myxospore isolate with its corresponding actinospore stage, *A. mississippiensis*, within a larger cluster of *Henneguya* spp. from ictalurid fishes in North America (Figure 4.4).

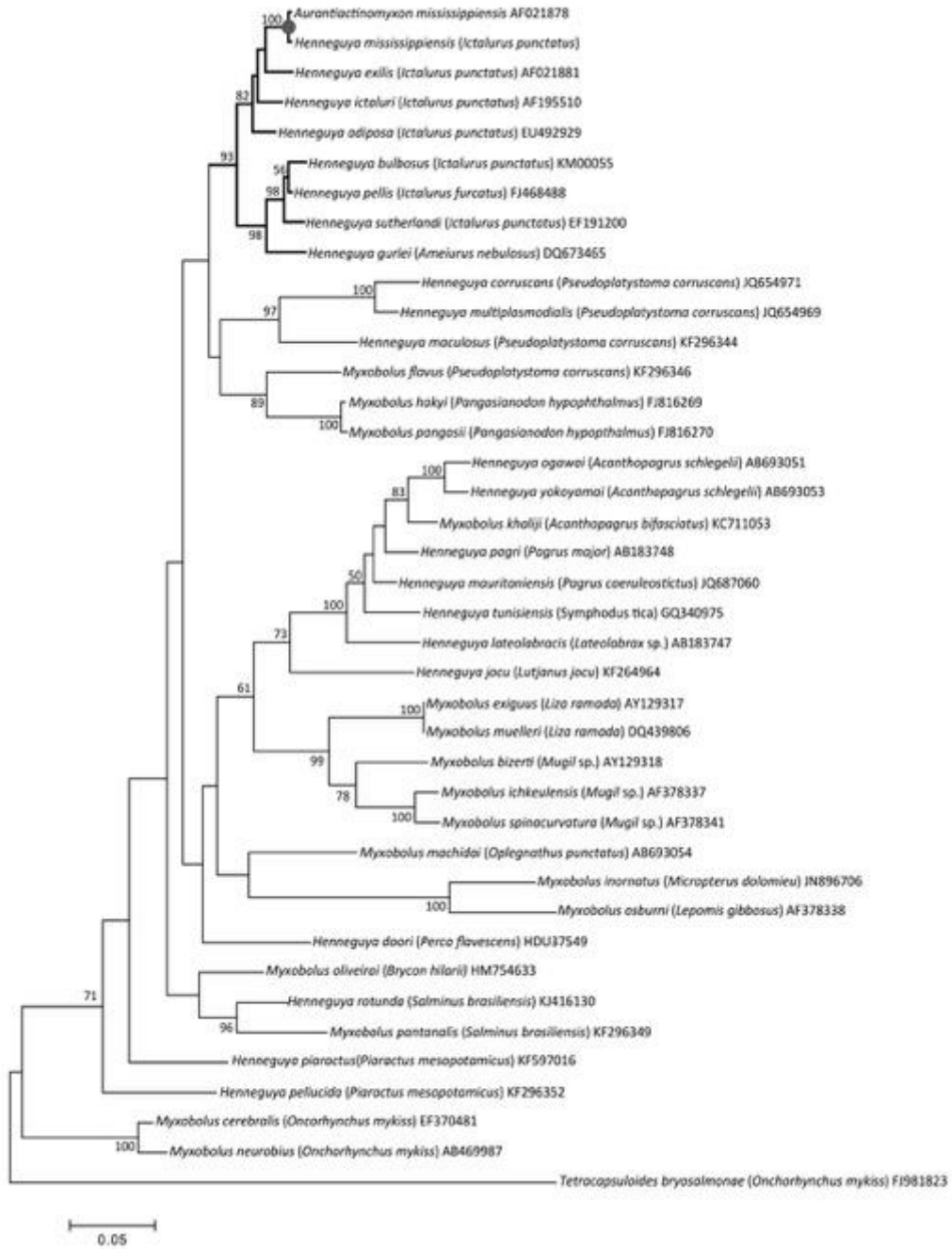

Figure 4.4 Maximum likelihood analysis of *Henneguya mississippiensis* 18S rRNA gene sequence.

Fish hosts are indicated in parentheses. Values at each node represent bootstrap confidence values (n=1,000 replicates). Circle represents the clustering of the *H. mississippiensis* n. sp. isolate in this paper with the previously sequenced actinospore isolate *Aurantiactinomyxon mississippiensis*. Bold branches represent the North American ictalurid infecting clade. Bootstrap values less than 50 have been omitted.

4.4.3 Taxonomic summary

Type species: *Henneguya mississippiensis* n. sp. (Myxozoa: Myxosporea)

Type host: *Ictalurus punctatus* (Rafinesque, 1818) (Siluriformes, Ictaluridae)

Site of infection: apical portion of the primary gill lamellae

Type locality: commercial catfish pond, Washington County, MS, USA

Materials deposited: holotype USNM 1270623, Smithsonian Institution, National Museum of Natural History, Washington DC, USA

4.5 Discussion

Henneguya mississippiensis n. sp. parasitizes the gills of commercially raised channel catfish, with pseudocysts forming toward the apical end of the primary lamellae. Myxozoans that form apical lamellar pseudocysts have been described in other fish species, but this is the first description in channel catfish. In South America plasmodia of *Henneguya azevedoi* are located primarily, but not limited to, the apical end of the lamellae of piava *Leporinus obtusidens* (Barassa et al. 2012). In a survey of Asian redtail catfish *Hemibagrus nemurus* from Malaysia, *Henneguya mystusia* was observed forming large plasmodia at the tip of gill filaments, deforming the lamellar structure. These large plasmodia were thought to be formed by the fusion of smaller neighboring plasmodia (Molnár et al. 2006). Lastly, *Myxobolus macrocapsularis* forms plasmodia in the afferent artery at the apical end of the gill filaments of white bream *Blicca bjoerkna* and common bream *Abramis brama* in Europe (Székely et al. 2002; Molnár et al. 2011).

The myxospores are morphologically unremarkable and bear similarities to other *Henneguya* species described from the channel catfish. Therefore, in order to more accurately describe the isolate, sequencing of the 18S rRNA gene was performed and

phylogenetic analysis conducted. The myxospore isolate shared >99.4 %(1970/1982 bp) sequence similarity with the aurantiactinomyxon actinospore *A. mississippiensis*, suggesting these stages are conspecific. This in line with the intraspecific variability reported for other myxozoans (Kent et al. 1998; Andree et al. 1999; Hallett et al. 2004; Easy et al. 2005; Whipps et al. 2006; Bartosova and Fiala 2011; Griffin et al. 2014; Scott et al. 2014). Following the guidelines for designating species names within the Myxozoa, the myxospore isolate has priority over the actinospore, even when the latter is described first (Kent et al. 1994). The actinospore genus name is suppressed and the myxospore genus name is adopted. As a result, we propose the adoption of the new taxon *H. mississippiensis* n. sp.

Phylogenetic analysis confirmed the placement of *H. mississippiensis* n. sp. among the other species of *Henneguya* parasitizing ictalurid fish species in North America with high bootstrap support (Figure 4.4). *Henneguya mississippiensis* represents the fourth *Henneguya* sp. life cycle to be described. Pote et al. (2000) elucidated the life cycle of *H. ictaluri* by exposing naïve channel catfish to aurantiactinomyxon actinospores isolated from naturally infected *D. digitata.* At 90 days post exposure, *Henneguya* myxospores were detected in the gills of infected fish. Furthermore when sequenced the 18S rRNA gene sequences of the actinospore and myxospore stages were identical (Pote et al. 2000). Similarly, Kallert et al. (2005) exposed naïve brown trout, brook trout, and common carp to triactinomyxon actinospores from naturally infected *T. tubifex* and observed *Henneguya* myxospores in connective tissue at 102 days post exposure. In addition, a 1417 bp fragment of the 18S rRNA gene was identical for both the actinospore and myxospore stages (Kallert et al. 2005). Lin et al. (1999) sequenced the

18S rRNA gene of an aurantiactinomyxon actinospore stage from *D. digitata* collected from commercial catfish ponds and the myxospore stages of *H. exilis* from the gill tissue of naturally infected channel catfish. Alignments of the two sequences from each stage demonstrated them to be conspecific (Lin et al. 1999).

Herein we unite the aurantiactinomyxon actinospore stage of *A. mississippiensis,* previously described from the oligochaete *Dero digitata,* with the corresponding myxospore stage of *H. mississippiensis* n. sp. from the gills of channel catfish using 18S rRNA gene sequences. Numerous researchers have employed this method to elucidate myxozoan life cycles, while others utilize experimental infection studies when capable (Andree et al. 1997; Lin et al. 1999; Pote et al. 2000; Kallert et al. 2005). As attested by Kallert et al. (2005), life cycle studies of myxozoan parasites are often labor intensive and require sources and maintenance of fish and oligochaete cultures for extended periods of time and require knowledge of both hosts involved in the life cycle. Currently, a laboratory model for maintaining *Henneguya* spp. from ictalurid fish and their oligochaete hosts does not exist. However culture methods for *D. digitata* have been established (Mischke and Griffin 2011). *Dero digitata* has been identified as an oligochaete host of at least 7 myxozoans associated with channel catfish ponds (Bellerud 1993; Rosser et al. 2014a). Future research will focus on experimental elucidation of these currently unknown life cycles. Understanding the life cycle dynamics of myxozoan parasites of farm raised catfish is the first step in developing management practices to minimize the impact of these parasites on catfish aquaculture.

4.6 References

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403–410.

Andree, K. B., S. J. Gresoviac, and R. P. Hedrick. 1997. Small subunit ribosomal RNA sequences unite alternate actinosporean and myxosporean stages of *Myxobolus cerebralis* the causative agent of whirling disease in salmonid fish. Journal of Eukaryotic Microbiology 44:208–215.

Andree, K. B., M. El-Matbouli, R. W. Hoffman, and R. P. Hedrick. 1999. Comparison of 18S and ITS-1 rDNA sequences of selected geographic isolates of *Myxobolus cerebralis.* International Journal for Parasitology 29:771–775.

Atkinson, S. D., and J. L. Bartholomew. 2009. Alternate spore stages of *Myxobilatus gasterostei*, a myxosporean parasite of three-spined sticklebacks (*Gasterosteus aculeatus*) and oligochaetes (*Nais communis*). Parasitology Research 104:1173–1181.

Barassa B., E. A. Adriano, N. S. Cordeiro, S. Arana, and P. S. Ceccarelli. 2012. Morphology and host-parasite interaction of *Henneguya azevedoi* n. sp., parasite of gills of *Leporinus obtusidens* from Mogi-Guaçu River, Brazil. Parasitology Research 110:887–894.

Barta, J. R., D. S. Marin, P. A. Liberator, M. Dshkevicz, J. W. Anderson, S. D. Feigner, A. Elbrecht, A. Perkins-Barrow, M. C. Jenkins, H. D. Danforth, M. D. Ruff, and H. Profous-Juchelka. 1997. Phylogenetic relationships among eight *Eimeria* species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. Journal of Parasitology 83:262–271.

Bartošová, P., and I. Fiala. 2011. Molecular evidence for the existence of cryptic species assemblages of several myxosporeans (Myxozoa). Parasitology Research 108:573–583.

Bartholomew, J. L., S. D. Atkinson, and S. L. Hallett. 2006. Involvement of *Manayunkia speciosa* (Annelida: Polychaeta: Sabellidae) in the life cycle of *Parvicapsula minibicornis*, a myxozoan parasite of Pacific salmon. Journal of Parasitology 92:742–748.

Bellerud, B. L. 1993. Etiological and epidemiological factors effecting outbreaks of proliferative gill disease on Mississippi channel catfish farms. Dissertation, College of Veterinary Medicine, Mississippi State University

Caffara, M., E. Raimondi, D. Florio, F. Marcer, F. Quaglio, and M. L. Fioravanti. 2009. The life cycle of *Myxobolus lentisuturalis* (Myxozoa: Myxobolidae), from goldfish (*Carassius auratus auratus*), involves a Raabeia-type actinospore. Folia Parasitologica 56:6–12.

Easy, R. H., S. C. Johnson, and D. K. Cone. 2005. Morphological and molecular comparison of *Myxobolus procerus* (Kudo, 1934) and *M. intramusculi* n. sp. (Myxozoa) parasitizing muscles of the trout-perch *Percopsis omiscomaycus*. Systematic Parasitology 61:115–122.

Eiras, J. C., and E. A. Adriano. 2012. A checklist of new species of *Henneguya* Thélohan, 1892 (Myxozoa: Myxosporea, Myxobolidae) described between 2002 and 2012. Systematic Parasitology 83:95–104.

El-Mansy, A., and K. Molnár. 1997a. Development of *Myxobolus hungaricus* (Myxosporea: Myxobolidae) in oligochaete alternate hosts. Diseases of Aquatic Organisms 31:227–232.

El-Mansy, A., and K. Molnár. 1997b. Extrapiscine development of *Myxobolus drjagini* Akhmerov, 1954 (Myxosporea: Myxobolidae) in oligochaete alternative hosts. Acta Veterinaria Hungarica 45:427–438.

El-Mansy, A., K. Molnár, and C. Székely. 1998. Development of *Myxobolus portucalensis* Saraiva and Molnár, 1990 (Myxosporea: Myxobolidae) in the oligochaete *Tubifex tubifex* (Müller). Systematic Parasitology 41:95–103.

Eszterbauer, E., C. Székely, K. Molnár, F. Baska. 2000. Development of *Myxobolus bramae* (Myxosporea: Myxobolidae) in an oligochaete alternate host, *Tubifex tubifex*. Journal of Fish Diseases 23:19–25.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the boostrap. Evolution 39:783–791.

Griffin, M. J., D. J. Wise, and L. M. Pote. 2009. Morphology and small-subunit ribosomal DNA sequence of *Henneguya adiposa* (Myxosporea) from *Ictalurus punctatus* (Siluriformes). Journal of Parasitology 95:1076–1085.

Griffin, M. J., L. M. Pote, D. J. Wise, T. E. Greenway, M. J. Mauel, and A. C. Camus. 2008. A novel *Henneguya* species from channel catfish described by morphological, histological, and molecular characterization. Journal of Aquatic Animal Health 20:127–135.

Griffin, M. J., S. Quiniou, C. Ware, L. Bogdanovic, and E. Soto. 2014. *Kudoa thunni* from blackfin tuna (*Thunnus atlanticus*) harvested off the island of St. Kitts, West Indies. Journal of Parasitology 100:110–116.

Hallett, S. L., S. D. Atkinson, C. Erséus, and M. El-Matbouli. 2004. Molecular methods clarify morphometric variation in triactinomyxon spores (Myxozoa) released from different oligochaete hosts. Systematic Parasitology 57:1–14.

Hanson, L. A., D. Lin, L. M. W. Pote, and R. Shivaji. 2001. Small subunit rRNA gene comparisons of four actinosporean species to establish a polymerase chain reaction test for the causative agent of proliferative gill disease in channel catfish. Journal of Aquatic Animal Health 13:117–123.

Holzer, A. S., C. Sommerville, and R. Wootten. 2004. Molecular relationships and phylogeny in a community of myxosporeans and actinosporeans based on their 18S rDNA sequences. International Journal for Parasitology 34:1099–1111.

Holzer, A. S., C. Sommerville, and R. Wootten. 2006. Molecular identity, phylogeny and life cycle of *Chloromyxum schurovi* Shul'man & Ieshko 2003. Parasitology Research 99:90–96.

Iwanowicz, L. R., D. D. Iwanowicz, L. M. Pote, V. S. Blazer, and W. B. Schill. 2008. Morphology and 18S rDNA of *Henneguya gurlei* (Myxosporea) from *Ameiurus nebulosus* (Siluriformes) in North Carolina. Journal of Parasitology 94:46–57.

Kallert, D. M., E. Eszterbauer, M. El-Matbouli, C. Erséus, and W. Haas. 2005. The life cycle of *Henneguya nuesslini* Schuberg & Schröder, 1905 (Myxozoa) involves a triactinomyxon-type actinospore. Journal of Fish Diseases 28:71–79.

Kent, M. L., K. B. Andree, J. L. Bartholomew, M. El-Matbouli, S. S. Desser, R. H. Devlin, S. W. Feist, R. P. Hedrick, R. W. Hoffmann, J. Khattra, S. L. Hallett, R. J. G. Lester, M. Longshaw, O. Palenzeula, M. E. Siddall, and C. Xiao. 2001. Recent advances in our knowledge of the Myxozoa. Journal of Eukaryotic Micriobiology 48:395–413.

Kent, M. L., Khattra J., D. M. L. Hervio, and R. H. Devlin. 1998. Ribosomal DNA sequence analysis of isolates of the PKX myxosporean and their relationship to members of the genus *Sphaerospora*. Journal of Aquatic Animal Health 10:12–21.

Kent, M. L., L. Margolis, and J. O. Corliss. 1994. The demise of a class of protists: taxonomic and nomenclatural revisions proposed for the protist phylum Myxozoa Grassé, 1970. Canadian Journal of Zoology 72:932–937.

Køie, M., E. Karlsbakk, and A. Nylund. 2008. The marine herring myxozoan *Ceratomyxa auerbachi* (Myxozoa: Ceratomyxidae) uses *Chone infundibuliformis* (Annelida: Polychaeta: Sabellidae) as invertebrate host. Folia Parasitologica 55:100–104.

Kudo, R. R. 1920. Studies on Myxosporidia: a synopsis of genera and species of Myxosporidia. Illinois Biological Monographs 5:1–265.

Kudo, R. 1929. Histozoic myxosporidia found in freshwater fishes in Illinois, USA. Archiv für Protistenkunde 65:364–378.

Lin, D., L. A. Hanson, and L. M. Pote. 1999. Small subunit ribosomal RNA sequence of *Henneguya exilis* (Class Myxosporea) identifies the actinosporean stage from an oligochaete host. Journal of Eukaryotic Microbiology 46:66–68.

Lom J, and I. Dyková. 2006. Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. Folia Parasitologica 53:1–36.

Markiw, M. E., and K. Wolf. 1983. *Myxosoma cerebralis* (Myxozoa: Myxosporea) etiologic agent of salmonid whirling disease requires tubificid worm (Annelida: Oligochaeta) in its life cycle. Journal of Protozoology 30:561–564.

Meglitsch, P. A. 1937. On some new and known Myxosporidia of the fishes of Illinois. Journal of Parasitology 23:467–477.

Minchew, C. D. 1977. Five new species of *Henneguya* (Protozoa: Myxosporida) from Ictalurid fishes. Journal of Protozoology 24:213–220.

Mischke, C. C., and M. J. Griffin. 2011. Laboratory mass culture of the freshwater oligochaete *Dero digitata*. North American Journal of Aquaculture 73:13–16.

Molnár, K. 2002. Site preference of fish myxosporeans in the gill. Diseases of Aquatic Organisms 48:197–207.

Molnár, K., A. El-Mansy, C. Székely, and F. Baska. 1999. Development of *Myxobolus dispar* (Myxosporea: Myxobolidae) in an oligochaete alternate host, *Tubifex tubifex*. Folia Parasitologica 46:15–21.

Molnar, K., C. Székely, K. Mohamed, and F. Shaharom-Harrison. 2006. Myxozoan pathogens in cultured Malaysian fishes. II. Myxozoan infections of redtail catfish *Hemibagrus nemurus* in freshwater cage cultures. Diseases of Aquatic Organisms 68:219–226.

Molnár, K., G. Cech, and C. Székely. 2011. Histological and molecular studies of species of *Myxobolus* Bütschli, 1882 (Myxozoa: Myxosporea) in the gills of *Abramis, Blicca* and *Vimba* spp. (Cyprinidae), with the redescription of *M. macrocapsularis* Reuss, 1906 and *M. bliccae* Donec & Tozyyakova, 1984. Systematic Parasitology 79:109–121.

Nigrelli, R. F., and G. M. Smith. 1940. A papillary cystic disease affecting the barbels of *Ameiurus nebulosus* (la Sueur), caused by the myxosporidian *Henneguya ameiurus* sp. nov. Zoologica 25:89–94.

Pote, L. M., L. A. Hanson, and R. Shivaji. 2000. Small subunit ribosomal RNA sequences link the cause of proliferative gill disease in channel catfish to *Henneguya* n. sp. (Myxozoa: Myxosporea). Journal of Aquatic Animal Health 12:230–240.

Rosser, T. G., M. J. Griffin, S. M. Quiniou, T. E. Greenway, L. H. Khoo, D. J. Wise, and L. M. Pote. 2014a. Molecular and morphological characterization of myxozoan actinospore types from a commercial catfish pond in the Mississippi Delta. Journal of Parasitology 100:828–839.

Rosser, T. G., M. J. Griffin, S. M. A. Quiniou, L. H. Khoo, and L. M. Pote. 2014b. 18S rRNA gene sequencing identifies a novel species of *Henneguya* parasitizing the gills of the channel catfish (Ictaluridae) Parasitology Research 113:4651– 4658.

Scott, S. J., M. J. Griffin, S. Quiniou, L. Khoo, and T. K. Bollinger. 2014. *Myxobolus neurophilus* Guilford (Myxosporea: Myxobolidae): A common parasite infecting yellow perch *Perca flavacens* (Mitchell, 1814) in Saskatchewan, Canada. Journal of Fish Diseases 38:355–364.

Székely, C., A. El-Mansy, K. Molnár, and F. Baska. 1998. Development of *Thelohanellus hovorkai* and *Thelohanellus nikolskii* (Myxosporea: Myxozoa) in oligochaete alternate hosts. Fish Pathology 33:107–114.

Székely, C., K. Molnár, E. Eszterbauer, and F. Baska. 1999. Experimental detection of the actinospores of *Myxobolus pseudodispar* (Myxosporea: Myxobolidae) in oligochaete alternate hosts. Diseases of Aquatic Organisms 38:219–224.

Székely, C., K. Molnár, and O. Rácz. 2001. Complete developmental cycle of *Myxobolus pseudodispar* (Gorbunova) (Myxosporea: Myxobolidae). Journal of Fish Diseases 24:461–468.

Székely, C., O. Rácz, K. Molnár, and E. Eszterbauer. 2002. Development of *Myxobolus macrocapsularis* (Myxosporea: Myxobolidae) in an oligochaete alternate host, *Tubifex tubifex*. Diseases of Aquatic Organisms 48:117–123.

Székely, C., S. L. Hallett, S. D. Atkinson, and K. Molnár. 2009. Complete life cycle of *Myxobolus rotundus* (Myxosporea: Myxobolidae), a gill myxozoan of common bream *Abramis brama*. Diseases of Aquatic Organisms 85:147–155.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6 Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30:2725–2729.

Tamura, K., and M. Nei. 1993. Estimation of the number of nuecleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10:512–526.

Yokoyama, H. 1997. Transmission of *Thelohanellus hovorkai* Achmerov, 1960 (Myxosporea: Myxozoa) to common carp *Cyprinus carpio* through the alternate oligochaete host. Systematic Parasitology 36:79–84.

CHAPTER V

MORPHOLOGICAL, HISTOLOGICAL AND MOLECULAR DESCRIPTION OF *UNICAUDA FIMBRETHILAE* N. SP. (CNIDARIA: MYXOSPOREA: MYXOBOLIDAE) FROM THE INTESTINAL TRACT OF CHANNEL CATFISH *ICTALURUS PUNCTATUS*

5.1 Abstract

The channel catfish *Ictalurus punctatus* is a known host for 10 species of *Henneguya*, but few other myxozoan genera are described from channel catfish. *Unicauda* is a genus of myxozoan parasites within the family Myxobolidae that consists of 10 valid species from freshwater fish. Herein, we describe a novel species of *Unicauda* from the intestinal tract of farm-raised channel catfish in Mississippi, USA. Myxospores were consistent with the genus *Unicauda*, but exhibited a unique branching at the terminal end of the caudal process that has not previously been reported. Myxospores measured 90.39 ± 14.97 µm (mean ± SD; range=70.88–126.02 µm) in total length. The spherical spore body measured 7.31 ± 0.26 µm (6.75–7.84 µm) in length and 7.01 ± 0.63 µm (6.1–8.01 µm) in width. The two polar capsules measured 3.45 ± 0.33 µm (3.02–4.03 µm) in length and 2.65 ± 0.32 µm (2.18–3.11 µm) in width. The single caudal process measured 82.98 ± 14.97 µm (63.39–118.63 µm) in length from the base of the spore body to the end of the most terminal projection. Terminal projections measured 26.83 ± 8.8 µm (12.34–42.29 µm) in length and 0.95 ± 0.23 µm (0.52–1.6 µm) in width.

The 18S rRNA gene sequence obtained did not match any published sequences. Given the uniqueness of the myxospore morphology, histological presentation, and gene sequence data, we describe this as an unreported species, *Unicauda fimbrethilae* n. sp.

5.2 Introduction

Myxozoans are common metazoan parasites of freshwater and marine fish worldwide, with a few exceptions existing in higher vertebrates (Kent et al. 2001; Lom and Dyková 2006). The typical myxozoan life cycle involves the actinospore stage released by an annelid worm and the myxospore stage which develops in the fish host. The most extensively studied myxozoans are associated with disease in economically important cultured and wild fishes, and are predominantly members of the family Myxobolidae (Lom and Dyková 2006). Of the myxobolids, the genera *Myxobolus* (Eiras et al. 2014) and *Henneguya* (Eiras and Adriano 2012) are the most comprehensively studied and have the most described species.

Unicauda species are also classified within the Myxobolidae, but are poorly represented. Recent studies have determined there to be only 10 valid species (Cone and Melendy 2000; Fiala et al. 2015). The myxospores of *Unicauda* are characterized by a spherical spore body containing two polar capsules located at the apex of the spore and a single caudal process. To date, *Unicauda* has only been described from freshwater fish species, primarily of the order Cypriniformes. *Unicauda pelteobagrus* from *Pelteobagrus fulvidraco, P. nitigus,* and *P. vachelli* in China is the only valid species described from the order Siluriformes (Chen and Ma 1998; Cone and Melendy 2000). Many of these species exist as single records, with the majority lacking histopathological descriptions of associated infections and 18S rRNA sequence data (Cone and Melendy

2000). At present, an actinospore stage from an alternate host has not been identified for any species within the genus.

Herein we describe the first species of *Unicauda* parasitizing channel catfish. The unique morphological description, histological presentation, and 18S rRNA gene sequence support this as a new species, *Unicauda fimbrethilae* n. sp., found parasitizing the intestinal tract of naturally infected channel catfish.

5.3 Materials and methods

5.3.1 Case history and histology

Channel catfish from a production pond in Eastern Mississippi were submitted in late March 2015 to the Aquatic Diagnostic Laboratory at the Mississippi State University College of Veterinary Medicine. The pond was experiencing increased mortalities in adult fish. Specimens were subjected to a full examination, including gill clips and mucus wet mounts, as well as internal examination. The spleen and kidney (mesonephros) were swab cultured for aerobic bacteria. A full complement of tissues was fixed in 10% neutral buffered formalin. Tissues were trimmed and embedded in paraffin for standard microtome sectioning and staining with hematoxylin and eosin (H & E). Select sections were also histochemically stained using standard periodic acid-Schiff (PAS) and Giemsa staining methods.

5.3.2 Fish collection

Initial diagnosis identified the presence of myxozoan parasites in the esophagus, warranting further investigation. Twenty-one live moribund, market-size channel catfish (~0.5-0.75 kg) were collected from the banks of the affected pond and transported live to

the Mississippi State University College of Veterinary Medicine for necropsy. Fish were euthanized with an overdose of MS-222 (tricaine methanesulfonate, Tricaine-S®, Western Chemical Inc., Ferndale, Washington) and examined both internally and externally for myxozoan parasites. Fish were opened along the ventral surface by sharp dissection and the gastrointestinal tract (esophagus to anus) was removed. The esophagus and anus were opened longitudinally and scrapings at each site were made using a sterile scalpel blade, placed on a clean glass microscope slide with physiological saline and coverslipped. Smears were viewed using a BH-2 Olympus microscope (Olympus Optical Co Ltd, Tokyo, Japan) for detection of myxozoans.

5.3.3 Myxospore collection and morphological characterization

Myxospores observed on intestinal scraping smears were washed into 50-ml conical centrifuge tubes and fixed in 70% molecular biology grade ethanol. Myxospores were later photographed using a BX-50 Olympus microscope with an Olympus DP72 camera with the DP-2-Twain/cellSens software (Olympus Optical Co Ltd, Tokyo, Japan). Measurements were made from digital images taken of ethanol fixed myxospores in accordance with previous descriptions of *Unicauda* species (Minchew, 1981; Cone and Melendy, 2000).

5.3.4 Molecular characterization of myxospores

Myxospores were pelleted by centrifugation at 10,000 x g for 5 min. Genomic DNA was extracted from pelleted myxospores using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, California). The 18S rRNA gene was amplified by polymerase chain reaction (PCR) using universal (Barta et al. 1997) and myxozoan specific primers

(Kent et al. 2000; Hallett and Diamant, 2001; Hanson et al. 2001; Fiala 2006; Griffin et al. 2008) (Table 5.1). The 25-µl reactions consisted of 22 µl of Platinum Taq Supermix (Invitrogen, Carlsbad, California), 10 pmol of each primer, 1 µl of genomic DNA template and nuclease-free water to volume. The following primers were paired in the PCRs: ERIB1 and ACT1R, MyxospecF and MyxospecR, Myxo1F and Myxgen3R, Genmyxo4 and ERIB10, and H2 and H9. All amplifications were performed on an MJ Research PTC-200 thermocycler (GMI, Ramsey, Minnesota) with a cycling protocol consisting of an initial denaturation of 95ºC for 10 min, followed by 35 cycles of 95ºC for 1 min, 52ºC for 1 min, 72ºC for 2 min, and a final extension at 72ºC for 10 min. Amplicons were electrophoresed through 1.2% agarose gels in the presence of ethidium bromide (0.5 mg/ml) and visualized under UV light. Band sizes were estimated by comparison with a concurrently run molecular weight marker (HyperLadder™ 50 bp, Bioline, London, United Kingdom) to confirm the presence of the appropriate sized bands. Amplicons were purified using the QIAquick PCR Purification Kit (QIAGEN, Valencia, California) and sequenced directly (Eurofins MWG Operon LLC, Huntsville, Alabama). Individual sequencing reads were assembled into a single contiguous sequence using the SeqMan™ utility of the Lasergene package (DNASTAR, Madison, Wisconsin). The assembled 18S rDNA sequence was compared to somewhat similar sequences deposited into the National Center for Biotechnology Information non-redundant nucleotide database using a BLASTn search (Altschul et al. 1990). Phylogenetic histories were inferred from an alignment of 18S rRNA gene sequences of closely related myxozoans identified by the BLASTn search and downloaded from the NCBI database. Only published sequences greater than 1500-bp were used in the

alignment. Alignment and phylogenetic analysis were conducted in MEGA6 (Tamura et al. 2013). The final dataset contained 1170 positions and phylogenetic placement of the novel *Unicauda* species was determined by maximum likelihood using the Tamura-Nei model (Tamura and Nei 1993). The initial tree was obtained by maximum parsimony and the final tree was constructed from 1000 bootstrap replicates (Felsenstein 1985).

Table 5.1 Primers used in ampflication of the 18S rRNA gene.

Primer	Sequence (5'-3')	Reference
ACT1R	AATTTCACCTCTCGCTGCCA	Hallett & Diamant (2001)
ERIB1	ACCTGGTTGATCCTGCCAG	Barta et al. (1997)
ERIB10	CCTCCGCAGGTTCACCTACGG	Barta et al. (1997)
Genmyxo4	GGATGTTGGTTCCGTATTGG	Griffin et al. (2008)
H2	CGACTTTTACTTCCTCGAAATTGC	Hanson et al. (2001)
H9	TTACCTGGTCCGGACATCAA	Hanson et al. (2001)
Myxo1F	CTGCCCTATCAACTWGTT	Kent et al. 2000
Myxogen3R	TGCCTTCGCATTYGTTAGTCC	Kent et al. 2000
MyxospecF	TTCTGCCCTATCAACTWGTTG	Fiala (2006)
MyxospecR	GGTTTCNCDGRGGGMCCAAC	Fiala (2006)

5.4 Results

5.4.1 Myxospore morphology

Myxozoan plasmodia were not observed grossly or histologically. Myxospores were morphologically consistent with the genus *Unicauda* (Davis 1944), but differed from all previously described species in that many myxospores possessed a single caudal process that differentiated terminally into many root-like projections of varying length and number, thought to signify maturity (Figures 5.1 and 5.2). Less mature myxospores (Figures 5.1C) bore resemblance to previous descriptions of the genus (Lom and Dyková 2006). Spore body, spherical 7.31 ± 0.26 µm (mean ± standard deviation; range = 6.75–7.84 µm) in length and 7.01 ± 0.63 µm (6.1–8.01 µm) in width. Polar capsules, two, 3.45

± 0.33 µm (3.02–4.03 µm) in length and 2.65 ± 0.32 µm (2.18–3.11 µm) in width. Caudal process, one, 82.98 ± 14.97 µm (63.39–118.63 µm) in length from the base of the spore body to the end of the most terminal projection. Projections from the caudal process (Figures 5.1D-E) were variable, 26.83 ± 8.8 µm (12.34–42.29 µm) in length and 0.95 ± 0.23 µm (0.52–1.6 µm) in width. Total spore length 90.39 ± 14.97 µm (70.88–126.02 µm). Coils in polar filament, four, when observable. Measurements derived from 20 myxospores. Immature spores were more variable in length of the caudal process and did not have the unique branching projections but ended bluntly.

Morphologically the spores shared overlapping features with several species of *Unicauda* described from freshwater fish (Table 5.2). The total spore length of myxospores of *Unicauda fimbrethilae* n. sp. was slightly less on average, but shared overlapping ranges with *Unicauda magna* (90.39 µm vs 109.6 µm) described from *Pimephales promelas* (Minchew, 1981). Similarly a vacuole was visible in some myxospores, but other morphological features were not consistent and differed considerably. Indeed the most notable feature of the spores of *U. fimbrethilae* n. sp. is the terminal branches of the caudal process, a feature that to our knowledge has not been described for any member of the Myxobolidae.

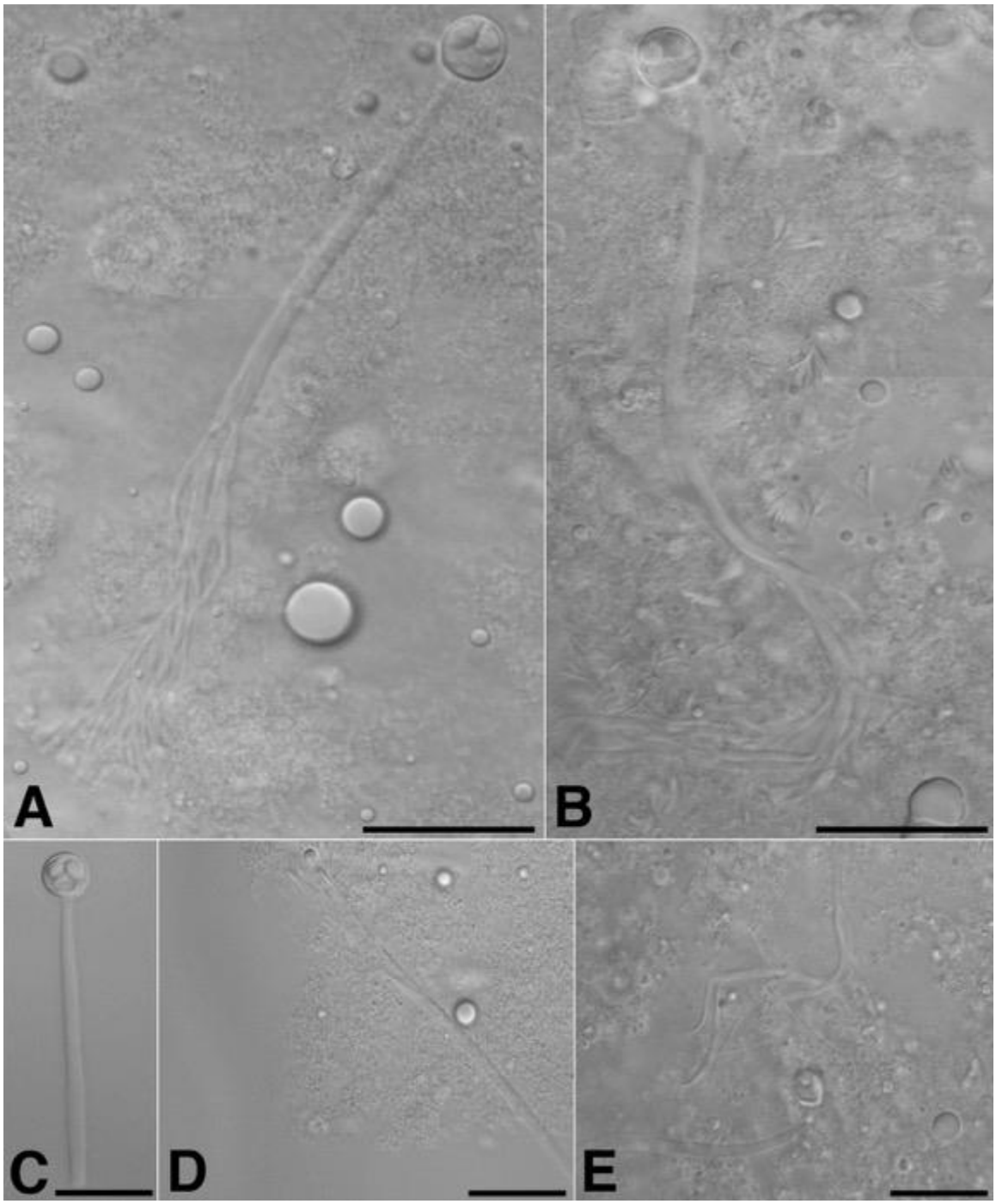

Figure 5.1 Photomicrographs of myxospores of *Unicauda fimbrethilae* n. sp.

(A, B) Mature spores of *Unicauda fimbrethilae* n. sp. (C) Immature spore of *U. fimbrethilae* n. sp. with blunted caudal process. (D, E) Variability in the morphology of the terminal projections of mature spores. Scale bars represent 20 µm.

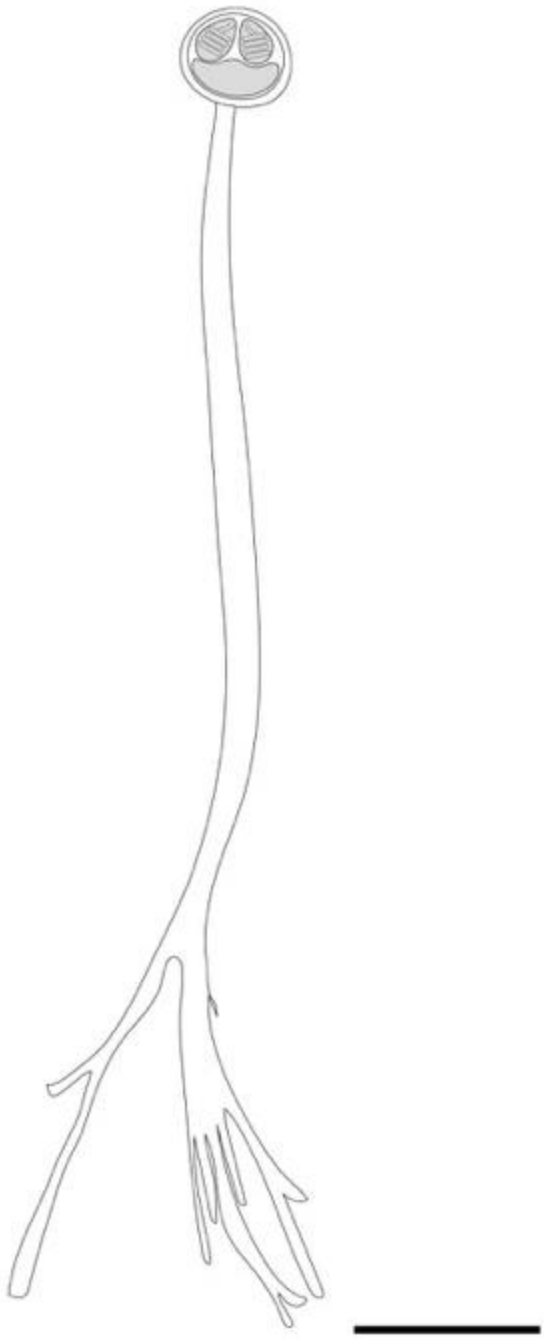

Figure 5.2 Line drawing of mature *Unicauda fimbrethilae* n. sp. myxospore. Scale bar represents 20 µm.

Table 5.2 Morphological comparisons to other species of *Unicauda* described from freshwater fishes worldwide.

Species	TSL	SBL	SBW	PCL	PCW	CPL	CPW	SV	CPF	Fish Host	Location	Reference
U. fimbrethilae	90.39 (70.88-126.02)	7.31 (6.75-7.84)	7.01 (6.1-8.01)	3.45 (3.02-4.03)	2.65 (2.18-3.11)	82.98 (63.39-118.63)	2.53 (2.08-3.23)	6.1 (5.52-6.97)	4	*Ictalurus punctatus*	Intestinal tract	This paper
Unicauda sp.	15.38 (12-18)	9.53 (7-11)	5.08 (4-6)	3.78 (3-5)	1.56 (1-2)	6.54 (6-10)	–	–	6	*Gnathonemus petersii*	Intestinal tract, kidney, liver	Caffara et al. (2007)
U. aristichthyalis	(20-24)	(9.6-11.2)	(5.6-6.3)	(4.4-4.8)	(2.0-2.4)	11.0	–	5.6	–	*Aristichthydis nobilis*	Urinary bladder	Chen and Ma (1998)
U. brachyura	–	(10-11.5)	(8.0-8.8)	(3.0-4.0)	2.0	up to 17.0	–	(4.0-5.0)	–	*Notropis anogenus*	Cartilaginous fin rays	Ward (1919)
U. clavicauda	(20.0-30.0)	(10.5-11.5)	(8.5-9.5)	(5.0-5.5)	(2.5)	(20.0-30.0)	(3.0-6.5)	6.0		*Notropis blennius*	Subdermal connective tissue	Kudo (1934)
U. crassicauda	(12.0-14.5)	(8.5-10.5)	(6.0-7.0)	(5.0-6.0)	(3.0-3.5)	(40.0-55.0)	(3.5-4.5)	(6.0-7.0)		*Campostoma anomalum*	Subcutaneous connective tissue	Kudo (1934)
U. lumae	13.4 (10.0-15.0)	11.0 (8.5-15.0)	8.5 (8.5-10.0)	6.1 (5.0-6.5)	3.0 (1.5-3.5)	(2-8.4)	–	(8.5-15.0)	5	*Barbus grypus*	Liver	Rahemo (1976)
U. macrura	–	(10.0-11.0)	(6.0-8.0)	–	–	(30-40)	–	4.0	–	*Hybognathus nuchalis*	Subcutaneous tissue of the head	Gurley (1893)
U. magna	109.6 (75-170)	15.5 (14-17)	12.1 (10-13)	8.6 (7.5-9.5)	4.1 (3.0-5.0)	94.1 (60-154)	–	–		*Pimephales promelas*	Pectoral, pelvic, and caudal fins	Minchew (1981)
U. pelteobragus	38.2 (31.2-39.2)	13.58 (12.8-14.2)	7.38 (7.0-8.0)	5.68 (4.8-6.4)	2.36 (2.1-2.5)	5.68 (4.8-6.4)	2.36 (2.1-2.5)	5.46 (5.2-5.60	6-7	*Pelteobagrus fulvidraco*	Gills	Chen & Ma, (1998)
U. wuhanensis	–	(4.6-5.1)	(2.8-3.3)	(3.7-4.2)	(2.0-2.3)	(10.0-18.0)	–	(5.8-6.4)	–	*Carassius auratus gibelio*	Nares and surface of body	Chen and Ma (1998)

TSL, total spore length; SBL, spore body length; SBW, spore body width; PCL, polar capsule length; PCW, polar capsule width; CPL, caudal process length; CPW, caudal process width; SV, spore body thickness in sutural view; CPF, coils in polar filament; –, data not available.

5.4.2 Gross and histopathology

Fish exhibited severe skin ulceration with secondary oomycete infection (Winter Kill Syndrome) as well as a systemic *Aeromonas sobria* infection. Examination of the rectum revealed numerous intraepithelial myxozoan myxospores and developmental stages within the epithelium. Severe inflammation with epithelial injury and erosion affected greater than 50% of the rectal mucosa and submucosa (Figure 5.3A). The epithelium was irregular and highly vacuolated due to parasitophorous vacuoles. As a result of significant erosion, epithelial cells were hyperplastic, being large and pleomorphic with large nuclei and nucleoli. The superficial lamina propria was expanded by many large macrophages which were replaced by lymphocytes in the deeper tissues (Figure 5.3B). Within epithelial cells, mature spores inhabited spacious clear vacuoles, while developmental stages were tightly clustered in smaller vacuoles (Figure 5.3C). Occasional small granulomas were present in the outer muscularis of severely affected segments; no parasites were identified within. Small numbers of intraepithelial organisms were present in the mucosa of the esophagus and rarely in the intestine.

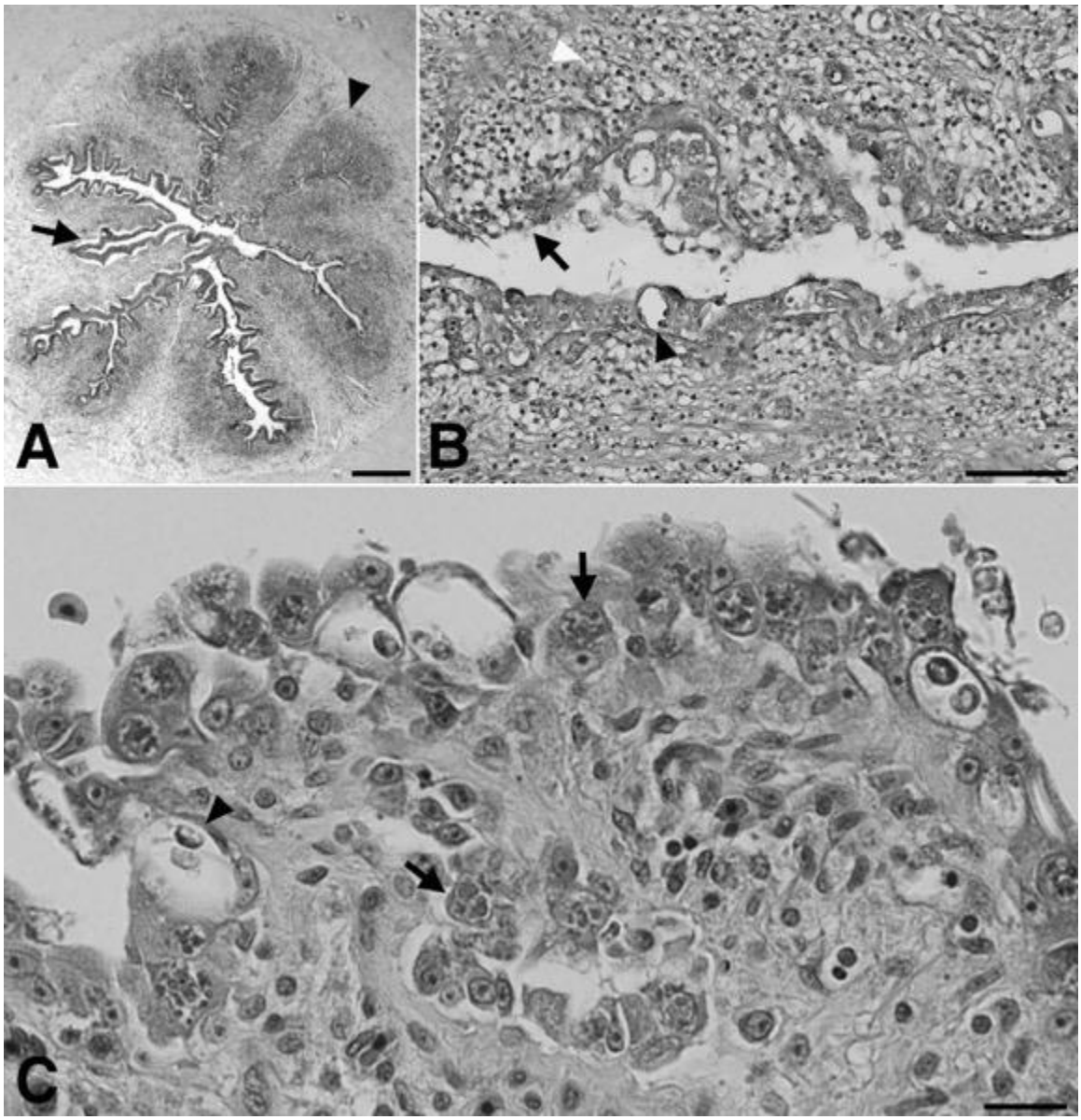

Figure 5.3 Photomicrographs of the rectum of a channel catfish with a *Unicauda fimbrethilae* n. sp. infection.

(A) Cross section of the rectum. Approximately 50% of the mucosa is normal (arrow), while the remaining tissues have a thin epithelium with abundant cellularity (inflammation) in the lamina propria (arrowhead). Giemsa 20x. Scale bar = 500 µm. (B) The epithelium is eroded (arrow), and many cells are distended by large vacuoles containing spores (black arrowhead). Many macrophages and lymphocytes are present in the lamina propria (white arrowhead). Periodic acid-Schiff 200x. Scale bar = 50 µm. (C) Epithelial cells are markedly distended by mature spores within spacious vacuoles (arrowhead) or developmental stages (arrows). In this section almost all cells are parasitized. Hematoxylin and eosin 400x. Scale bar = 20 µm.

5.4.3 Molecular characterization of myxospores

The 1908-bp 18S rRNA gene sequence (KT072742) was compared to other closely related myxozoan species deposited in the NCBI nr/nt database. *Unicauda fimbrethilae* n. sp. was the closest match to *Unicauda pelteobagrus* (84.5% sequence identity; 98.0% coverage; KC193254) from the muscle of *Pelteobagrus fulvidraco* in China. The next closest match was *Myxobolus aureus* (84.6% sequence identity; 92.0% coverage; KF296348) from the liver of *Salminus brasiliensis* from Brazil and an unpublished sequence of *Henneguya mystusia* (89.7% sequence identity; 72.0% coverage; EU732603) from the gills of *Hemibagrus nemurus* from Malaysia. Phylogenetic placement of the isolate was within a clade containing the single species of *Unicauda* in the NCBI database (Figure 5.4) sister to other myxobolid myxozoans, confirming placement within the family Myxobolidae.

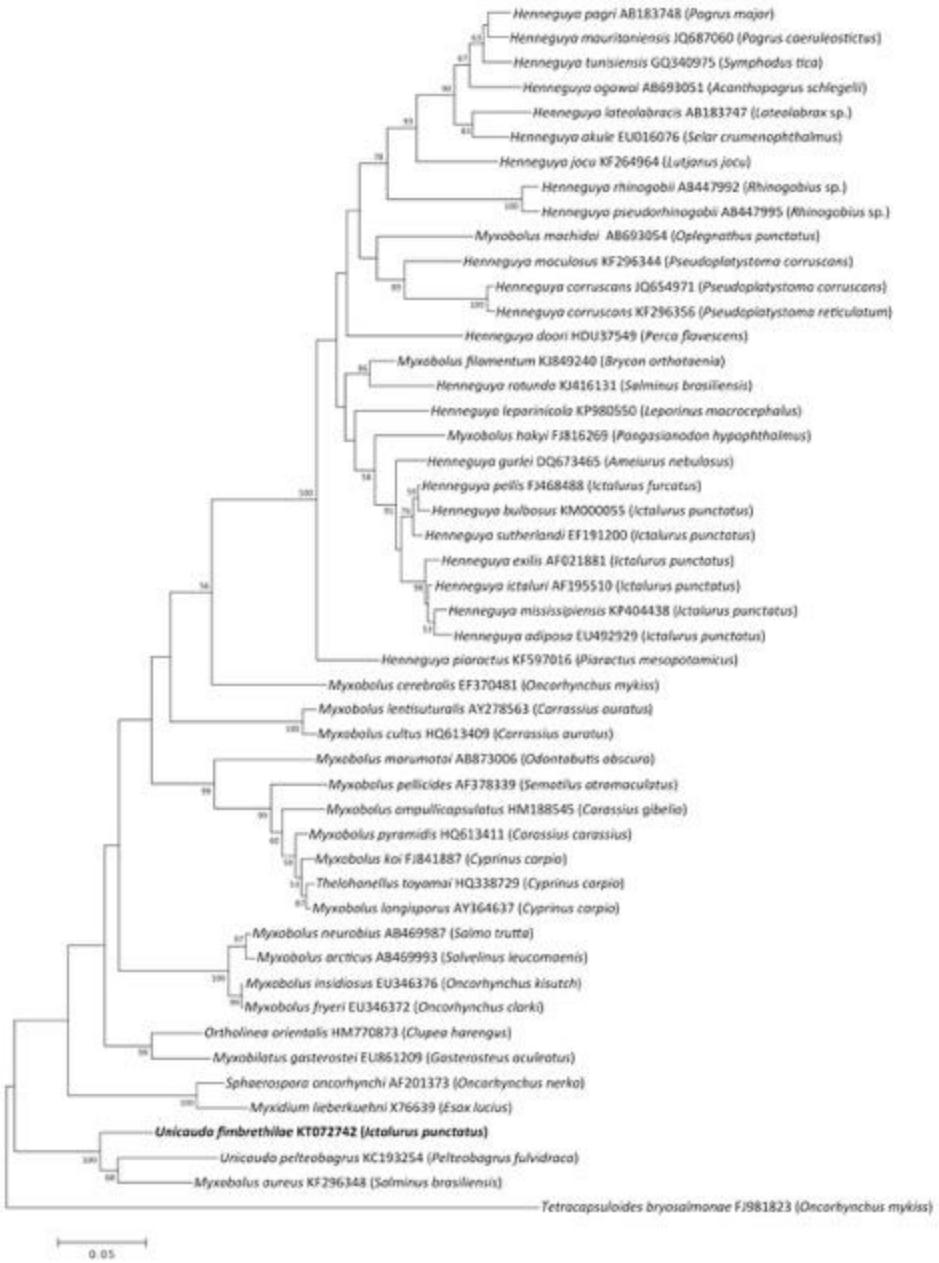

Figure 5.4 Maximum likelihood analysis of the 18S SSU rRNA gene sequence of *Unicauda fimbrethilae* n. sp.

Maximum likelihood tee of the 18S SSU rRNA gene sequence of *Unicauda fimbrethilae* n. sp. and relevant myxozoan sequences (fish host) obtained by a blast search of the NCBI nr/nt database and rooted at *Tetracapsuloides bryosalmonae*. Numbers at the nodes represent bootstrap confidence values (n=1,000 replicates).

5.4.4 Taxonomic summary

Species: Unicauda fimbrethilae n. sp. (Cnidaria: Myxosporea: Myxobolidae)

Type host: Ictalurus punctatus (Rafinesque, 1818) (Siluriformes: Ictaluridae)

Site of infection: throughout intestinal tract

Prevalence: 12 of 21 fish (57.14%)

Locality: Commercial catfish pond, Brooksville, MS, Noxubee County

Specimens deposited: holotype USNM 1283045; paratypes USNM 1283046–1283047 Smithsonian Institution, National Museum of Natural History, Washington DC, USA

Etymology: In reference to the root-like projections from the caudal process, the species is named after a fictional character, Fimbrethil of the tree-like race of Ents, from J.R.R. Tolkien's epic trilogy *The Lord of the Rings*.

5.5 Discussion

The genus *Unicauda* was first erected by Davis (1944) to include *Henneguya*-like species that have a single caudal process. Cone and Melendy (2000) reviewed the members of the genus and concluded that only 10 of the 26 previously described species were valid. Furthermore Cone and Melendy (2000) considered *U. plasmodia* (Davis 1922, 1944; synonymous with *Henneguya plasmodia*) from the gills and *U. limatula* (Meglitsch 1937; Davis, 1944; synonymous with *Henneguya limatula*) from the gall bladder of the channel catfish as poorly described and invalid members of the genus. Indeed the spore body of *H. limatula* is lanceolate shaped and some spores were acknowledged to have bifurcated caudal processes consistent with the genus *Henneguya*

(Meglitsch 1937). The original description of *Henneguya plasmodia* is most likely of immature spores of a *Henneguya* species and has not been reported since its discovery. The majority of the valid members of the genus *Unicauda* are described from fish in the order Cypriniformes from China (Chen and Ma 1998), Georgia (Cone and Melendy, 2000), Iraq (Rahemo 1976), Spain (Cone and Melendy 2000), the US (Gurley 1893; Ward 1919; Kudo 1934; Minchew 1981) and a single species from a siluriform fish in China (Chen and Ma 1998). Morphologically *U. fimbrethilae* n. sp. shared several overlapping features with *U. magna* from the fins of fathead minnow from Pennsylvania (Minchew 1981). However, myxospores of *U. magna* typically had finely tapered caudal processes of varying lengths, compared to the elaborate branching pattern of the caudal process of mature spores of *U. fimbrethilae* n. sp. (Minchew 1981).

A single unidentified species of *Unicauda* has been reported from the intestinal tract of the elephantnose fish (*Gnathonemus petersii*) imported from Nigeria (Caffara et al. 2007). Infected fish exhibited anorexia, lethargy, cachexia, and weight loss. While plasmodia of this species were detected histologically in the tunica muscularis and tunica serosa, free myxospores were found in the lumen. Some areas of multifocal epithelial necrosis were also observed. Myxospores were also observed on wet mount slide preparations of the intestinal wall, kidney and liver (Caffara et al. 2007).

The catfish in this case were dying from causes other than *U. fimbrethilae* n. sp., however the degree of infection and inflammation contributed, perhaps significantly, to disease. Infection was restricted to the intestine, with a predominance in the rectum and esophagus. In our catfish, the degree of damage in the rectum due to infection was severe,

with mucosal erosion and thickening of the lamina propria by inflammation, effectively narrowing the gut lumen.

Myxozoan species that primarily infect the intestinal epithelium, such as *Enteromyxum leei* in warmwater marine culture species, are known to cause significant disease in aquaculture species in the form of severe enteritis and emaciation. *Enteromyxum leei* induces cachexia by causing anorexia in addition to impaired nutrient uptake and osmoregulation from intestinal damage (Sitjà-Bobadilla and Palenzuela, 2012). Severe infection with *E. leei* in sea bream (*Sparus aurata*) is seen throughout the intestine, including severe disease in the rectum (Fleurance et al. 2008). The *Unicauda* reported from Caffara et al. (2007) although present in the blood vessels and lamina propria, were not identified within enterocytes.

Phylogenetic analysis clusters *U. fimbrethilae* n. sp. with *U. pelteobagrus* from China, confirming placement within the Myxobolidae. Molecularly *U. fimbrethilae* n. sp. does not match any deposited myxozoan sequence in the NCBI nr/nt database. In addition, 18S rRNA gene sequences of actinospore stages from a recent survey of oligochaetes from commercial catfish ponds did not match the 18S rRNA gene sequence of *U. fimbrethilae* n. sp. (Rosser et al. 2014b).

In the southeastern United States, and more specifically Mississippi, catfish aquaculture is an economically important industry (Hargreaves and Tucker, 2004). Infectious diseases are a major hindrance to production, especially the myxozoan *Henneguya ictaluri,* which is the causative agent of proliferative gill disease (PGD) in channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*) x channel catfish hybrids (Pote et al. 2000; Bosworth et al. 2003; Griffin et. al., 2010). *Henneguya*

ictaluri is the most commonly diagnosed parasitic infection and the third most common infectious disease associated with catfish aquaculture in the southeastern United States. In addition to the pathogenic *H. ictaluri*, there are at least 9 other *Henneguya* species that have been identified in commercially raised channel catfish. Pseudocysts of *Henneguya* species are a routine finding in diagnostic case submissions to the Aquatic Research and Diagnostic Laboratory of the Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS but are often not associated with disease (Pote et al. 2012; Rosser et al. 2014a, 2015).

Limited information is available on other myxozoan genera that infect channel catfish. However there are records of other myxozoans parasitizing the channel catfish in North America. *Myxidium bellum* has been identified from the gall bladder of infected channel catfish from Illinois (Meglitsch 1937). *Sphaerospora ictaluri* was described from the kidney of channel catfish from California (Hedrick et al. 1990). Since their descriptions *M. bellum* and *S. ictaluri* have not been described or reported elsewhere. Interestingly no *Myxobolus* sp. has been identified in the channel catfish to date, as the predominant myxozoan species that infect cultured catfish belong to the genus *Henneguya*.

This is the first account of a species of *Unicauda* infecting channel catfish, as well as the first record of a myxozoan that develops in the intestinal tract of channel catfish. Based on the structure of the caudal process and spherical spore body characteristic of the genus, we propose the isolate described here represents a previously undescribed species within the myxozoan family Myxobolidae, *Unicauda fimbrethilae* n. sp.

5.6 References

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403–410.

Andree, K. B., C. Székely, K. Molnár, S. J. Gresoviac, and R. P. Hedrick. 1999. Relationships among members of the genus *Myxobolus* (Myxozoa: Bivalvulidae) based on small subunit ribosomal DNA sequences. Journal of Parasitology 85:68–74.

Barta, J. R., D. S. Marin, P. A. Liberator, M. Dashkevicz, J. W. Anderson, S. D. Feigner, A. Elbrecht, A. Perkins-Barrow, M. C. Jenkins, H. D. Danforth Et Al. 1997. Phylogenetic relationships among eight *Eimeria* species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. Journal of Parasitology 83:262–271.

Bosworth, B. G., D. J. Wise, J. S. Terhune, and W. R. Wolters. 2003. Family and genetic group effects for resistance to proliferative gill disease in channel catfish, blue catfish and channel catfish x blue catfish backcross hybrids. Aquaculture Research. 34:569–573.

Bowser, P. R., and J. D. Conroy. 1985. Histopathology of gill lesions in channel catfish associated with *Henneguya*. Journal of Wildlife Diseases 21:177–179.

Burtle, G. J., L. R. Harrison, And E. L. Styer. 1991. Detection of a triactinomyxid myxozoan in an oligochaete from ponds with proliferative gill disease in channel catfish. Journal of Aquatic Animal Health 3: 281–287.

Caffara, M., V. De Vita, D. Florio, A. Gustinelli, F. Quaglio, and M. L. Fioravanti. 2007. Infections due to *Unicauda* spp. (Myxozoa, Myxobolidae) in elephantnose fish (*Gnathonemus petersii*) imported to Italy. Bulletin of the European Association of Fish Pathologists 27:142–147.

Carriero, M. M., E. A. Adriano, M. R. M. Silva, P. S. Ceccarelli, and A. A. M. Maia. 2013. Molecular phylogeny of the *Myxobolus* and *Henneguya* genera with several new South American species. PLoS One 8:e73713.

Chen, C. and C. Ma. 1998. Fauna Sinica. Myxozoa. Myxosporea. Science Press, Beijing, 993 pp.

Cone, D. K. and J. S. Melendy. 2000. Infections of *Unicauda clavicauda* (Kudo, 1934) (Myxozoa) in the skin of *Notropis hudsonius* (Cyprinidae) from Montana, with a synopsis of the genus *Unicauda*, 1944. Folia Parasitologica 47: 273–278.

Davis H. S. 1922. A new myxosporidian parasite of the channel catfish, *Ictalurus punctatus*. Journal of Parasitology 8:118-122.

Davis H. S. 1944. A review of the genus *Henneguya* (Myxosporidia) with descriptions of two new species. Transactions of the American Microscopical Society 63: 311-320.

Eiras, J. C. and E. A. Adriano. 2012. A checklist of new species of *Henneguya* Thélohan, 1892 (Myxozoa: Myxosporea, Myxobolidae) described between 2002 and 2012. Systematic Parasitology 83: 95–104.

Eiras, J. C., J. Zhang, and K. Molnár. 2014. Synopsis of the species of *Myxobolus* Bütschli, 1882 (Myxozoa: Myxosporea, Myxobolidae) described between 2005 and 2013. Systematic Parasitology 88: 11–36.

Eszterbauer, E. 2004. Genetic relationship among gill-infecting *Myxobolus* species (Myxosporea) of cyprinids: molecular evidence of importance of tissue-specificity. Diseases of Aquatic Organisms 58: 35–40.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Fiala, I. 2006. The phylogeny of Myxosporea (Myxozoa) based on small subunit ribosomal RNA gene analysis. International Journal of Parasitology 36: 1521–1534.

Fiala, I., P. Bartošová-Sojková, and C. M. Whipps. 2015. Classification and Phylogenetics of Myxozoa. *In* Myxozoan evolution, ecology and development. Okamura, B., A. Gruhl, and J. L. Bartholomew (eds). Springer International Publishing, Switzerland, p.85–110.

Fleurance, R., C. Sauvegrain, A. Marques, A. Le Breton, C. Guereaud, Y. Cherel, and M. Wyers. 2008. Histopathological changes caused by *Enteromyxum leei* infection in farmed sea bream *Sparus aurata*. Diseases of Aquatic Organisms 79: 219–228.

Griffin, M. J., A. C. Camus, D. J. Wise, T. E. Greenway, M. J. Mauel, and L. M. Pote. 2010. Variation in susceptibility to *Henneguya ictaluri* infection by two species of catfish and their hybrid cross. Journal of Aquatic Animal Health 22: 21–35.

Griffin, M. J., L. M. Pote, D. J. Wise, T. E. Greenway, M. J. Mauel, AND A. C. Camus. 2008. A novel *Henneguya* species from channel catfish described by morphological, histological and molecular characterization. Journal of Aquatic Animal Health 20: 127–135.

Hallett, S. L., and A. Diamant. 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. Diseases of Aquatic Organisms 46: 197–212.

Hanson L. A., D. Lin, L. M. Pote, AND R. Shivaji. 2001. Small subunit rRNA gene comparisons of four actinosporean species to establish a polymerase chain reaction test for the causative agent of proliferative gill disease in channel catfish. Journal of Aquatic Animal Health 13: 117–123.

Hargreaves, J. A., and C. S. Tucker. 2004. Industry Development. *In* Biology and culture of the channel catfish, C. S. Tucker and J. A. Hargreaves (eds.). Elsevier B. V., Amsterdam, The Netherlands, p. 1–14.

Hedrick, R. P., T. McDowell, and J. M. Groff. 1990. *Sphaerospora ictaluri* n. sp. (Myxosporea: Sphaerosphoridae) observed in the kidney of channel catfish, *Ictalurus punctatus* Rafinesque. Journal of Protozoology 37: 107–112.

Kent, M. L., K. B Andree, J. L. Bartholomew, M. El-Matbouli, S. S. Desser, R. H. Devlin, S. W. Feist, R. P. Hedrick, R. W. Hoffmann, J. Khattra et al. 2001. Recent advances in our knowledge of the Myxozoa. Journal of Eukaryotic Microbiology 48: 395–413.

Lin, D., L. A. Hanson, AND L. M. Pote. 1999. Small subunit ribosomal RNA sequence of *Henneguya exilis* (class Myxosporea) identifies the actinosporean stage from an oligochaete host. Journal of Eukaryotic Microbiology 46: 66–68.

Lom, J. and I. DykovÁ. 2006. Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. Folia Parasitologica 53: 1–36.

Nei, M. and S. Kumar. 2000. Molecular evolution and phylogenetics. Oxford University press, New York, New York, 333 p.

Pote, L. M., B. Bellerud, D. L. Lin, and E. F. Chenney. 1994. The isolation and propagation of *Dero digitata* infected with *Aurantiactinomyxon* sp. Journal of the World Aquaculture Society 25: 303–307.

Pote, L. M., L. A. Hanson, and R. Shivaji. 2000. Small subunit ribosomal RNA sequences link the cause of proliferative gill disease in channel catfish to *Henneguya* n. sp. (Myxozoa: Myxosporea). Journal of Aquatic Animal Health 12: 230–240.

Pote, L. M., L. Khoo, and M. Griffin. 2012. *Henneguya ictaluri*. *In* Fish parasites pathobiology and protection, P. T. K. Woo and K. Buchmann (eds.). CAB International, Oxfordshire, U. K., p. 177–192.

Rosser T. G., M. J. Griffin, S. M. A. Quiniou, L. H. Khoo, T. E. Greenway, D. J. Wise, and L. M. Pote. 2015. Small subunit ribosomal RNA sequence links the myxospore stage of *Henneguya mississippiensis* n. sp. from channel catfish *Ictalurus punctatus* to an actinospore released by the benthic oligochaete *Dero digitata*. Parasitology Research 114: 1595–1602.

Rosser T. G., M. J. Griffin, S. M. A. Quiniou, L. H. Khoo, AND L. M. Pote. 2014a. 18S rRNA gene sequencing identifies a novel species of *Henneguya* parasitizing the gills of the channel catfish (Ictaluridae). Parasitology Research 113: 4651–4658.

Rosser T. G., M. J. Griffin, S. M. A. Quiniou, T. E. Greenway, L. H. Khoo, D. J. Wise, and L. M. Pote. 2014b. Molecular and morphological characterization of myxozoan actinospore types from a commercial catfish pond in the Mississippi Delta. Journal of Parasitology 100: 828–839.

Sitjà-Bobadilla, A., and O. Palenzuela. 2012. *Enteromyxum* Species. *In* Fish parasites pathobiology and protection, P. T. K. Woo and K. Buchmann (eds.). CAB International, Oxfordshire, U. K., p. 163–176.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30: 2725–2729.

Tucker, C. S., J. L. Avery, and D. Heikes. 2004. Culture Methods. *In* Biology and culture of the channel catfish, C. S. Tucker and J. A. Hargreaves (eds.). Elsevier B. V., Amsterdam, The Netherlands, p. 166–195.

Wise, D. J., A. Camus, T. Schwedler, and J. Terhune. 2004. Health Management. *In* Biology and culture of the channel catfish. Tucker, C. S., and J. A. Hargreaves (eds). Elsevier B. V., Amersterdam, The Netherlands, p.444–502.

CHAPTER VI

VERRUCUOUS DERMAL HENNEGUYOSIS ASSOCIATED WITH *HENNEGUYA EXILIS* (KUDO, 1929) (CNIDARIA: MYXOBOLIDAE) A PARASITE OF THE CHANNEL CATFISH *ICTALURUS PUNCTATUS* (RAFINESQUE, 1818)

6.1 Abstract

Henneguya exilis is a myxozoan parasite commonly reported from the gills of channel catfish in North America. Limited reports exist of *H. exilis* parasitizing the skin of catfish, but these lack morphologic and molecular confirmation. Herein we provide the first report of *H. exilis* parasitizing the skin of a commercially raised channel catfish using histology supplemented with morphological and molecular data. The 18S rDNA gene sequence of the present *H. exilis* isolate was a 100% match (1959/1959 bp) to the previous published sequence. Histopathological examination revealed the epidermis in the affected area had been completely replaced by layers of pseudocysts of varying sizes (~ 25-200µm in diameter) containing myxospores of different stages of maturation. In some areas, the pseudocysts even extended past the dermis and were present in the fascial planes separating the muscle bundles or were present in the tissue between the bony elements of the lepidotrichia. The effects of parasitizing multiple tissue sites on the health of catfish are unknown and further research is needed to determine what triggers the maturation of this parasite in non-branchial sites.

6.2 Introduction

Henneguya spp. are cosmopolitan myxozoan parasites found in various organ systems of marine and freshwater fish. They represent the second largest genus of myxozoans, with several species pathogenic to the fish host (Eiras and Adriano 2012). Catfish producers in the southeastern United States struggle with seasonal outbreaks of proliferative gill disease (PGD) caused by the myxozoan *Henneguya ictaluri* (Pote et al. 2000; Pote et al. 2012). At present, three *Henneguya* spp. associated with catfish aquaculture, *H. exilis, H. ictaluri* and *H. mississippiensis,* have had the myxospore stage in the fish linked to an actinospore stage in the benthic oligochaete, *Dero digitata* (Lin et al. 1999; Pote et al. 2000; Rosser et al. 2015). In addition to these three, seven other species of *Henneguya* have been reported from channel catfish (Minchew 1977; Pote et al. 2012; Rosser et al. 2014b, 2015), and a recent molecular survey of actinospore stages found in catfish ponds suggests the existence of several more (Rosser et al. 2014a).

While most of the *Henneguya* spp. associated with North American ictalurid fishes are limited to the gills, several have been reported from other tissues. *Henneguya diversis* was described from liver, kidneys and connective tissues of muscle and fins (Minchew 1977). *Henneguya exilis* has been described from both gills and the epidermis of the channel catfish (Kudo 1929; Meglitsch 1937). *Henneguya pellis* and *H. sutherlandi* infect the epidermis of blue catfish (*Ictalurus furcatus*) and channel catfish, respectively, forming large epidermal pseudocysts (Minchew 1977; Griffin et al. 2008, 2009a). *Henneguya adiposa* forms pseudocysts on the adipose fin of channel catfish (Minchew 1977; Griffin et al. 2009b) and *H. gurlei* forms plasmodia on the dorsal, pectoral and anal fins of the brown bullhead (*Ameiurus nebulosus*) (Iwanowicz 2008).

6.3 Materials and Methods

6.3.1 Fish collection

In the spring of 2015, five channel catfish fingerlings (~6-9 cm in length) from a commercial catfish farm were submitted to the Aquatic Research and Diagnostic Laboratory because of mortalities. Diagnostic workup revealed that all 5 fish had severe proliferative gill disease, based on microscopic examination of gill biopsies. In addition, one fish cultured positive for *Edwardsiella ictualuri*, the causative agent of Enteric Septicemia of Catfish (Hawke et al. 1981). One of the fingerlings was noted to have an extensive, yellowish tan, soft, growth with a nodular surface covering approximately one-sixth of the body at the level of the adipose fin. The adipose fin did not appear to be affected (Figure 6.1). This growth extended on both sides of the body affecting the distal third of the ventral fin. Portions of the ventral fin were eroded with loss of tissue and exposing tips of several of the lepodotrichia or soft fin rays.

Figure 6.1 Gross appearance of the affected fish showing the extensive nature of the lesion.

6.3.2 Histopathological and morphological characterization of myxospores

Biopsies of infected tissue were fixed in 70% molecular grade ethanol for molecular characterization and 10% neutral buffered formalin for histological analysis. Ethanol-fixed myxospores were mounted on slides and coverslipped, and representative images were digitally captured using a BX-50 Olympus microscope (Olympus Optical Co Ltd, Tokyo, Japan) mounted with an Olympus DP72 camera and DP-2-Twain/cellSens software (Olympus Optical Co Ltd, Tokyo, Japan). Characters used for the description of *Henneguya* spp. were measured from photomicrographs of 25 myxospores.

6.3.3 Molecular characterization of myxospores

Genomic DNA was isolated from myxospores using the DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany). The 18S small subunit rRNA gene was amplified using combinations of myxozoan sequencing primers used in previous studies (Hanson et al. 2001; Fiala 2006; Griffin et al. 2008; Rosser et al. 2015), but following the methods of Rosser et al. (2015). Amplification products were electrophoresed through 1.2% agarose gels in the presence of ethidium bromide (0.5 µg/ml) and viewed under ultraviolet light. All amplicons were compared to a concurrently run molecular weight ladder (HyperLadder™ 50bp, Bioline, London, United Kingdom) to confirm the presence of appropriate sized bands. Amplicons were excised from the agarose and purified using the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, California). Purified products were sequenced commercially (Eurofins MWG Operon LLC, Huntsville, Alabama, USA) and sequences were edited and aligned in SeqMan™ (DNAStar, Madison, Wisconsin). The contiguous sequence was used in a Blastn search of the NCBI nr/nt database (Altschul et al. 1990) to identify somewhat similar sequences.

6.4 Results

6.4.1 Morphological description of *Henneguya exilis* myxospores

The myxospores in this current study were morphologically consistent with characters from the original descriptions of *H. exilis* by Kudo (1929) and a later description by Minchew (1977), with the exception that caudal processes were shorter than previously documented, Table 6.1 (Figure 6.2). Conventionally, morphology has been used to describe myxozoans species, although evidence has suggested morphological descriptions are limited as a stand-alone method of classification. In cases

where phenotypic characters are subjective, especially where morphologically ambiguous species occupy the same host niche, molecular confirmation provides a more accurate identification (Hallett et al. 2002, 2004; Eszterbauer et al. 2006; Atkinson and Bartholomew 2009; Urawa et al. 2011).

Table 6.1 Morphologic comparison of *Henneguya exilis* myxospores.

	Henneguya exilis		
Reference	This study	Kudo 1929	Minchew 1977
Host	*Ictalurus punctatus*	*Ictalurus punctatus*	*Ictalurus punctatus*
Locality	Mississippi, USA	Illinois, USA	Mississippi, USA
Total spore			
Length	51.5±3.5 (44.4–61.1)	(60.0–70.0)	69.9 (60.0–90.0)
Spore body			
Length	18.5±1.1 (16.4–20.7)	(18.0–20.0)	17.6 (16.0–19.0)
Width	4.3±0.4 (3.8–5.4)	(4.0–5.0)	4.9 (4.0–5.0)
Polar capsule			
Length	6.7±0.4 (6.1–7.5)	(8.0–9.0); 6.7 (6.0–8.0)	8.5 (7.0–9.0)
Width	1.5±0.1 (1.3–1.7)	(1.0–1.5)	(1.5–2.0)
Caudal process			
Length	34.0 ±2.8 (27.2–38.6)	34.6 (25.0–47.0)	52.3 (41.0–73.0)

All values are reported in micrometers.

Figure 6.2 Photomicrograph of a representative ethanol-fixed *Henneguya exilis* myxospore.

Wet mount. Scale bar = 20 µm..

6.4.2 Molecular characterization of *H. exilis* myxospores

The 1959-bp contiguous sequence was a 100% match to *H. exilis* (AF021881; Hanson et al. 2001) suggesting the gill infecting myxospores of *H. exilis* and the tail fin myxospores reported in this study are conspecific. While reports of *H. exilis* parasitizing

the fins of channel catfish exist, there are no morphologic, histologic, or molecular data confirming these accounts (Meglitsch 1937).

6.4.3 Histological description of the *H. exilis* lesions

Histologically the papillomatous forms were characterized by hyperplasia of the squamous epithelium and goblet cells (McCraren et al. 1975). Histopathological examination of the affected fish in this case revealed that the epidermis in the affected area had been completely replaced by layers of pseudocysts of varying sizes (~ 25-200 µm in diameter) containing myxospores of different stages of maturation (Figure 6.3). It was very difficult to discern any normal epidermal tissue within this sea of pseudocysts. In some areas, the pseudocysts even extended past the dermis and were present in the fascial planes separating the muscle bundles (Figure 6.4). The pseudocysts were sometimes present in the tissue between the bony elements of the lepidotrichia (Figure 6.5). The inflammatory response within the affected area was relatively mild and only a few inflammatory cells presents between the pseudocysts particularly in areas that were ulcerated.

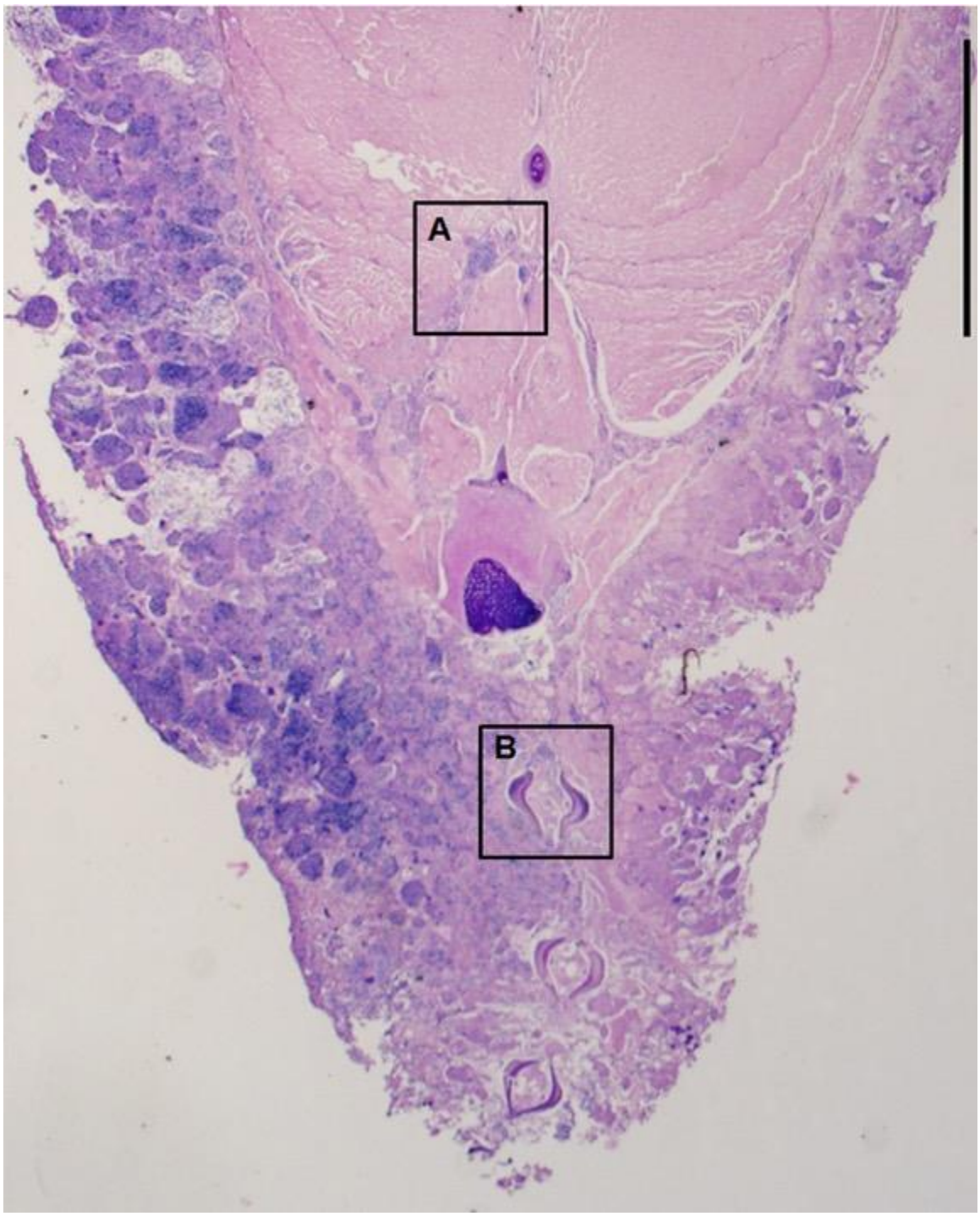

Figure 6.3 Cross section through the body of the fish showing the effacement of the epidermis by the layers of pseudocysts.

Giemsa stain (bar ~1000 µm). Box A and B represent areas highlighted in Figures 6.4 and 6.5.

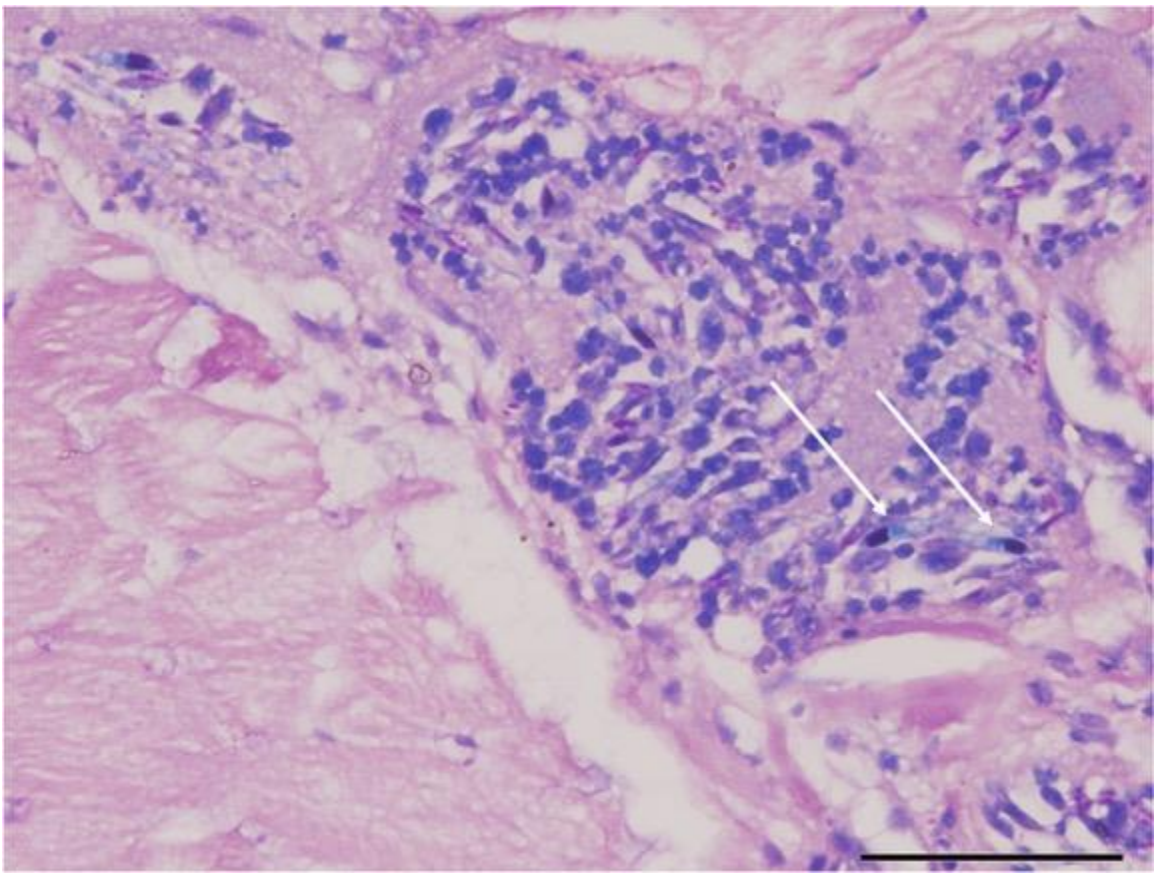

Figure 6.4 Higher magnification of the area in box A in Figure 6.3 showing the presence of the pseudocysts within the fascial plane separating the muscle bundles.

Note the mature spores within the pseudocyst (arrows). Giemsa stain (bar ~50 μm).

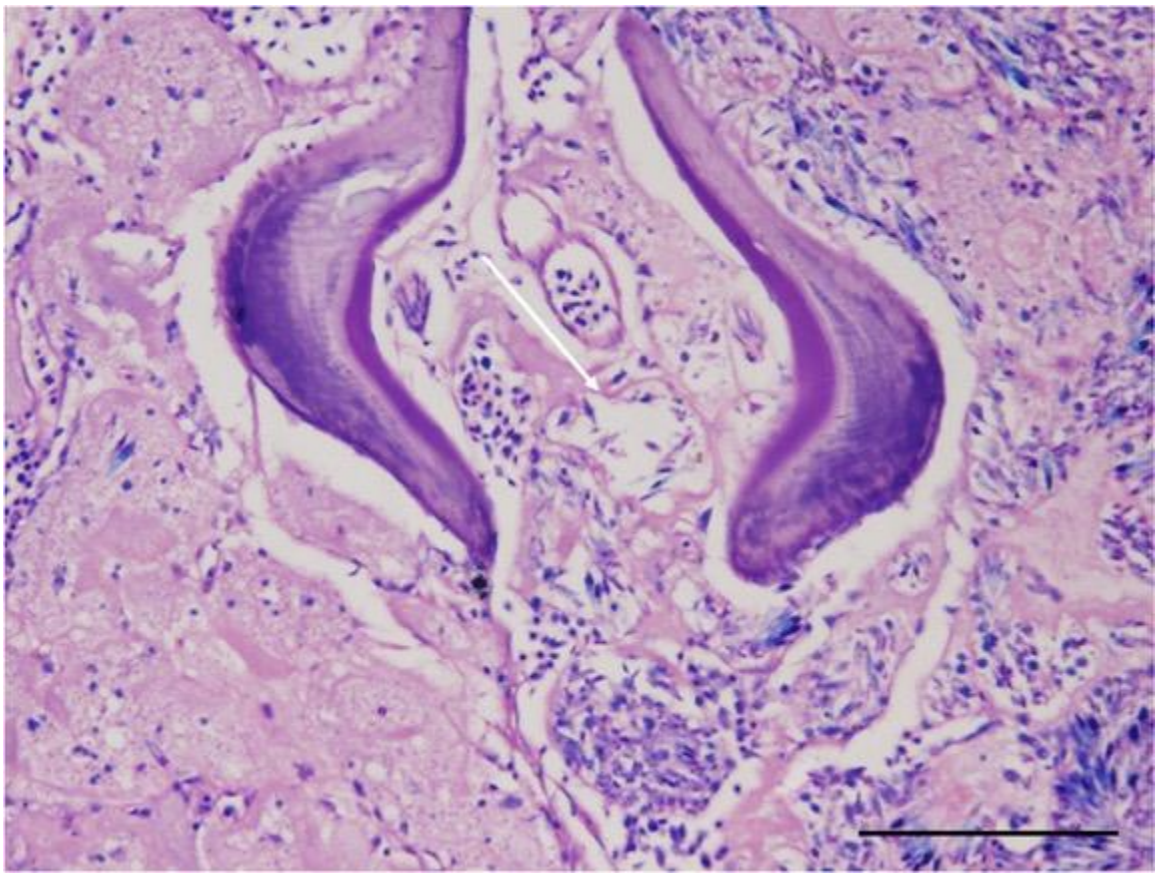

Figure 6.5 Higher magnification of the area in box B in Figure 6.3 revealing the presence of the pseudocysts.

Pseudocysts between the bony elements of the lepidotrichia (arrow points to pseudocyst with several mature spores). Giemsa stain (bar ~100 µm).

6.5 Discussion

Dermal infections of *H. exilis* have been reported from the channel catfish (Kudo 1929; Meglitsch 1937). McCraren et al. (1975) described seven different forms of *Henneguya* sp. infections in the channel catfish based on morphology and histopathology. Two cutaneous forms were described: papillomatous and cutaneous cysts. The papillomatous forms are tumor-like and appear on the fins and occasionally the caudal peduncle of cultured channel catfish with lesions measuring up to 1 cm in diameter.

Some lesions are reported to have completely covered the dorsal fin (McCraren et al. 1975). In comparison to the case described here, tumor-like growths occur on the caudal peduncle. Though these forms are likely *H. exilis*, no molecular or morphologic data of spores were recorded for these forms to confirm this.

Historical accounts of *H. exilis* have described the parasite as having affinity for gill tissue, but reports also exist of *Henneguya* species morphologically identified as *H. exilis* from the skin of catfish (Kudo 1929; Meglitsch 1937; Minchew 1977). However, many of these reports lack confirmation of species by molecular techniques and evidence suggests that morphology alone can be ambiguous especially when multiple closely related and morphologically indistinguishable myxozoan species parasitize the same host and tissue site (Eszterbauer 2002, 2004). The mechanisms that affect tissue tropism among myxozoans have yet to be clarified and it is unsure if multiple species occupying the same tissue site influence the spread of a species to other ectopic or aberrant locations. This represents the first molecularly confirmed account of *H. exilis* developing as mature myxospores in a second tissue site, the skin.

6.6 References

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403–410.

Atkinson, S. D. and J. L. Bartholomew. 2009. Alternate spore stages of *Myxobilatus gasterostei*, a myxosporean parasite of three-spined sticklebacks (*Gasterosteus aculeatus*) and oligochaetes (*Nais communis*). Parasitology Research 104:1173–1181.

Eiras, J. C. and E. A. Adriano. 2012. A checklist of new species of *Henneguya* Thélohan, 1892 (Myxozoa: Myxosporea, Myxobolidae) described between 2002 and 2012. Systemic Parasitology 83:95–104.

Eszterbauer, E. 2002. Molecular biology can differentiate morphologically indistinguishable myxosporean species: *Myxobolus elegans* and *M. hungaricus*. Acta Veterinaria Hungarica 50:59–62.

Eszterbauer, E. 2004. Genetic relationship among gill-infecting *Myxobolus species* (Myxosporea) of cyprinids: molecular evidence of importance of tissue-specificity. Diseases of Aquatic Organisms 58:35–40.

Eszterbauer, E., S. Marton, O. Z. Rácz, M. Lenteyei, and K. Molnár. 2006. Morphological and genetic differences among actinosporean stages of fish-parasitic myxosporeans (Myxozoa): Difficulties of species identification. Systematic Parasitology 65:97–114.

Griffin, M. J., L. H. Khoo, L. Torrans, B. G. Bosworth, S. M. Quiniou, P. S. Gaunt, and L. M. Pote. 2009a. New data on *Henneguya pellis* (Myxozoa: Myxobolidae), a parasite of blue catfish *Ictalurus furcatus*. Journal of Parasitology 95:1455–1467.

Griffin, M. J., L. M. Pote, D. J. Wise, T. E. Greenway, M. J. Mauel, and A. C. Camus. 2008. A novel *Henneguya* species from channel catfish described by morphological, histological, and molecular characterization. Journal of Aquatic Animal Health 20:127–135.

Griffin, M. J., D. J. Wise, and L. M. Pote. 2009b. Morphology and small-subunit ribosomal DNA sequence of *Henneguya adiposa* (Myxosporea) from *Ictalurus punctatus* (Siluriformes). Journal of Parasitology 95:1076–1085.

Hallett, S. L., S. D. Atkinson, and M. El-Matbouli. 2002. Molecular characterization of two aurantiactinomyxon (Myxozoa) phenotypes reveals one genotype. Journal of Fish Diseases 25:627–631.

Hallett, S. L., S. D. Atkinson, C. Erséus, and M. El-Matbouli. 2004. Molecular methods clarify morphometric variation in triactinomyxon spores (Myxozoa) released from different oligochaete hosts. Systematic Parasitology 57:1–14.

Hanson, L. A., D. Lin, L. M. W. Pote, and R. Shivaji. 2001. Small subunit rRNA gene comparisons of four actinosporean species to establish a polymerase chain reaction test for the causative agent of proliferative gill disease in channel catfish. Journal of Aquatic Animal Health 13:117–123.

Hawke, J. P., A. C. McWhorter, A. G. Steigerwalt, and D. J. Brenner. 1981. *Edwardsiella ictaluri* sp. nov., the causative agent of Enteric Septicemia of Catfish. International Journal of Systematic and Evolutionary Microbiology 31: 396–400.

Iwanowicz, L. R., D. D. Iwanowicz, L. M. Pote, V. S. Blazer, and W. B. Schill. 2008. Morphology and 18S rDNA of *Henneguya gurlei* (Myxosporea) from *Ameiurus nebulosus* (Siluriformes) in North Carolina. Journal of Parasitology 94:46–57.

Lin, D., L. A. Hanson, and L. M. Pote. 1999. Small subunit ribosomal RNA sequence of *Henneguya exilis* (Class Myxosporea) identifies the actinosporean stage from an oligochaete host. Journal of Eukaryotic Microbiology 46:66–68.

McCraren, J. P., M. L. Landolt, G.L Hoffman, and F. P. Meyer. 1975. Variation in response of channel catfish to *Henneguyai* sp. infections (Protozoa: Myxosporidea). Journal of Wildlife Diseases 11:2–7.

Meglitsch, P. A. 1937. On some new and known Myxosporidia of the fishes Illinois. Journal of Parasitology 23:467–477.

Minchew, C. D. 1977. Five new species of *Henneguya* (Protozoa: Myxosporida) from ictalurid fishes. Journal of Protozoology 24:213–220.

Pote, L. M., L. A. Hanson, and R. Shivaji. 2000. Small subunit ribosomal RNA sequences link the cause of proliferative gill disease in channel catfish to *Henneguya* n. sp. (Myxozoa: Myxosporea). Journal of Aquatic Animal Health 12:230–240.

Rosser, T. G., M. J. Griffin, S. M. A. Quiniou, L. H. Khoo, T. E. Greenway, D. J. Wise and L. M. Pote. 2015. Small subunit ribosomal RNA sequence links the myxospore stage of *Henneguya mississippiensis* n. sp. from channel catfish *Ictalurus punctatus* to an actinospore released by the benthic oligochaete *Dero digitata*. Parasitology Research 114:1595–1602.

Rosser, T. G., M. J. Griffin, S. M. A. Quiniou, T. E. Greenway, L. H. Khoo, D. J. Wise, and L. M. Pote. 2014a. Molecular and morphological characterization of myxozoan actinospore types from a commercial catfish pond in the Mississippi delta. Journal of Parasitology 100:828–839.

Rosser, T. G., M. J. Griffin, S. M. A. Quiniou, L. H. Khoo, and L. M. Pote L.M. 2014b. 18S rRNA gene sequencing identifies a novel species of *Henneguya* parasitizing the gills of the channel catfish (Ictaluridae). Parasitology Research 113:4651–4658.

Urawa, S., M. A. Freeman, S. C. Johnson, S. R. M. Jones, and H. Yokoyama. 2011. Geographical variation in spore morphology, gene sequences, and host specificity of *Myxobolus arcticus* (Myxozoa) infecting salmonid nerve tissues. Diseases of Aquatic Organisms 96**:**229–237.

CHAPTER VII

MYXOBOLUS ICTIOBUS N. SP. AND *MYXOBOLUS MINUTUS* N. SP. (CNIDARIA: MYXOBOLIDAE) FROM THE GILLS OF SMALLMOUTH BUFFALO *ICTIOBUS BUBALUS* (CYPRINIFORMES: CATOSTOMIDAE) POLYCULTURED IN COMMERCIAL CATFISH PONDS

7.1 Abstract

The smallmouth buffalo *Ictiobus bubalus* Rafinesque (Catostomidae) is native to North American waterways and occasionally grown in pond aquaculture. Species of *Myxobolus* Bütschli, 1882 have been reported from the gills, integument, and intestinal tract of buffalo fish, although there is ambiguity in some host records. In the summer of 2013, thirteen adult smallmouth buffalo were seined from a 0.1-acre (0.04-hectare) experimental research pond at the Thad Cochran National Warmwater Aquaculture Center in Stoneville, Mississippi, USA. Smallmouth buffalo were examined for the presence of parasitic infection. Two previously unknown *Myxobolus* species were observed parasitizing the gills. Plasmodia of the two species differed from each other in both size and shape. Morphologically the two species were distinct from one another and from other *Myxobolus* spp. previously reported from buffalo fish. Myxospores of *Myxobolus ictiobus* n. sp. were spherical and measured 12.7–14.5 (13.9±0.4) µm in length and 10.7–13.6 (12.5±0.7) µm in width with a thickness of 10.3–14.8 (12.6±2.3) µm. Polar capsules measured 5.6–7.4 (6.6±0.4) µm in length and 3.7–4.9 (4.5±0.8) µm

in width and each contained a coiled polar filament with 5–6 turns. *Myxobolus minutus* n. sp. myxospores were circular in shape and measured 7.4–9.6 (8.6±0.7) µm in length and 7.5–9.9 (8.8±0.7) µm in width with a thickness of 6.5–7.3 (6.7±0.3) µm. Polar capsules measured 3.6–4.9 (4.3±0.3) µm in length and 2.8–3.8 (3.3±0.3) µm and each contained a coiled polar filament with 5–6 turns. Supplemental 18S rRNA gene sequencing identified unique sequences for each isolate. Phylogenetic analysis of 18S rRNA sequence demonstrated a strong clustering of both isolates with other species of *Myxobolus* from Cypriniform fish.

7.2 Introduction

Species of the genus *Myxobolus* Bütschli, 1882 are cosmopolitan metazoan parasites of freshwater and marine fish. With > 900 members, the genus has the most described species of all known myxozoans (Eiras et al. 2014). Many are described only by morphological characters, which alone can be ambiguous and subjective, especially if multiple species display similar tissue tropism or a single species occurs in multiple hosts (Eszterbauer 2002; Ferguson et al. 2008; Griffin et al. 2014). Currently, novel species are described using a combination of morphological characterization supplemented with sequencing of the small subunit ribosomal RNA gene.

Smallmouth buffalo *Ictiobus bubalus* Raffinesque (Catostomidae) is native to North America, inhabiting the waterways of the Mississippi River drainage system and other water bodies within this range. Myxozoans have been reported from buffalo fish (*Ictiobus* spp.) in North America, although all reports are from wild-caught fish. Recently researchers attempted to employ the benthivorous capacity of smallmouth buffalo as biological control of proliferative gill disease (PGD) in farm-raised catfish

(Mischke et al. 2016). It is speculated that opportunistic foraging on the benthos in catfish aquaculture ponds would result in a reduction of *Dero digitata* Müller, the oligochaete host of *Henneguya ictaluri* Pote, Hanson, and Shivaji, 2000, the causative agent of PGD in channel and hybrid catfish (Bosworth et al. 2003; Griffin et al. 2010).

A subsample of harvested smallmouth buffalo that had been raised in polyculture with channel catfish as part of a biological control study, were screened to determine if smallmouth buffalo carried parasites that could infect channel catfish. Two previously uncharacterized species of *Myxobolus* were identified. Herein we describe *Myxobolus ictiobus* n. sp. and *Myxobolus minutus* n. sp. from the gills of smallmouth buffalo raised in polyculture with channel catfish in experimental research ponds.

7.3 Materials and methods

7.3.1 Myxospore isolation and morphologic characterization

Thirteen smallmouth buffalo, seined from 0.1-acre channel catfish research ponds at the Thad Cochran National Warmwater Aquaculture Center in the spring of 2013, were necropsied and examined for internal and external parasites. Wet mounts of gill tissues were taken, revealing the presence of myxozoan pseudocysts. Gill arches were removed and preserved in 70% molecular biology grade ethanol for further analysis.

Pseudocysts were excised by sharp dissection and placed onto clean glass microscope slides, diluted with saline, and covered with a coverslip. Photomicrographs of ethanol fixed myxospores were obtained using an Olympus BX-50 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) with an Olympus DP72 camera and the DP-2-Twain/cellSens software package (Olympus Optical Co. Ltd., Tokyo, Japan). Measurements of myxospores were obtained from digital images and line drawings were

made from photomicrographs with the aid of a camera lucida. Comparisons of these isolates were made to other *Myxobolus* spp. parasitising the gills of buffalo fish and catostomid fish. It is important to note that measurements of these isolates, while averaged from multiple spores, may be lower than those of fresh spores due to shrinkage associated with ethanol fixation (Kudo 1921; Parker and Warner 1970). However, these isolates are supplemented with molecular sequence data that would allow for more accurate identification moving forward. All measurements are in micrometres unless otherwise indicated and are given in the text and tables as the range followed by the mean and standard deviation in parentheses. Measurements are from 30 myxospores preserved in 70% ethanol.

7.3.2 Myxospore DNA extraction and molecular characterization

Myxospores were rinsed from microscope slides into 1.5 ml microcentrifuge tubes and centrifuged at 10,000 x g for 10 minutes. The supernatant was removed and genomic DNA (gDNA) was extracted using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, California). All gDNA was stored at -20°C until further use.

The 18S rRNA gene was amplified using previously published primers (Table 7.1). Each 25-µl PCR reaction contained 22 µl of Platinum Taq Supermix (Invitrogen, Carlsbad, California), 10 pmol of each primer, and 1 µl of gDNA. Primers were paired as follows to generate overlapping sequences: ERIB1/ACT1R, H9/ERIB10, Myxo1F/Myxgen3R, H2/H9, MyxospecF/MyxospecR. The thermal cycling program consisted of 95°C for 10 minutes, 35 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 2 min, and a final extension step at 72° C for 10 min. Amplification of the 18S rRNA gene was performed using an MJ Research PTC-200 thermocycler (GMI, Ramsey,

Minnesota). All amplicons were imaged in 1.2% agarose gels stained with ethidium bromide (0.5 µg/ml) under UV light alongside a concurrently run molecular weight DNA marker (HyperLadder™ 50 bp, Bioline, London, U.K.). Appropriately sized products were either purified directly using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, California) or bands were gel excised and purified using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, California). Purified amplicons were sequenced commercially using the corresponding primers (Eurofins MWG Operon LLC, Huntsville, Alabama). Sequencing reads were edited and aligned using SeqMan™ (DNAStar, Madison, Wisconsin). A single contiguous sequence was obtained for each species and compared to other myxozoan sequences in the National Center for Biotechnology Information non-redundant nucleotide database using a BLASTn search (Altschul et al. 1990).

Table 7.1 Primers used in the amplification of the 18S rRNA gene of *Myxobolus ictiobus* n. sp. and *Myxobolus minutus* n. sp.

Primer ID	Sequence (5'-3')	Reference
ACT1R	AATTTCACCTCTCGCTGCCA	Hallett & Diamant 2001
ERIB1	ACCTGGTTGATCCTGCCAG	Barta et al. 1997
ERIB10	CCTCCGCAGGTTCACCTACGG	Barta et al. 1997
Genmyxo4	GGATGTTGGTTCCGTATTGG	Griffin et al. 2008
H2	CGACTTTTACTTCCTCGAAATTGC	Hanson et al. 2001
H9	TTACCTGGTCCGGACATCAA	Hanson et al. 2001
Myxo1F	CTGCCCTATCAACTWGTT	Kent et al. 2000
Myxogen3R	TGCCTTCGCATTYGTTAGTCC	Kent et al. 2000
MyxospecF	TTCTGCCCTATCAACTWGTTG	Fiala 2006
MyxospecR	GGTTTCNCDGRGGGMCCAAC	Fiala 2006

The 18S rRNA gene sequences for each species were analyzed against other published myxozoan sequences available in the NCBI database. Published myxozoan sequences > 1,500 nt were downloaded and used in phylogenetic analysis performed in

Molecular Evolutionary Genetic Analysis 6.0 (MEGA6) (Tamura et al. 2013). Sequences were aligned with ClustalW. Using the Akaike Information Criterion the model of best fit was TN93+G+I (Nei & Kumar, 2000). Phylogenetic placement was inferred with the maximum likelihood method using the Tamura-Nei model (Tamura and Nei 1993) with 1,000 bootstraps.

7.4 Results

Myxospores typical of the genus *Myxobolus* were observed on gill wet mounts of 3 out of 13 (23.1%) smallmouth buffalos examined. These were further differentiated morphologically and molecularly as two previously undescribed species of *Myxobolus*.

7.4.1 Taxonomic summary

Species: *Myxobolus ictiobus* n. sp. (Cnidaria: Myxosporea: Myxobolidae)

Type host: *Ictiobus bubalus* (Rafinesque, 1818) (Cypriniformes: Catostomidae)

Type locality: catfish aquaculture pond, Washington County, Mississippi, USA

Site of infection: gill filaments

Prevalence: 2/13 fish; 15.38%

Materials deposited: 18S rRNA gene sequence, accession number KU232371 National Center for Biotechnology Information

Etymology: The specific epithet is in reference to the host genus *Ictiobus*.

7.4.2 Remarks

Pseudocyst, round, 148 in length and 122 in width and intralamellar. Myxospore, spherical, 12.7–14.5 (13.9±0.4) in length, 10.7–13.6 (12.5±0.7) in width and 10.3–14.8 (12.6±2.3) thick. Polar capsules 2, pyriform, 5.6–7.4 (6.6±0.4) in length, 3.7–4.9

(4.5±0.8) in width, each containing a coiled polar filament with 5–6 coils (Figures 7.1A, 7.1C and 7.2A).

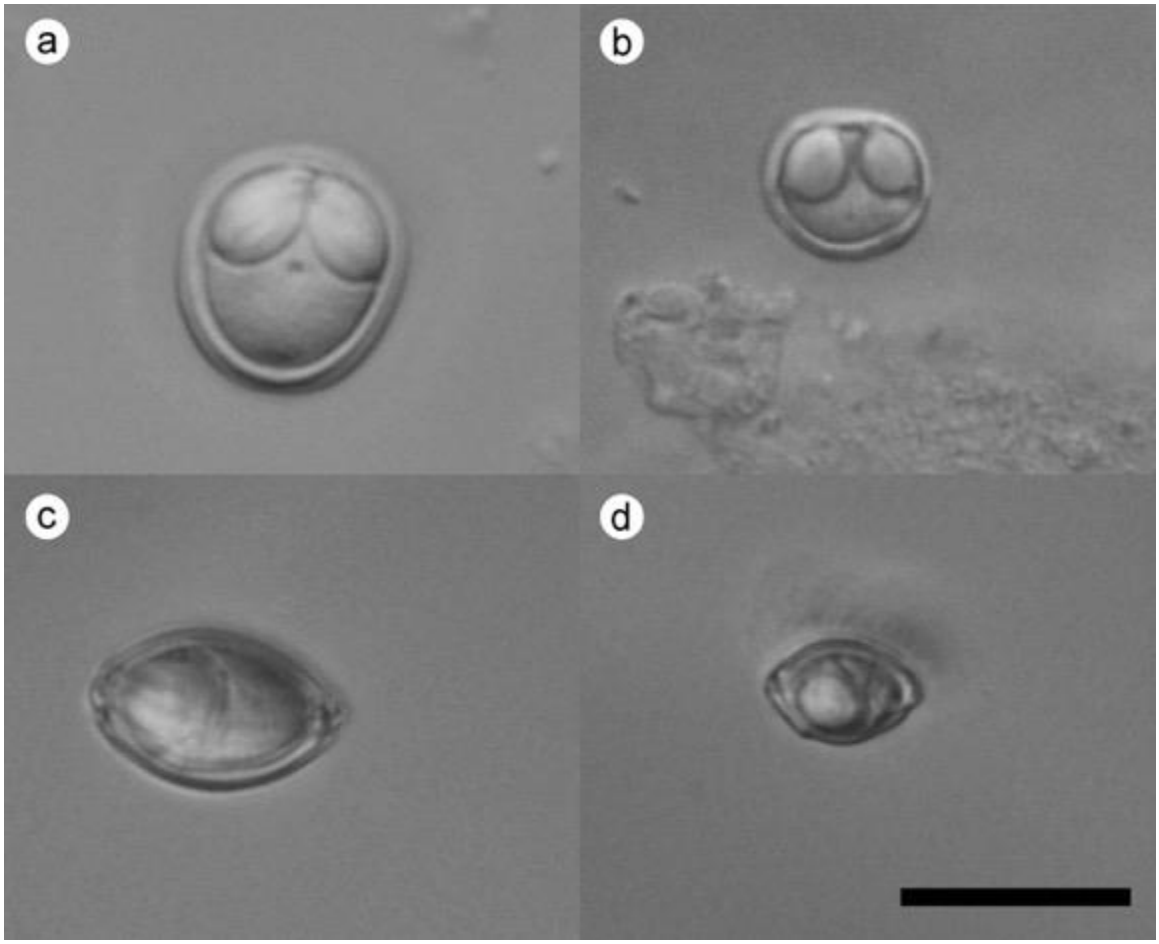

Figure 7.1 Photomicrographs of myxospores of *Myxobolus ictiobus* n. sp. and *Myxobolus minutus* n. sp.

Valvular view of *Myxobolus ictiobus* n. sp. (a) and *Myxobolus minutus* n. sp. (b). Sutural view of myxospores of *Myxobolus ictiobus* n. sp. (c) and *Myxobolus minutus* n. sp. (d). Scale bar 10 µm.

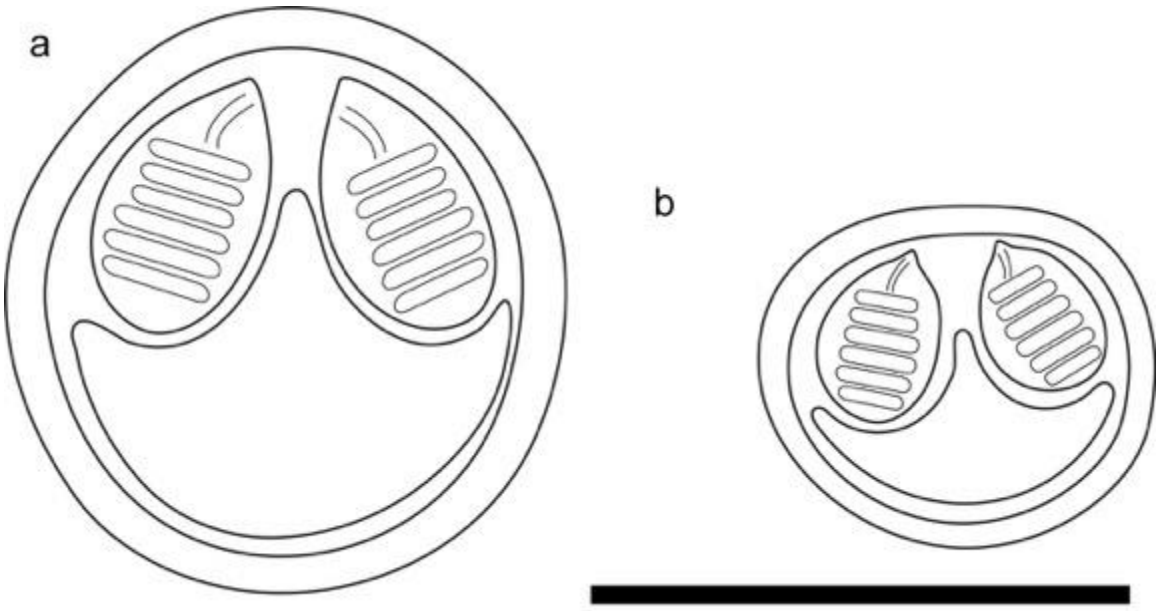

Figure 7.2 Line drawings of myxospores of *Myxobolus ictiobus* n. sp. (a) and *Myxobolus minutus* n. sp. (b).

Scale bar 10 µm.

When compared to other *Myxobolus* spp. from buffalo fish, *M. ictiobus* n. sp. shared several features overlapping with previously identified *Myxobolus* species from the gills of smallmouth buffalo from other localities, but differed greatly in several measured features suggesting this is a uniquely described species. The most marked variation occurred with spore thickness observed in sutural view (Figure 7.1 C). *Myxobolus ictiobus* n. sp. was much thicker (12.6 µm) compared to the other species reported from *Ictiobus* spp. (<11.0 µm; Table 7.2). Also, pseudocysts of *M. ictiobus* n. sp. were smaller (148 x 122 µm) compared to the other species of *Myxobolus* reported from buffalo fish. Although similar to *Myxobolus bibullatus* Kudo, 1934 from the integument and the gills of white sucker *Catostomus commersoni* Lacépède in Nova Scotia, polar capsules were smaller than those of *M. ictiobus* (see Kudo, 1934).

Table 7.2 Morphological comparison of *Myxobolus* species from buffalo fish *Ictiobus* spp.

Species	SL	SW	ST	PCL	PCW	PF	PL	PW	Host	Tissue site	Locality	Reference
Myxobolus bubalis	(13.1–14.7)	(10.2–11.7)		(5.8–6.3)	(2.2–2.9)	(6–7)	438	–	*Ictiobus bubalus*	Intestine	Iowa, USA	Otto and Jahn 1943
Myxobolus discrepans	(11.4–13.5); 15.5 14.3	(9.5–11.0); 13.9	–	(5.5–6.0); 5.4	(3.5–4.0); 3.9 5.5	(8–10)	–	–	*Carpiodes difformis; Ictiobus bubalus*	Gills	Iowa, USA	Kudo 1920; Rice and Jahn 1943
Myxobolus endovasus	(13.1–15.4) 14.4	13 (12.2–13.8) 11.1	8.5	8.7 (7.7–9.6)	(5.0–6.2) 4.8	6 (5–7)	1.2 mm	1.3 mm	*Ictiobus cyprinellus*	Gills	Illinois, USA	Lom and Cone 1996
Myxobolus enoblei	(13.5–15.0) 13.9	(10.5–11.5) 12.5	7.5 12.6	8.3 (7.9–8.5)	(4.5–5.0) 4.5	7	1.5 mm	0.3 mm	*Ictiobus bubalus*	Gills	Illinois, USA	Lom and Cone 1996
Myxobolus ictiobus	(12.7–14.5)	(10.7–13.6)	(10.3 – 14.8)	6.6 (5.6–7.4)	(3.7–4.9)	(5-6)	148	122	*Ictiobus bubalus*	Gills	Mississippi, USA	This paper
Myxobolus filamentus	13.2	16.3	–	7.8	6.3 3.3	–	(175–200)	–	*Ictiobus bubalus*	Gills	Iowa	Rice and Jahn 1943
Myxobolus minutus	8.6 (7.4–9.6)	8.8 (7.5–9.9)	6.7 (6.5–7.3)	4.3 (3.6–4.9)	(2.8–3.8) 3.7	(5-6)	1.3 mm	0.4 mm	*Ictiobus bubalus*	Gills	Mississippi, USA	This paper
Myxobolus morrisonae	10 (9.6–10.5)	9.5 (9.1–10.3)	5.0	5.5 (5.3–5.8)	(3.3–4.0) 2.25	6	1.5 mm	0.3 mm	*Ictiobus bubalus*	Gills	Illinois, USA	Lom and Cone 1996
Myxobolus multiplicatum	12.0 (12-12.5); 10.9	9.5 (9.25–9.5); 9.3	6.0 (6.0–6.5); –	4.0 (3.5–4.5); 4.7	(2.0–2.5); 3.1	(7–9)	–	–	*Idus melanotus; I. bubalus*	Muscle, Gills	Russia; Iowa, USA	Reuss 1909; Rice and Jahn 1943
Myxobolus ovalis	(15.0–17.0)	15.0	11.0	(8.0–9.0)	6.0	(5–6)	(500–700)	–	*I. bubalus, I. cyprinellus*	Gills	Iowa, USA	Davis 1923
Myxobolus ovatus	(11.5–13.0)	(9.0–10.0)	7.0	(5.5–6.5)	(2.5–3.0)	–	(1.5–2mm)	1 mm	*Ictiobus bubalus*	Integument	Illinois, USA	Kudo 1934

Measurements are all reported in µm unless otherwise stated with ranges being represented in parentheses. For species with multiple references the measurements are presented chronologically and separated by a semicolon. Spore length (SL), spore width (SW), spore thickness (ST), polar capsule length (PCL), polar capsule width (PCW), coils in polar filament (PF), plasmodium length (PL), plasmodium width (PW), and no data reported (–).

The 1,713 nt long 18S rRNA gene sequence of *Myxobolus ictiobus* n. sp. shared a 95.1% (1,626/1,710 nt) sequence identity (99% coverage) with a triactinomyxon type actinospore from *Limnodrilus hoffmeisteri* Claparède from Oregon, USA (AY997026, unpublished sequence), 88.6% (98% coverage) with *M. bibullatus* from *C. commersoni* in Canada (AF378336; Kent et al. 2001), 85.1% (99% coverage) and 84.9% (99% coverage) with an antonactinomyxon type (AF378355) and a synactinomyxon type (AF378354) actinospore from *Limnodrilus hoffmeisteri* from Canada, respectively (see Kent et al. 2001).

7.4.3 Taxonomic summary

Species: *Myxobolus minutus* n. sp. (Cnidaria: Myxosporea: Myxobolidae)

Type host: *Ictiobus bubalus* (Rafinesque, 1818) (Cypriniformes: Catostomidae)

Type locality: catfish aquaculture pond, Washington County, Mississippi, USA

Site of infection: gill filaments

Prevalence: 1/13 fish; 7.69%

Materials deposited: 18S rRNA gene sequence, Accession number KU232372 National Center for Biotechnology Information

Etymology: The specific epithet is in reference to the small size of the myxospores.

7.4.4 Remarks

Pseudocyst, elongate, approximately 1.3 mm in length and 0.4 mm in width along the edge of the primary lamellae. Myxospore, ovoid, 7.4–9.6 (8.6±0.7) in length, 7.5–9.9 (8.8±0.7) in width, and 6.5–7.3 (6.7±0.3) thick. Pyriform polar capsules, 2, 3.6–4.9

(4.3±0.3) in length and (2.8–3.8) (3.3±0.3) in width, and each containing a coiled polar filament with 5–6 coils when visible.

Morphologically *M. minutus* n. sp. shared similar features with other species of *Myxobolus* from buffalo fish (Table 2). The pseudocyst dimensions of *M. minutus* n. sp. (1.3 × 0.4 mm) are similar to *Myxobolus enoblei* Lom & Cone, 1996 (1.5 × 0.3 mm) and *Myxobolus morrisonae* Lom & Cone, 1996 (1.5 × 0.3 mm). However the myxospores vary considerably from these species and are greatly reduced in size (8.6 × 8.8 µm) compared with the other species of Myxobolus described from catostomid fish from North America. *Myxobolus minutus* n. sp. differs notably from the morphologically similar *M. morrisonae* when comparing the thickness of the myxospores in sutural view and the overall shape of the myxospores. Myxospores of *Myxobolus minutus* n. sp. were considerably thicker on average than those of *M. morrisonae* (6.7 vs 5.0). The myxospores of *M. minutus* were more ovoid as well. The minute size and greater thickness of myxospores of *M. minutus* n. sp. suggests this is also a unique species.

The 1,970 nt long 18S rRNA gene sequence of *Myxobolus minutus* n. sp. shared a 91.5% (1,810/1,979 nt) sequence identity (99% coverage) with *Myxobolus bibullatus* from the cyprinid *C. commersoni* from Canada (AF378336; Kent et al. 2001), 88.5% (99% coverage) with a triactinomyxon type actinospore from *L. hoffmeisteri* from Oregon, USA (AY997026, unpublished sequence), 85.8% (97% coverage) and 85.4% (97% coverage) with a synactinomyxon type (AF378354) and an antonactinomyxon type (AF378355) actinospore from *L. hoffmeisteri* from Canada, respectively (Kent et al. 2001).

Phylogenetic analysis positioned both *M. ictiobus* n. sp. and *M. minutus* n. sp. sister to *M. bibullatus* from the gills of the white sucker *C. commersoni* in Canada (Kent et al. 2001) with high bootstrap support (Figure 7.3). *Myxobolus ictiobus* n. sp. was also sister to an unidentified myxozoan triactinomyxon type actinospore from the oligochaete *L. hoffmeisteri* (see Kent et al. 2001).

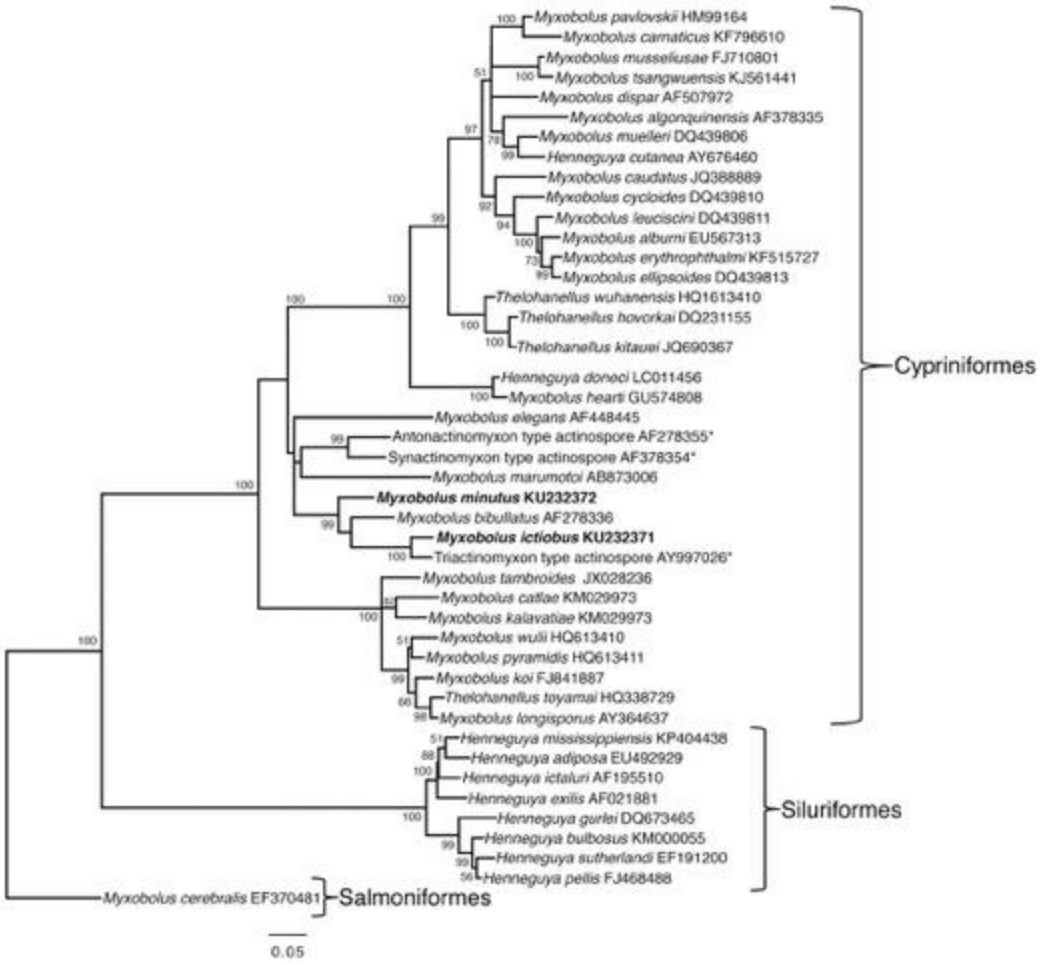

Figure 7.3 Maximum likelihood tree containing *Myxobolus ictiobus* n. sp. and *Myxobolus minutus* n. sp. (bolded) and closely related myxozoans and those parasitizing ictalurid fish from North America.

Values at the nodes represent bootstrap confidence values based on 1,000 replicates. (*) Indicates sequence from an annelid actinospore stage not yet identified as a myxospore stage in a fish host.

7.5 Discussion

Myxobolus is the most species-rich genus of myxozoan parasites to date. Davis (1923) first reported *Myxobolus* sp. from the gills of smallmouth buffalo and largemouth buffalo *Ictiobus cyprinellus* Valenciennes in Iowa, USA, and described the differing stages of development and sporulation of a gill infecting myxosporean, *Myxobolus ovalis* Davis, 1923 (syn. *Lentospora ovalis*). Following Davis, Kudo (1934) described *Myxobolus ovatus* Kudo, 1934 from the integument of a smallmouth buffalo during a survey of myxozoan species of freshwater fish of Illinois. Otto and Jahn (1943) described spherical plasmodia of *Myxobolus bubalis* Otto and Jahn, 1943 in the intestinal tract of *I. bubalus* in Iowa, while Rice and Jahn (1943) reported *Myxobolus multiplicatum* Kudo, 1933 from the gill filaments and arches of *I. bubalus* in lakes of Iowa. The authors acknowledged this as a new host and geographical record for this species and discussed discrepancies between their measurements and with those originally reported from ide *Leuciscus idus* Linnaeus, from the Volga, Russia (Reuss 1906). Similarly, Rice and Jahn (1943) also reported *Myxobolus transovalis* Gurley, 1893, a myxobolid originally described from beneath the scales of rosyside dace *Clinostomus funduloides* Girard, from a tributary of the Potomac River in Virginia (Gurley 1893).

Grinham and Cone (1990) described several new myxozoan species from the white sucker *C. commersoni* from Nova Scotia and provided a thorough review of the literature concerning species of *Myxobolus* parasitizing catostomid fishes. They considered the smallmouth buffalo to have seven valid species, including *M. multiplicatum* and *M. transovalis* (see Grinham and Cone 1990). Lom and Cone (1996) later reported three new species of myxozoan from *I. bubalus* in Illinois; however, the

authors consistently used the common name of bigmouth buffalo when reporting these species, rather than smallmouth buffalo, which is the appropriate common name of *I. bubalus* (Page et al. 2013).

Two previously unidentified species of *Myxobolus* from the gills of smallmouth buffalo from catfish production ponds in Mississippi are described herein using morphological and molecular techniques. These two species were morphologically distinct from all reported species from *Ictiobus* spp. In recent years, molecular techniques, largely sequencing of the 18S rRNA gene, have exposed the limitations of using phenotypic characters alone to describe novel species of myxozoans, especially when a species has been reported to have a wide host range (Eszterbauer 2004; Molnár et al. 2009). The historical records of myxozoans from buffalo fish are from before the advent of molecular techniques; therefore molecular comparisons could not be made with these species. Phylogenetic analysis clusters *M. ictiobus* n. sp. and *M. minutus* n. sp. with other species of *Myxobolus* reported from cyprinid fish (Figure 7.3) and is in agreement with previous reports of species of *Myxobolus* and *Henneguya* Thélohan, 1892 group according to host order and family (Carriero et al. 2013).

Currently the life histories of these two species are unknown, but it is speculated they involve an annelid definitive host endemic to catfish production ponds. Most myxospores take less than six months to develop in the fish host (Székely et al. 2001; Kallert et al. 2005; Székely et al. 2009). Given the time the smallmouth buffalo were in the pond (12–18 months), the presence of mature myxospores in the gills suggests the invertebrate host required to complete these life-cycles was present in the culture system. It is unknown whether these parasites were introduced into the system with the

smallmouth buffalo or if they were already present in the pond and cycling through other non-catfish hosts. Since myxozoan species usually parasitize closely related fish hosts, it is unlikely these parasites of cypriniform fish would infect pond-raised catfish and to date morphologically or molecularly similar species have not been reported from Siluriformes. In addition to the morphological descriptions, the 18S rRNA gene sequencing data obtained for both species will be useful in elucidating the life-cycles of *M. ictiobus* n. sp. and *M. minutus* n. sp. once the corresponding actinospore stage is characterized from an annelid host.

7.6 References

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403–410.

Barta, J. R., D. S. Marin, P. A. Liberator, M. Dashkevicz, J. W. Anderson, S. D. Feigner, A. Elbrecht, A. Perkins-Barrow, M. C. Jenkins, H. D. Danforth, M. D. Ruff, and H. Profous-Juchelka. 1997. Phylogenetic relationships among eight *Eimeria* species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. Journal of Parasitology 83:262–271.

Carriero, M. M., E. A. Adriano, M. R. M. Silva, P. S. Ceccarelli, and A. A. M. Maia. 2013. Molecular phylogeny of the *Myxobolus* and *Henneguya* genera with several new South American species. PLoS One 8:e73713.

Davis, H. S. 1923. Studies on sporulation and development of the cysts in a new species of myxosporidia, *Lentospora ovalis*. Journal of Morphology 37:425–455.

Eiras, J. C., K. Molnár, and Y. S. Lu. 2005. Synopsis of the species of *Myxobolus* (Bütschli, 1882, (Myxozoa: Myxosporea: Myxobolidae). Systematic Parasitology 61:1–46.

Eiras, J. C., J. Zhang, and K. Molnár. 2014. Synopsis of the species of *Myxobolus* Bütschli, 1882 (Myxozoa: Myxosporea, Myxobolidae) described between 2005 and 2013. Systematic Parasitology 88:11–36.

Eszterbauer, E. 2002. Molecular biology can differentiate morphologically indistinguishable myxosporean species: *Myxobolus elegans* and *M. hungaricus*. Acta Veterinaria Hungarica 50:59–62.

Eszterbauer, E. 2004. Genetic relationship among gill-infecting *Myxobolus* species (Myxosporea) of cyprinids: molecular evidence of importance of tissue-specificity. Diseases of Aquatic Organisms 58:35–40.

Ferguson, J. A., S. D. Atkinson, C. M. Whipps, and M. L. Kent. 2008. Molecular and morphological analysis of *Myxobolus* spp. of salmonid fishes with the description of a new *Myxobolus* species. Journal of Parasitology 94:1322–1334.

Fiala, I. 2006. The phylogeny of Myxosporea (Myxozoa) based on small subunit ribosomal RNA gene analysis. International Journal of Parasitology 36:1521–1534.

Griffin, M. J., L. M. Pote, D. J. Wise, T. E. Greenway, M. J. Mauel, and A. C. Camus. 2008. A novel *Henneguya* species from channel catfish described by morphological, histological and molecular characterization. Journal of Aquatic Animal Health 20:127–135.

Griffin, M., S. Quiniou, C. Ware, L. Bogdanovic, and E. Soto. 2014. *Kudoa thunni* from blackfin tuna (*Thunnus atlanticus*) harvested off the island of St. Kitts, West Indies. Journal of Parasitology 100:110–116.

Grinham, T. and D. K. Cone. 1990. A review of species of *Myxobolus* (Myxosporea) parasitizing catostomid fishes, with a redescription of *Myxobolus bibullatus* (Kudo, 1934) n. comb. and description of *Myxobolus lamellus* n. sp. from *Catostomus commersoni* in Nova Scotia. Canadian Journal of Zoology 68:2290–2298.

Gurley, R. R. 1893. On the classification of the Myxosporidia, a group of protozoan parasites infesting fishes. Bulletin of the U. S. Fish Commission 11:407–420.

Hallett, S. L. and A. Diamant. 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. Diseases of Aquatic Organisms 46:197–212.

Hanson, L. A., D. Lin, L. M. Pote, and R. Shivaji. 2001. Small subunit rRNA gene comparisons of four actinosporean species to establish a polymerase chain reaction test for the causative agent of proliferative gill disease in channel catfish. Journal of Aquatic Animal Health 13:117–123.

Kallert, D. M., E. Eszterbauer, M. El-Matbouli, C. Erséus, and W. Haas. 2005. The life cycle of *Henneguya nuesslini* Schuberg and Schröder, 1905 (Myxozoa) involves a triactinomyxon-type actinospore. Journal of Fish Diseases 28:71–79.

Kent, M. L., K. B. Andree, J. L. Bartholomew, M. El-Matbouli, S. S. Desser, R. H. Devlin, S. W. Feist, R. P. Hedrick, R. W. Hoffmann, J. Khattra, S. L. Hallett, R. J. G. Lester, M. Longshaw, O. Palenzeula, M. E. Siddall, and C. Xiao. 2001. Recent advances in our knowledge of the Myxozoa. Journal of Eukaryotic Microbiology 48:395–413.

Kudo, R. 1921. On the nature of structures characteristic of cnidosporidian spores. Transactions of the American Microscopical Society 40:59–74.

Kudo, R. R. 1934. Studies on some protozoan parasites of fishes of Illinois. Illinois Biological Monographs 13:7–44.

Page, L. M., H. Espinosa-Pėrez, L. T. Findley, C. R. Gilbert, R. N. Lea, N. E. Mandrak, R. L. Mayden and J. S. Nelson. 2013. Common and scientific names of fishes from the United States, Canada, and Mexico. American Fisheries Society Special Publication (34): 243 p.

Lom, J. and D. Cone. 1996. Myxosporeans infecting the gills of bigmouth buffalo (*Ictiobus bubalus*) in Illinois, USA. Folia Parasitologica 43:37–42.

Mischke, C. C., M. J. Griffin, D. J. Wise, and T. E. Greenway. 2016. Effects of co-stocking smallmouth buffalo, *Ictiobus bubalis*, with channel catfish, *Ictalurus punctatus*. Journal of the World Aquaculture Society. 47:212–219.

Molnár, K., C. Székely, S. L. Hallett, and S. D. Atkinson. 2009. Some remarks on the occurrence, host-specificity and validity of *Myxobolus rotundus* Nemeczek, 1911 (Myxozoa: Myxosporea). Systematic Parasitology 72:71–79.

Nei, M., and S. Kumar. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Otto, G. R., and T. L. Jahn. 1943. Internal myxosporidian infections of some fishes of the Okoboji region. Proceedings of the Iowa Academy of Sciences 50:323–335.

Parker, J. D., and M. C. Warner. 1970. Effects of fixation, dehydration and staining on dimensions of myxosporidan and microsporidan spores. Journal of Wildlife Diseases 6:448–456.

Reus, H. 1906. Neue Myxoboliden von Süsswasserfischen. Bulletin de l'Académie Impériale des Sciences de Saint-Pétersbourg, 25:199–206.

Rice, V. J., and T. L. Jahn. 1943. Myxosporidian parasites from the gills of some fishes of the Okoboji region. Proceedings of the Iowa Academy of Sciences 50:313–321.

Székely, C., S. L. Hallett, S. D. Atkinson, and K. Molnár. 2009. Complete life cycle of *Myxobolus rotundus* (Myxosporea: Myxobolidae), a gill myxozoan of common bream *Abramis brama*. Diseases of Aquatic Organisms 85:147–155.

Székely, C., K. Molnár, and O. Rácz. 2001. Complete developmental cycle of *Myxobolus pseudodispar* (Gorbunova) (Myxosporea: Myxobolidae). Journal of Fish Disease 24:461–468.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30:2725–2729.

Tamura, K. and M. Nei. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10:512–526.

CHAPTER VIII

INVESTIGATIONS INTO THE DEVELOPMENT OF *HENNEGUYA ICTALURI* (CNIDARIA: MYXOBOLIDAE) IN CHANNEL CATFISH *ICTALURUS-PUNCTATUS*, BLUE CATFISH *ICTALURUS FURCATUS*, AND THEIR HYBRID CROSS

8.1 Abstract

Henneguya ictaluri is the etiologic agent of proliferative gill disease (PGD) in farm raised channel and hybrid catfish in the southeastern United States. The most prevalent parasitic disease in catfish aquaculture, PGD is attributed to significant yearly losses. Recent research has suggested the dynamics of *H. ictaluri* infection in blue catfish and channel catfish (♀) x blue catfish (♂) hybrids differs from channel catfish. Two separate experimental infectivity trials were carried out to investigate the development of *H. ictaluri* in channel catfish, blue catfish, and their hybrid cross. On two separate occasions using two different year classes of fish, fish were exposed to infectious pond water containing *H. ictaluri* actinospores and sampled weekly, for twelve weeks in Trial 1 and fourteen weeks in Trial 2. In Trial 1, the presence of *H. ictaluri* was evaluated histologically and by quantitative polymerase chain reaction (qPCR) on a complement of tissues, including gills, blood, anterior kidney, brain, heart, liver, posterior kidney, spleen, and stomach. Trial 1 demonstrated *H. ictaluri* DNA in significantly higher concentrations throughout multiple organ systems in the channel catfish compared

to hybrid catfish and blue catfish, with the gills having the highest levels of *H. ictaluri* DNA and being the site of pseudocyst development. Mature *Henneguya* spp. myxospores were observed in channel catfish as early as 8 weeks post-exposure. No mature myxospores were observed in either blue or hybrid catfish at any period during Trial 1. The second experimental trial focused on gills only and yielded results similar to Trial 1, with channel catfish having higher levels of *H. ictaluri* DNA across all time points. Again, mature *Henneguya* spp. myxospores were observed histologically in channel catfish beginning 6 weeks post-exposure, and were found in 36% (58/162) of channel catfish sampled from Week 6 through Week 14. Comparatively, myxospores and *H. ictaluri* DNA in hybrid catfish were sparse, with single myxozoan pseudocysts observed in only two hybrid catfish over the same span (1.2%), both at 14 weeks post-exposure. While this may suggest hybrid catfish are not completely refractory to *H. ictaluri* infection, these results imply significant arrested development of *H. ictaluri* in hybrid catfish. As such, propagation and culture of hybrid catfish may be an effective management strategy to minimize the burden of PGD on catfish aquaculture in the southeastern United States. Field studies investigating the impacts of these findings on a commercial scale are warranted.

8.2 Introduction

In the United States, the culture of ictalurid catfish is considered the largest component of the nation's aquaculture industry, with >50,000 water surface acres dedicated to production, yielding ~$352 million in total sales in 2014 (Hansen and Sites 2015) in spite of a significant industry contraction since its peak in 2003. Rising feed costs, increased competition from imported nonnative catfish species (e.g. *Pangasius* spp.

and *Pangasianodon* spp.) and more profitable land use alternatives threaten industry stability. Traditionally, production in the southeastern United States has focused on raising channel catfish *Ictalurus punctatus*, however there is continued interest in the channel catfish (♀) x blue catfish (♂) hybrid (hereafter hybrid catfish) (Dunham and Masser 2012). In addition to the hybrids superior production characteristics, inherent resistance to certain infectious diseases problematic for channel catfish culture has been reported and warrants further investigation (Wolters et al. 1996; Bosworth et al. 2003; Griffin et al. 2010; Brown et al. 2011; Dunham and Masser 2012).

Losses attributed to infectious disease accounts for nearly half of all losses in catfish aquaculture (Hawke and Khoo 2004). Farm-raised channel catfish *Ictalurus punctatus* have persistently been plagued by the myxozoan *Henneguya ictaluri*, the causative agent of proliferative gill disease (Bowser and Conroy 1985; Pote et al. 2000; Pote et al. 2012). The complex life cycle of *H. ictaluri* is perpetuated by actinospore stages released by the ubiquitous benthic oligochaete definitive host *Dero digitata* and myxospore stages in the gill tissue of the channel catfish (Pote et al. 2000), consistent with other myxozoans (Wolf and Markiw 1984; Kent et al. 2001). Seasonal outbreaks in the spring have been attributed to increased populations of *D. digitata* actively shedding infectious actinospore stages (Bellerud et al. 1995; Mischke et al. 2016). Unlike other myxozoan diseases of cultured and wild fish populations, clinical manifestation of PGD is associated with the initial penetration and proliferation of the parasite rather than mature myxospore stages (Wise et al. 2008).

Host specificity appears to play a significant role in *H. ictaluri* transmission. When exposed to pond water containing *H. ictaluri* actinospores, blue catfish

demonstrate significantly less gill damage than their channel and hybrid counterparts, with no observed developmental stages and limited detection of parasite DNA in gills and blood (Bosworth et al. 2003; Griffin et al. 2010). While hybrid catfish exposed under similar conditions suffer gill damage on par with channel catfish, *H. ictaluri* DNA in gill tissues was significantly lower during early stages of disease (1 week post-exposure), suggesting less efficient transmission and proliferation of the parasite in hybrid catfish (Griffin et al. 2010). In this current study, the period from initial infection to myxospore maturation was evaluated using a polyphasic approach, combining molecular and histological techniques. Herein we report the results of two separate infectivity trials covering the entire developmental period of *H ictaluri* in channel catfish to document the development of *H. ictaluri* in blue catfish and hybrid catfish and evaluate their putative role in the propagation of *H. ictaluri* in catfish production systems.

8.3 Materials and methods

8.3.1 Fish exposures

Two separate year classes (2010 and 2014) of channel, blue and hybrid catfish fingerlings (7-13 cm) were used in these studies. Fish were reared indoors for disease research at the Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS. Prior to the infectivity trials, fish were housed in 1000 L circular tanks in 500 L of flow-through well water (~26-27ºC) supplied at a rate of 3.8 L/min with constant aeration.

Two separate infectivity trials were performed to assess the development of *H. ictaluri* in channel catfish, blue catfish and hybrid catfish. In line with previous studies, infectious water (pond water containing actinospores) collected from ponds harboring

active PGD outbreaks in the resident fish population were used for disease challenges (Wise et al. 2008). The presence of *H. ictaluri* in pond water was verified by *H. ictaluri*-specific qPCR (Griffin et al. 2009) and infectious pond water was transported to the Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS in a 2000-L live hauler. Fish for all trials were stocked into 115-L flow-through aquaria, holding ~35 liters of well water (~27ºC) supplied at a flow rate of 1.9 L per minute together with supplemental aeration. During fish exposure, water flow was suspended, the water level was lowered to approximately ~30 L, and aquaria were filled with freshly collected pond water to near full capacity (~115 L). Fish were held in infectious pond water for 4 hours, after which the flow of well water was resumed. Exposures were repeated every day for 4 consecutive days.

In Trial 1 (Spring 2011), triplicate groups of 45 channel, blue, and hybrid catfish were placed in discrete 115-L aquaria were exposed to infectious pond water containing *H. ictaluri* actinospores as described above. Three additional aquaria for each fish group were maintained similarly, but were not exposed to infectious pond water. Actinospore levels were determined using by qPCR assay Griffin et al. (2009) of averaging three separate 500 ml water samples collected from the infectious pond water in the live hauler. In trial one, the qPCR estimated infectious dose was 25–100 (4-day average=~80) spores per liter over the course of the four-day exposure period.

This procedure was repeated in Trial 2 (Spring 2015), using sextuplicate groups of 50 channel, blue and hybrid catfish housed in 115-L aquaria, respectively. Fish were exposed to infectious pond water containing *H. ictaluri* actinospores as described

previously. The qPCR estimated infectious dose for Trial 2 was 10–25 (4-day average=~20) spores per liter over the four-day exposure.

8.3.2 Fish necropsies, tissue collection and histology

During Trial 1, fish were maintained up to 12 weeks post exposure. Three fish were sampled weekly from each aquarium for Weeks 1–4 and Weeks 8–12. Due to subtle fish losses throughout the trial, fish sampling was forgone for Weeks 5–7 in an effort to ensure enough fish remained until maturation of pseudocysts (~90 days post exposure, Pote et al. 2000). Fish were euthanized with an overdose of tricaine methanesulfonate, MS-222 (Tricaine-S®, Western Chemical Inc., Ferndale, Washington). Of the three fish sampled, two were necropsied and organs were aseptically collected for molecular analysis. The remaining fish was opened ventrally by sharp dissection, the opercula were removed and the entire fish was fixed in 10% neutral buffered formalin for histopathologic evaluation. Approximately 100 µl of blood was collected from the caudal vein into 1.5-ml microcentrifuge tubes. From the necropsied fish, approximately 100 mg each of anterior kidney, brain, gill, heart, liver, posterior kidney, spleen, and stomach tissue were collected into 1.5-ml microcentrifuge tubes. All tissues were frozen at -80ºC until further processing. Presence of PGD lesions and developing myxospores were assessed by examining gill clip wet mounts of ~40-80 filaments taken from the right gill arch according to the lesion scoring system established by Wise et al. (2008).

During Trial 2, for Week 1–Week 4, two fish were sampled from each aquarium. One fish was processed as described above for molecular analysis and the other was fixed in 10% neutral buffered formalin for histopathologic evaluation. Starting at Week 5 and continuing until the end of the study at Week 14, three fish were sampled from each

aquarium. Two of these were fixed in 10% neutral buffered formalin and the remaining fish were processed for molecular analysis. Based on results of Trial 1, only gill tissues were analyzed in Trial 2. Again, PGD severity and myxospore development was assessed from fresh gill wet mounts according to Wise et al. (2008).

Tissues fixed in 10% neutral buffered formalin were trimmed routinely and processed by dehydration through a graded series of ethanol solutions, cleared in xylene, embedded in paraffin blocks and sectioned at 5 µm. Slides were stained with hematoxylin and eosin or Giemsa. Photomicrographs were captured using a BX-50 Olympus microscope (Olympus Optical Co., Ltd., Tokyo, Japan) with an Olympus DP72 camera and the corresponding DP-2-Twain/cellSens software (Olympus Optical Co., Ltd.).

8.3.3 Genomic DNA extraction from catfish tissues

Total genomic DNA (gDNA) was extracted from catfish tissues collected during the infectivity trials. Briefly, 600 µl of Cell Lysis Solution (Gentra® Puregene®, QIAGEN, Valencia, California) and 3 µl of proteinase K (20 mg/ml) was added to thawed tissues prior to overnight incubation at 55ºC. Isolation of gDNA then progressed according to the manufacturer's suggested protocol. Isolated gDNA was suspended in 200 µl of DNA Hydration Solution (DHS; 10 mM Tris, 1 mM EDTA, pH 7-8; Gentra® Puregene®, QIAGEN) and stored at -20ºC until further processing.

Blood was thawed at room temperature before gDNA was extracted following the Gentra® Puregene® Blood DNA kit (QIAGEN) manufacturer's suggested protocol. Extracted gDNA from blood was suspended in 100 µl of DHS and stored at -20ºC until further use.

8.3.4 Quantitative PCR

Spectrophotometric quantification of gDNA extracts was performed using a NanoDrop spectrophotometer (Nanodrop, Wilmington, Delaware). All gDNA extracts were standardized by dilution to 10 ng/µl with DHS and stored at -20ºC until PCR analysis. All diluted gDNA extracts from tissue samples collected from Trial 1 and gill tissue samples from Trial 2 were analyzed using qPCR assays targeting a 104-bp product of the *H. ictaluri* 18S rRNA gene (Griffin et al. 2008).

Each 15-µl reaction consisted of 7.75µl of IQ™ Supermix (Bio-Rad, Hercules, California, USA), 20 pmols of each primer, 2 pmols of probe (Table 8.1), 50 ng of genomic DNA template and nuclease free water to volume. Amplifications were carried out using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The thermal cycling program consisted of 95ºC for 2 minutes, followed by 40 cycles of 95ºC for 5 seconds and a combined 60ºC annealing and extension for 10 seconds. Data collection was performed following the extension phase at the end of each cycle. All amplification assays contained concurrently run, serial dilutions of the purified 104 bp amplification product. All samples, as well as no-template negative controls were run in triplicate. Data were only considered valid from runs where the slope of the standard curve ranged from 3.1-3.6, equating to estimated reaction efficiencies between 90% and 110% (Taylor et al. 2010).

Table 8.1 *Henneguya ictaluri* specific primers and probe used in qPCR analysis.

Primer ID	Sequence (5'-3')	Reference
H. ictaluri-1	CAAAAGTTTCTGCTATCATTG	Whitaker et al. 2001
H. ictaluri-2	AGCGCACAGATTACCTCA	Whitaker et al. 2001
H. ictaluri TaqMan probe	[FAM]-TCAGCCTTGATGTTGCCACCTCA-[BHQ1]	Griffin et al. 2008

FAM, 6-carboxyfluorescein; BHQ1, Black Hole Quencher-1.

8.3.5 Statistical analysis

All statistical analyses were performed in SAS version 9.4 (SAS Institute Inc., Cary, North Carolina). Prior to statistical analyses, the estimated quantity of *H. ictaluri* DNA was subjected to a log (x+1) transformation. Data was not normally distributed on account of several samples being negative for *H. ictaluri* by qPCR and having values of 0.0. As such, the Kruskal-Wallis one-way analysis of variance was applied to nonparametric data to compare the mean log starting quantity of *H. ictaluri* DNA between channel catfish, blue catfish, hybrid catfish, and the naïve control groups at each week. The Steel-Dwass-Critchlow-Fligner test was employed to determine significance between groups ($\alpha<0.05$). Significant differences in the proportions of channel catfish with grossly visible cysts versus hybrid catfish with grossly visible cysts during Week 6–Week 14 of Trial 2 was tested using the N-1 Chi-squared test.

8.4 Results

8.4.1 Trial 1

8.4.1.1 Gill tissue

Henneguya ictaluri DNA was detected in the gill tissue of all channel and hybrid catfish sampled at Week 1. In channel catfish *H. ictaluri* DNA was detected in the gills (log starting quantity [copy number]±standard error; range, 5.84±0.11; 5.76–6.06) and

also in hybrid catfish (4.97±1.13; 3.30–6.27), but were not significant (P=0.68). No *H. ictaluri* DNA was detected in the gills of any blue catfish or control fish at Week 1. By Week 2 channel catfish gill tissue had significantly higher (P=0.02) levels of detectable *H. ictaluri* DNA (6.25±0.26; 5.56–7.03) compared to hybrid catfish (3.45±0.08; 3.20–3.70). Continually at Week 3 and Week 4, detectable levels of *H. ictaluri* DNA in channel and hybrid catfish gills declined, but remained significantly lower in hybrid than channels (Week 3, P=0.02; Week 4, P=0.01). By Week 4, *H. ictaluri* DNA was only detectable in a single hybrid catfish, while 100% of channel catfish sampled remained positive. Similarly, at Week 8, *H. ictaluri* DNA was detectable in only a single hybrid catfish compared to 67% (4/6) of channel catfish (P=0.20). At Week 9, *H. ictaluri* DNA was detected in only a single channel catfish and no hybrid catfish. Beginning at Week 10, there was an increase in *H. ictaluri* DNA in channel catfish gills (0.87±0.54; 0.0–2.43), peaking at Week 11 (1.71±0.76; 0.0–4.56) before dropping at Week 12 (1.09±0.69; 0.0–3.52). There was no detectable *H. ictaluri* in hybrid gill tissue after Week 9. Throughout Trial 1 there was no amplification of *H. ictaluri* DNA observed in control or blue catfish tissue extracts (Figure 8.1).

Figure 8.1 Line graph of plotted mean log starting quantities of *H. ictaluri* DNA detected in gill tissue for Trial 1.

Values are plotted as the mean (± standard error). No data for Weeks 5–Week 7 post exposure. Superscripts denote statistical significance between groups at each respective time point; a,channel vs hybrid; b,channel vs blue; c,channel vs controls; d,hybrid vs blue; e,hybrid vs controls; f,blues vs controls.

Gill clip wet mounts revealed areas of chondrolytic lesions consistent with PGD in both channel catfish and hybrid catfish (Figure 8.2) at Week 1. Channel catfish had a higher incidence of cartilage breaks (50%; 3/6) compared to hybrid catfish (33%; 2/6) at Week 1. Similarly, at Week 2 more channel catfish (67%; 4/6) had breaks in the filamental cartilage than hybrid catfish (17%; 1/6). No breaks or developing pseudocysts were observed on gill clip wet mounts at Week 8, but pseudocysts were observed at Week 9 and Week 10 in 33% (2/6) of channel catfish examined. At Week 11, no pseudocysts were observed in gill clip wet mounts of any fish examined. Finally, at Week 12, 17% (1/6) of channel catfish examined had pseudocysts evident in the gills.

Pseudocysts containing mature myxospores were not observed in the gills of any hybrid catfish, blue catfish or controls.

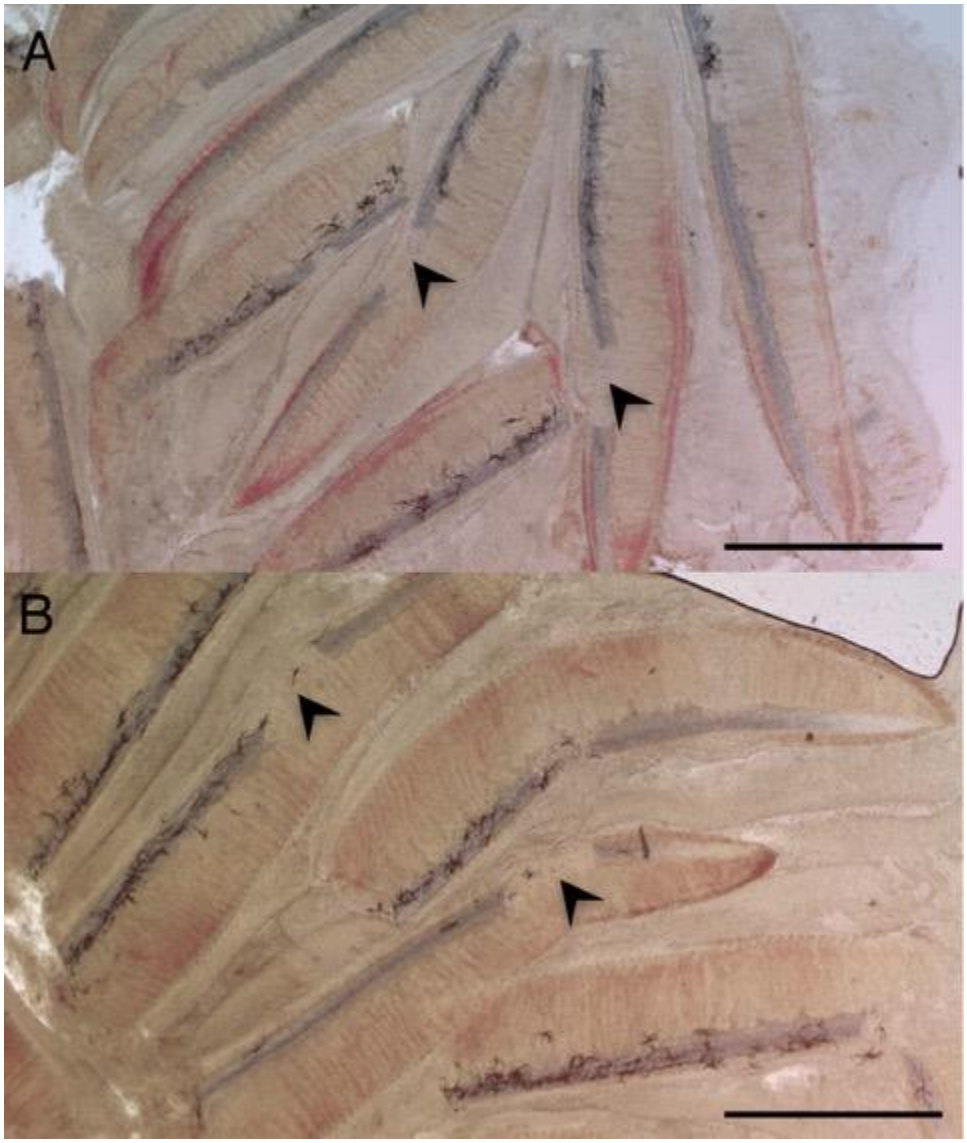

Figure 8.2 Gill clip wet mounts at Week1 post exposure for Trial 1.

Channel catfish (A) and hybrid catfish (B) gill filaments with chondrolytic lesions/breaks (arrow heads) consistent with acute PGD. Scale bar= 500 µm.

Histologically, inflammatory changes consistent with PGD were observed in the gills of channels and hybrid catfish (Figure 8.3A) and developing multinucleated basophilic plasmodia were evident in areas of chondrolysis and surrounding granulomatous inflammation (Figure 8.3B). The timeline of development is consistent with previous experimental studies of *H. ictaluri* infections where developing plasmodia were observed at 7 days post exposure (Pote et al. 2000; Wise et al. 2008; Griffin et al. 2010; Pote et al. 2012). By Week 2 inflammation was still present in gill tissue of channel and hybrid catfish, but there was evidence of recovery as callus formation was observed in areas where chondrolytic breaks occurred (Figure 8.4). Beginning at Week 8 (Figure 8.5A, B) developing *Henneguya* pseudocysts in the gills were observed in channel catfish. Moving forward, pseudocysts containing myxospores were seen continually in channel catfish (Figures 8.5C, D and Figure 8.6) until Week 12 when myxospores were no longer evident. Myxozoan plasmodia were not observed in any blue catfish, hybrid catfish or naïve controls throughout Trial 1. Similarly, no pseudocysts were observed grossly in any blue catfish, hybrid catfish or naïve controls throughout the trial.

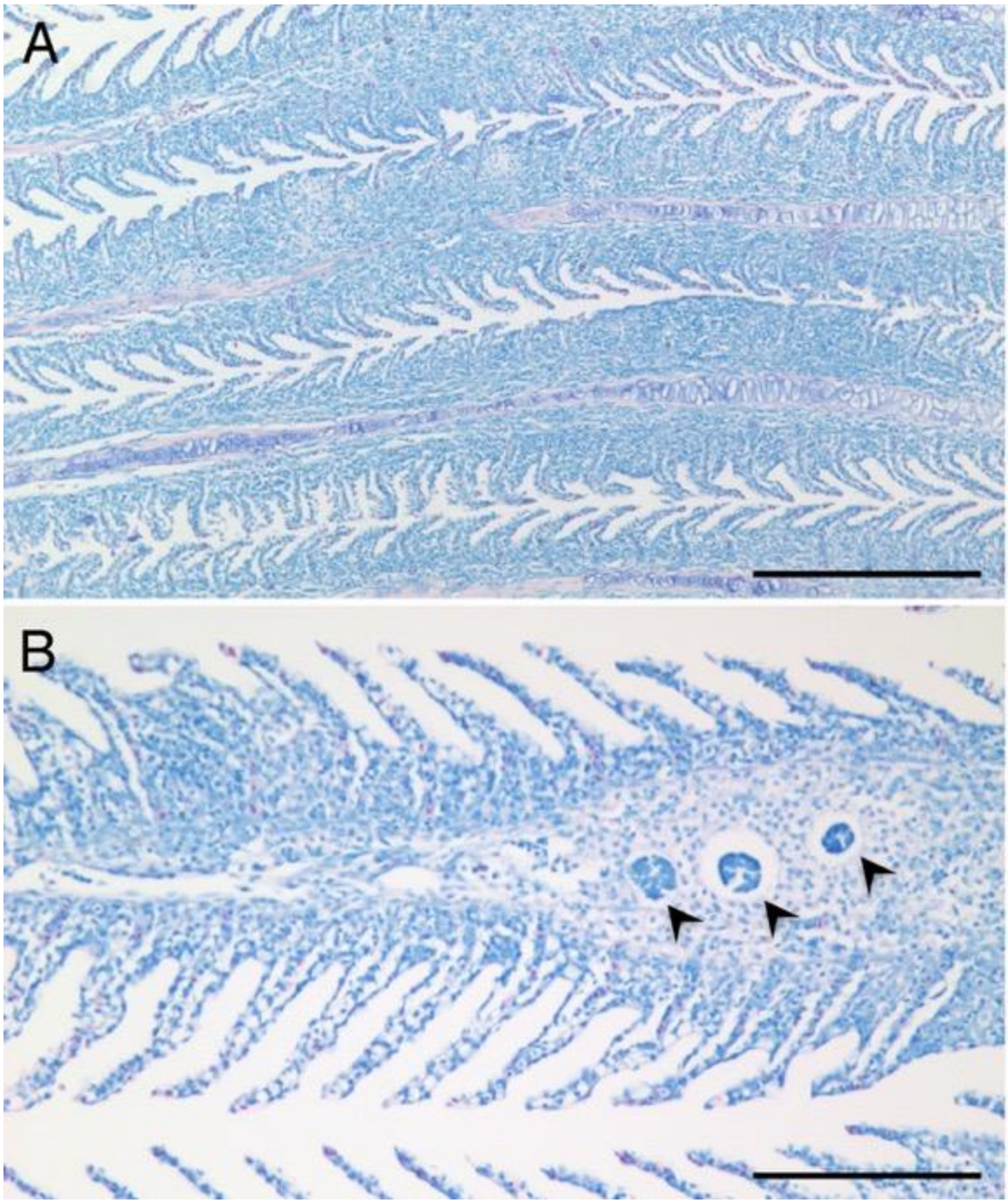

Figure 8.3 Gill histological changes at Week 1 post exposure for Trial 1.

Example of granulomatous inflammatory response at Week1 post exposure demonstrating multifocal areas of epithelial hyperplasia with occlusion of the lamellar troughs and thickening of the respiratory surfaces (A) and developing plasmodia (B; arrow heads) in areas of chondrolysis in the same channel catfish. Giemsa stain; bar in A= 200 µm, bar in B= 100 µm.

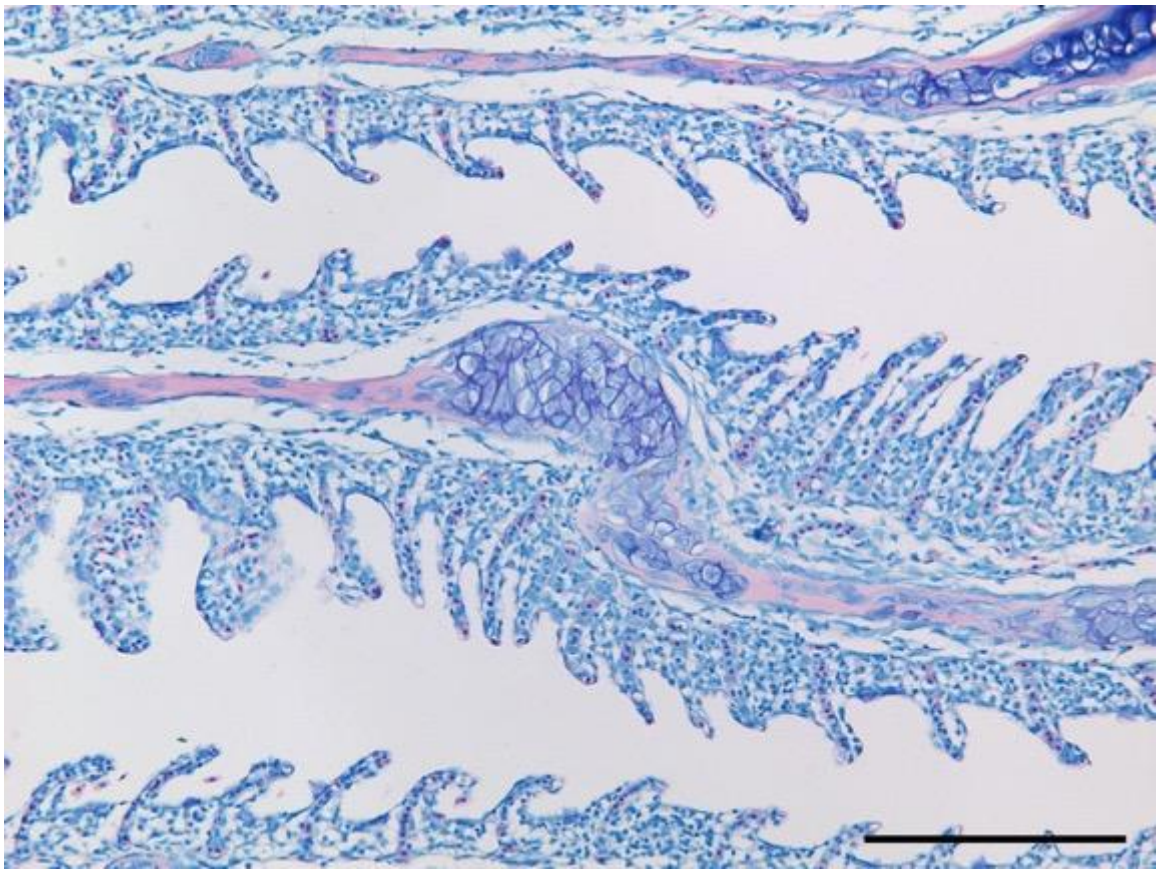

Figure 8.4 Gill histological changes at Week 2 post exposure for Trial 1.

Callus formation where previously a break in the filamental cartilage was located in the gill of a channel catfish. Giemsa stain; bar= 100 µm.

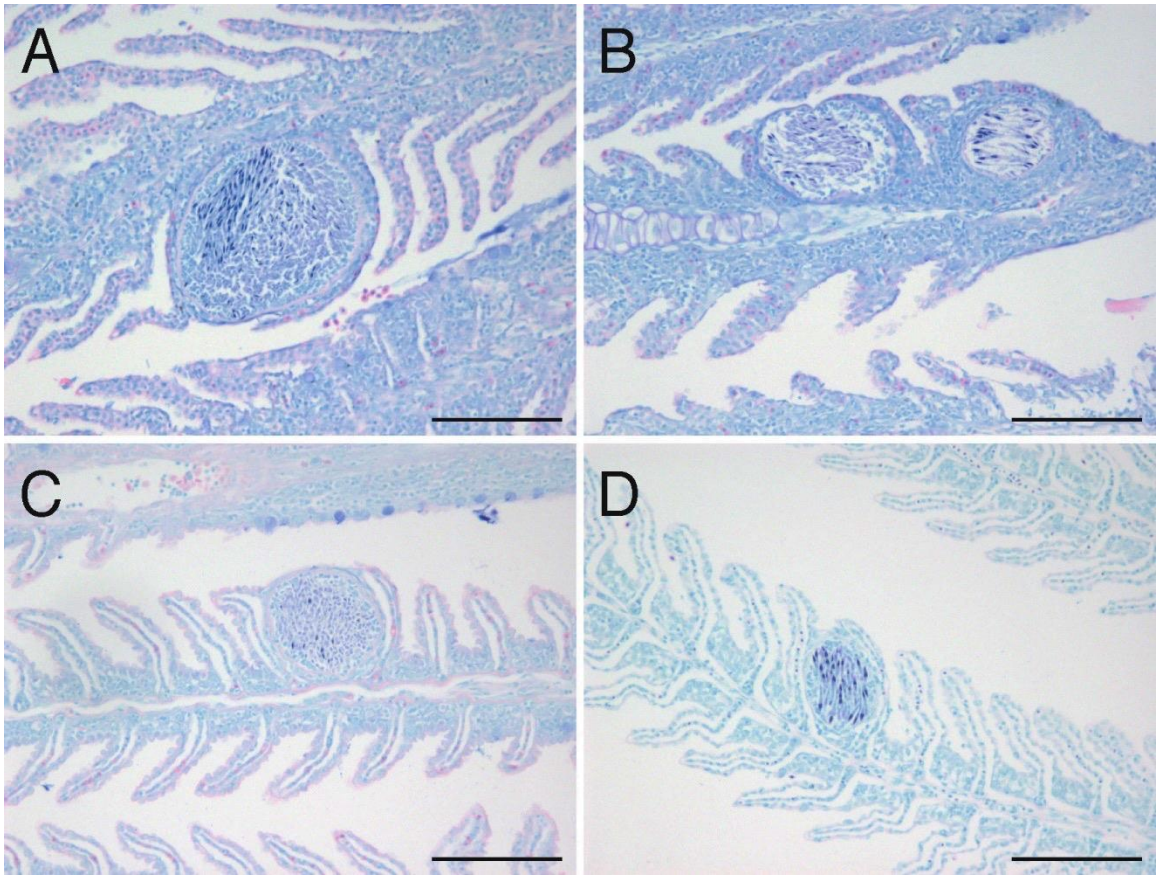

Figure 8.5 Gill histological changes at Week 8 and Week 9 post exposure for Trial 1.

Photomicrographs of channel catfish gill tissue demonstrating *Henneguya* pseudocysts at Week 8 (A & B) and Week 9 (C & D). Giemsa stain; bars=100 µm.

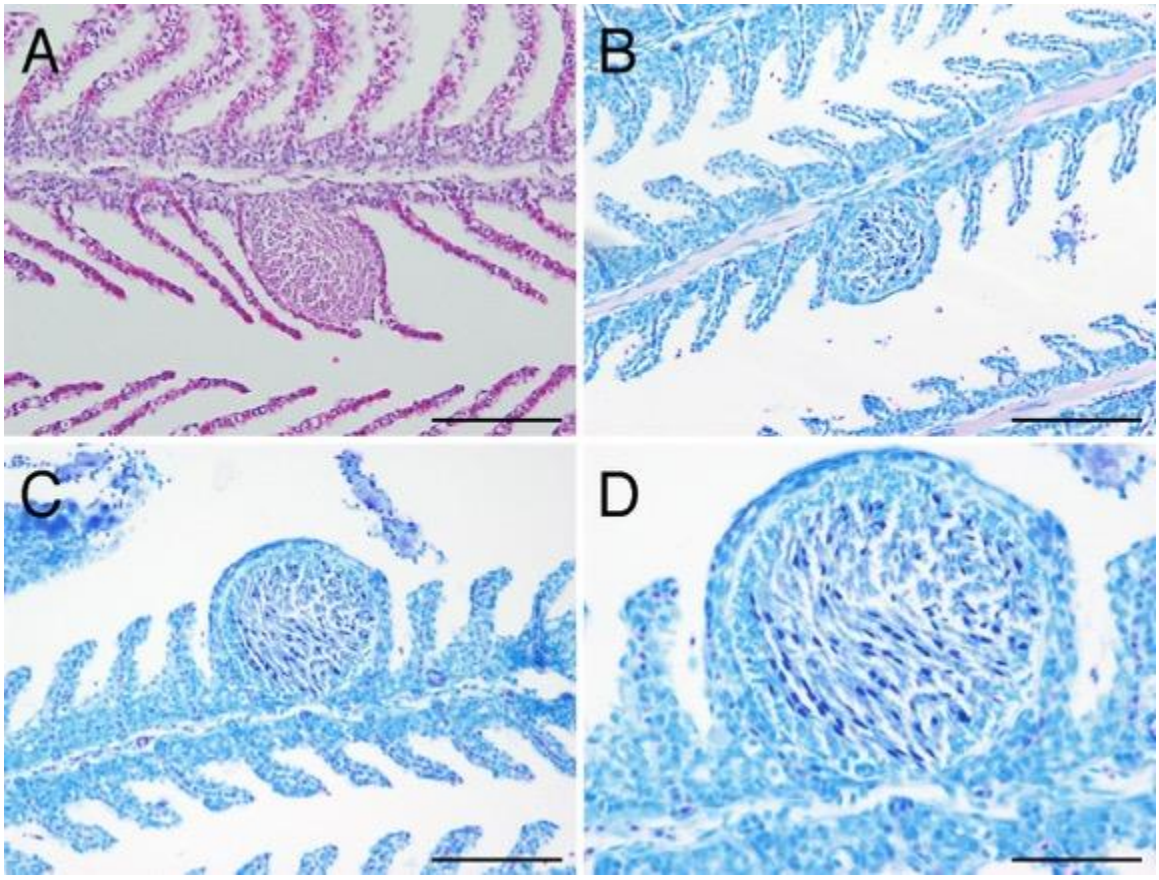

Figure 8.6 Gill histological changes at Week 10 and Week 11 post exposure for Trial 1.

Photomicrographs of channel catfish gill tissue demonstrating *Henneguya* pseudocysts at Week 10 (A & B) and Week 11 (C & D). H & E (A). Giemsa stain (B–D); Bars (A–C)= 100 µm. Bar (D)= 50 µm.

8.4.1.2 Non-gill tissue

Throughout Trial 1 there were no blood tissue extracts with detectable *H. ictaluri* DNA in any fish. Although Griffin et al. (2010) and Beecham et al. (2010) were able to detect *H. ictaluri* in blood and gill tissue of channel, blue, and hybrid catfish, their challenge models consisted of continuous in-pond exposures where fish were not removed from the source of infection until sampling. Therefore, it is likely that

circulating *H. ictaluri* stages were still present in the blood of these fish and detectable by PCR, but further work is needed to clarify this and investigate blood stages of *H. ictaluri*.

For brain, heart and stomach tissue extracts *H. ictaluri* DNA was detected in only channel catfish and hybrid catfish tissues during the acute stages of infection (Week 1–Week 2), but was undetectable in these tissues by Week 4 and was no longer detectable until the end of Trial 1 (Figure 8.7). No histologic lesions or parasite development was observed in the brain, heart, or stomach of any fish throughout Trial 1.

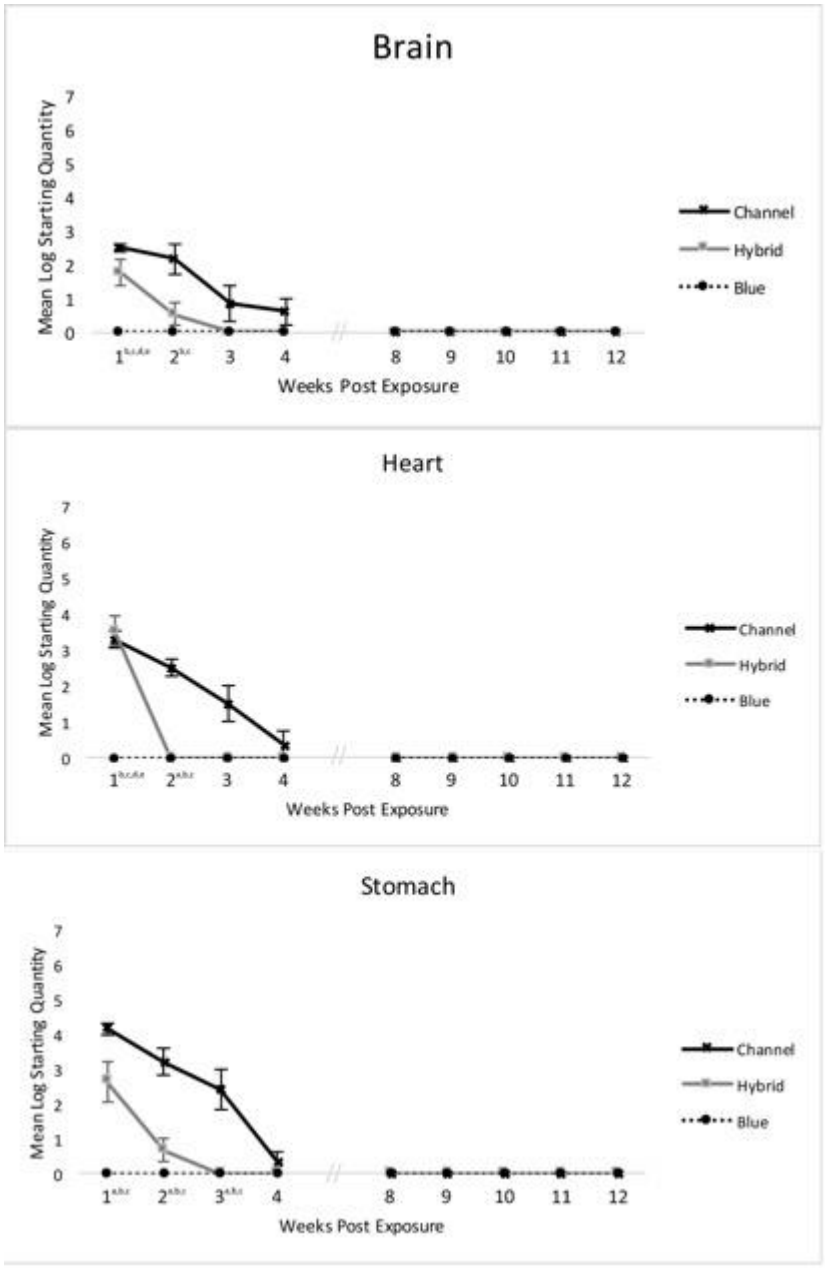

Figure 8.7 Line graph of plotted mean log starting quantities of *H. ictaluri* DNA detected in brain, heart and stomachl tissue for Trial 1.

Values are plotted as the mean (± standard error). No data for Weeks 5–Week 7 post exposure. Superscripts denote statistical significance between groups at each respective time point; a,channel vs hybrid; b,channel vs blue; c,channel vs controls; d,hybrid vs blue; e,hybrid vs controls; f,blues vs controls.

For anterior kidney, posterior kidney, spleen, and liver tissue extracts *H. ictaluri* DNA was detected during Week 1– Week 4 at varying levels, but was at undetectable levels following Week 4. However at Week 11, *H. ictaluri* DNA was detected in the anterior kidney, posterior kidney, spleen, and liver of only channel catfish at Week 11 (Figure 8.8).

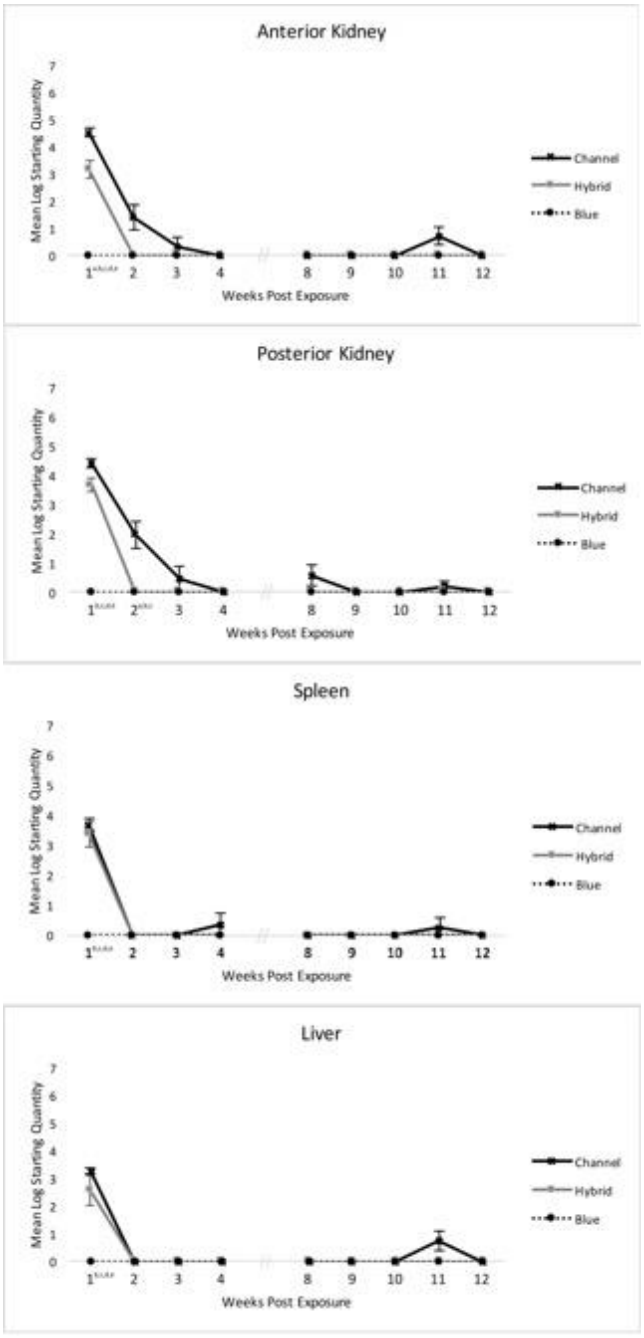

Figure 8.8 Line graph of plotted mean log starting quantities of *H. ictaluri* DNA detected in anterior kidney, posterior kidney, spleen and liver tissue for Trial 1.

Values are plotted as the mean (± standard error). No data for Weeks 5–Week 7 post exposure. Superscripts denote statistical significance between groups at each respective time point; a,channel vs hybrid; b,channel vs blue; c,channel vs controls; d,hybrid vs blue; e,hybrid vs controls; f,blues vs controls.

Histologically, no developing organisms or significant changes were observed in the anterior kidney and spleen for any fish throughout Trial 1. Developing multinucleated plasmodia were observed at Week 2 in sections of posterior kidney from channel catfish exposed to infectious pond water (Figure 8.8), although at present it is unknown if these plasmodia are *H. ictaluri* or another ictalurid infecting myxozoan introduced with the infectious pond water. No other significant changes were observed in the sections of posterior kidney tissue for any group.

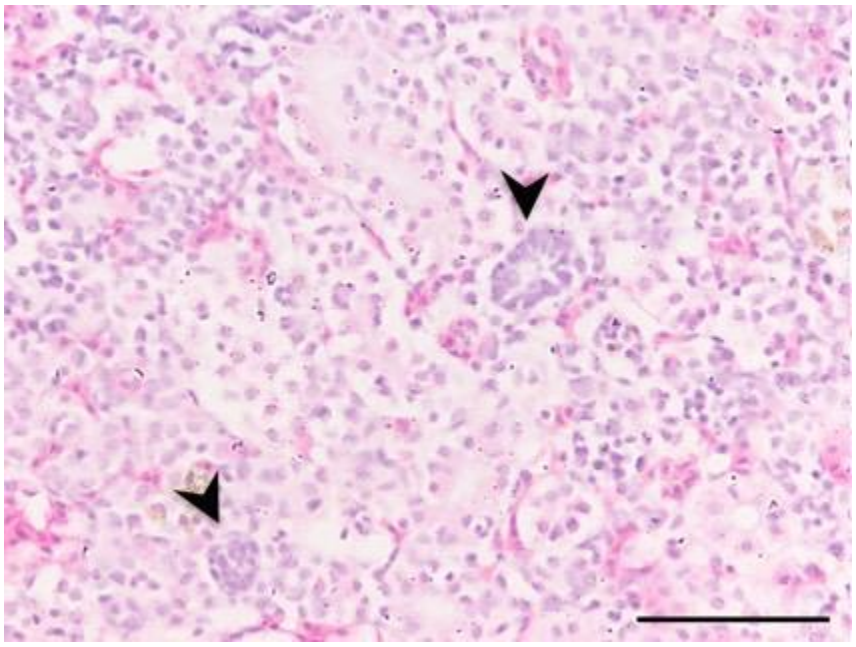

Figure 8.9 Posterior kidney histological changes at Week 2 post exposure for Trial 1. Suspect organisms (arrows) developing in the posterior kidney of a channel catfish at Week 2 post exposure. Bar= 50 µm.

Histologically, at Week 2 several developing plasmodia (Figure 8.14) were observed in sections of the liver of channel catfish. Similar to the stages seen in the posterior kidney, although *H. ictaluri* DNA was detected, verification of these stages

observed as *H. ictaluri* requires further study. No other significant changes were observed in the sections of liver tissue of any group.

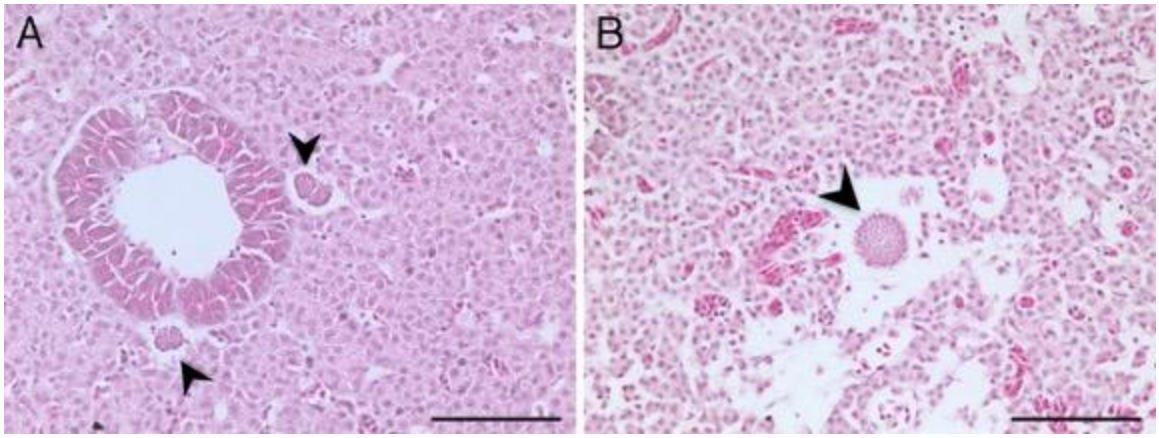

Figure 8.10 Liver histological changes at Week 2 post exposure for Trial 1.

Suspect organisms developing in the liver of a channel catfish at Week 2 post exposure. Bars (A–B)= 50 µm.

8.4.2 Trial 2

At Week 1, channel catfish had higher detectable levels of *H. ictaluri* DNA (2.59±0.85;0.0–4.88) compared to hybrid catfish (0.84±1.08;0.0–2.64), but was not significant (*P*=0.32). Continuing to Week 9 channel catfish had higher levels of *H. ictaluri* DNA when compared to hybrids, which at Week 9 had no detectable *H. ictaluri* DNA (Figure 8.11). However, at Week 11, a single hybrid catfish (17%; 1/6) had detectable *H. ictaluri* DNA, and there were no significant difference between channel and hybrid catfish (P=0.28). At Week 13 levels of *H. ictaluri* DNA detected in hybrid and channel catfish were low, with no hybrid catfish having detectable *H. ictaluri* DNA and only a single channel catfish (17% 1/6) positive by qPCR. This is likely due to the uneven distribution of pseudocysts in gill filaments taken for qPCR in the channel catfish

sampled. By Week 14 *H. ictaluri* DNA was detectable in 83% (5/6) of channel catfish sampled and 17% (1/6) hybrid catfish sampled. Although not significant (P=0.09), channel catfish (3.02±0.77; 0.0–5.15) had higher levels of *H. ictaluri* DNA when compared to hybrid catfish (0.38±0.38; 0.0–2.26) at Week 14.

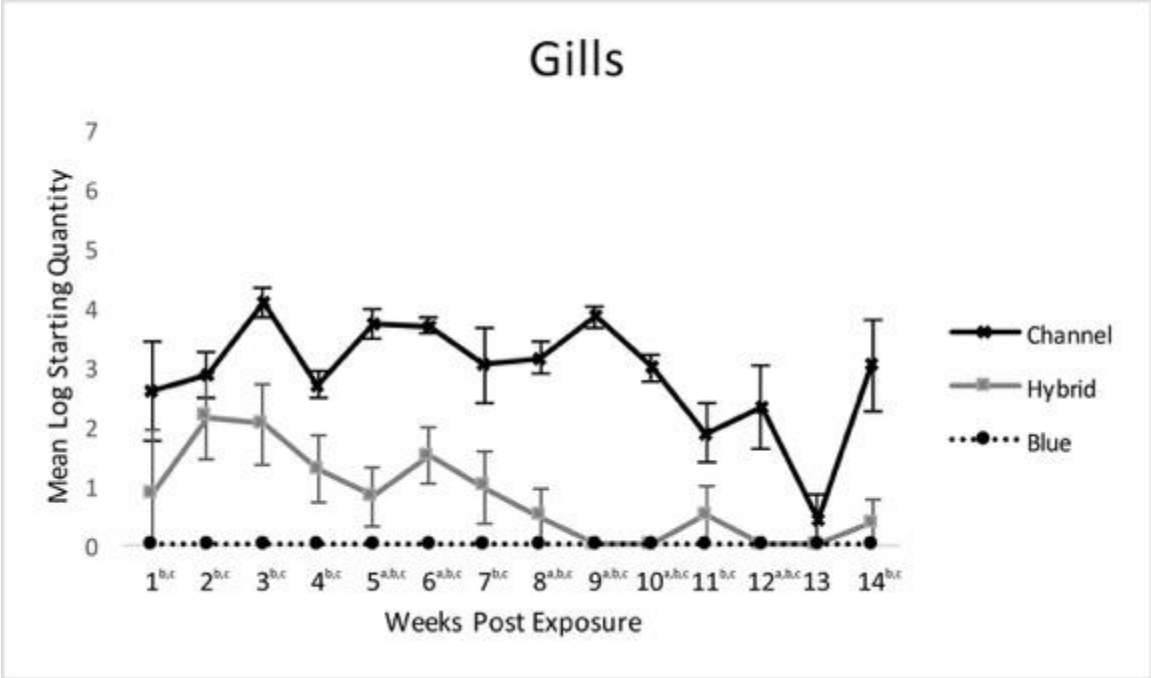

Figure 8.11 Line graph of plotted mean log starting quantities of *H. ictaluri* DNA detected in gill tissue for Trial 2.

Values are plotted as the mean (± standard error). No data for Weeks 5–Week 7 post exposure. Superscripts denote statistical significance between groups at each respective time point; a,channel vs hybrid; b,channel vs blue; c,channel vs controls; d,hybrid vs blue; e,hybrid vs controls; f,blues vs controls.

Gill clip wet mounts were taken each week from the same fish used for qPCR detection of *H. ictaluri*. At Week 1, 50% (3/6) channel catfish had minor breaks in the cartilaginous aspect of the gill filaments compared to 33% (2/6) of hybrid catfish sampled. Similarly, at Week 2, more channel catfish (50%; 3/6) sampled had breaks

indicative of PGD compared to hybrids (17%; 1/6). Cartilage breaks were not observed in gill clip wet mounts of any catfish from Week 3–Week 5. However, beginning at Week 6 evidence of myxospore development was observed in a single channel catfish where one pseudocyst was observed on gill clip wet mounts (Figure 8.12). The pseudocyst was small and of the interlamellar epithelial type as defined by Molnár (2002) and contained numerous developing *Henneguya* myxospores. Similarly, at Week 7 a single channel catfish had pseudocysts present in the gills. By Week 8, 33% of channel catfish examined had pseudocysts developing in the gills, which were larger in size and more numerous than in previous weeks (Figure 8.12). Continually, pseudocysts were observed in 33% of channel catfish gill clip wet mounts from Week 9–Week 11 (Figure 8.13). At Week 12, 50% of channel catfish had developing pseudocysts (Figure 8.19). At Week 13 a single channel catfish had pseudocysts, while at Week 14 33% of channel catfish examined had pseudocysts in the gills (Figure 8.14). No pseudocysts were detected in any gill clip wet mounts of hybrid catfish examined throughout Trial 2. No breaks in the filamental cartilage or pseudocyst were observed in any blue catfish or control fish throughout Trial 2. The proportion of plasmodia detected in channel catfish was significantly greater than in hybrid catfish from the time of appearance of plasmodia at Week 6 until the end of the trial at Week 14 ($p<0.0001$).

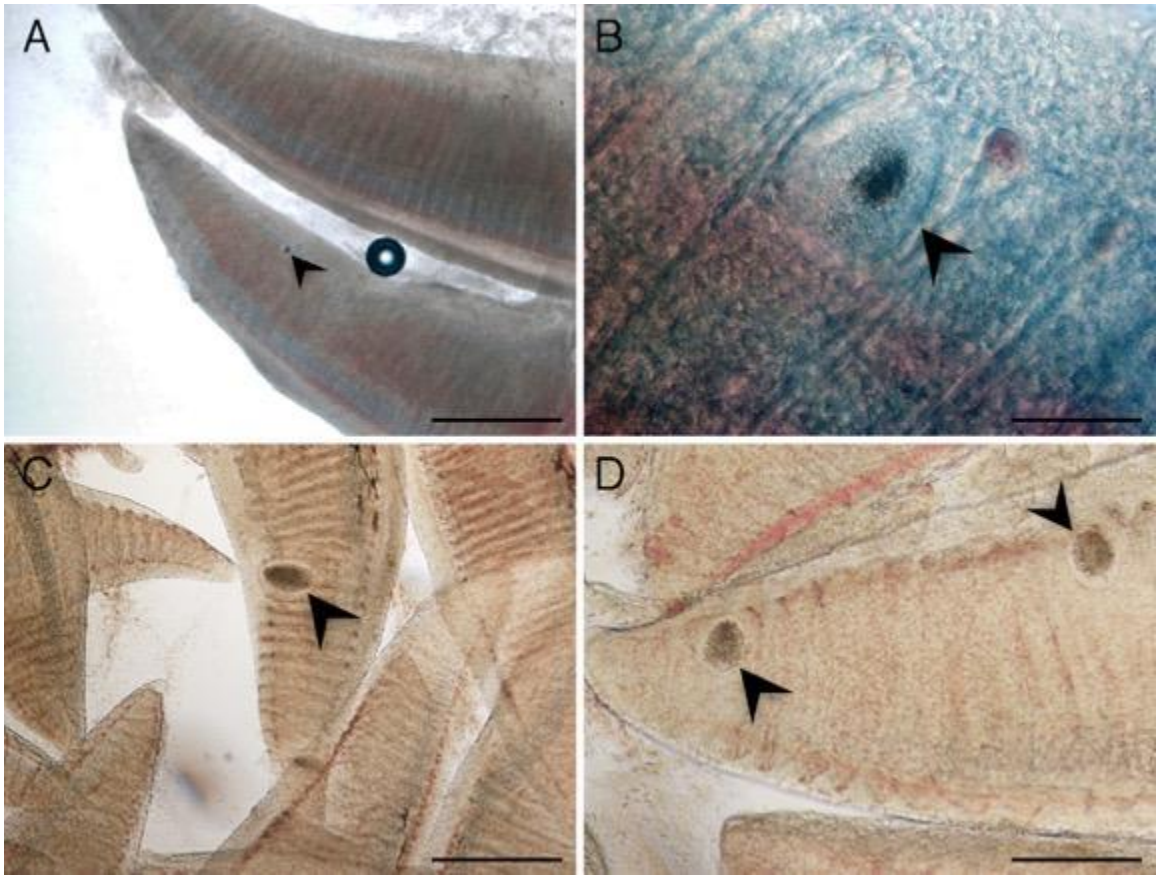

Figure 8.12 Gill clip wet mounts from channel catfish at Week 6 and Week 8 post exposure for Trial 2.

Photomicrographs of channel catfish gill filaments with *Henneguya* pseudocysts (arrowheads) at Week 6 (A–B) and Week 8 (C–D) post exposure. Bar (A & C)= 500µm. Bar (B)= 50 µm. Bar (D)= 200 µm.

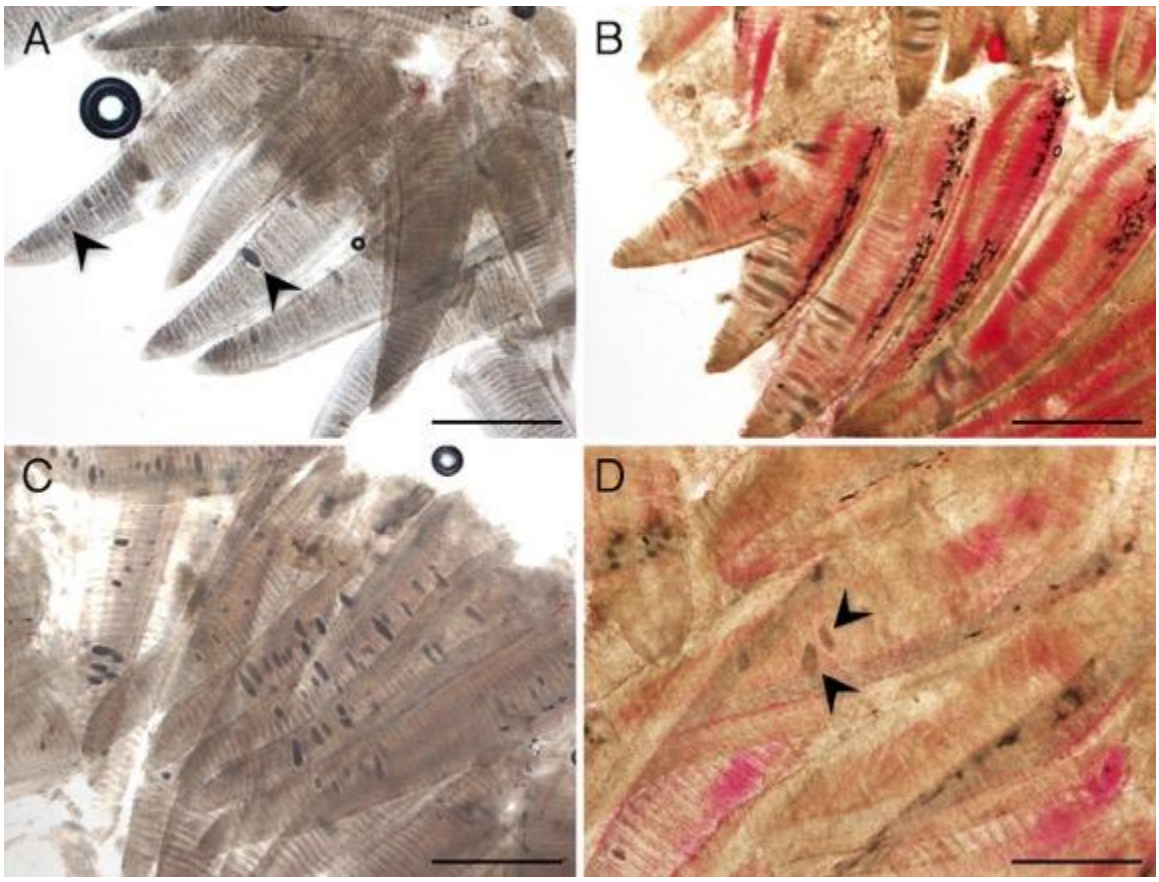

Figure 8.13 Gill clip wet mounts from channel catfish at Week 8–Week 11 post exposure for Trial 2.

Photomicrographs of channel catfish gill filaments with *Henneguya* pseudocysts (arrowheads) at Week 8 (A), Week 9 (B), Week 10 (C), and Week 11 post exposure. Bar (A–C) = 1 mm. Bar (D)= 500 µm. Hemorrhage in B and D.

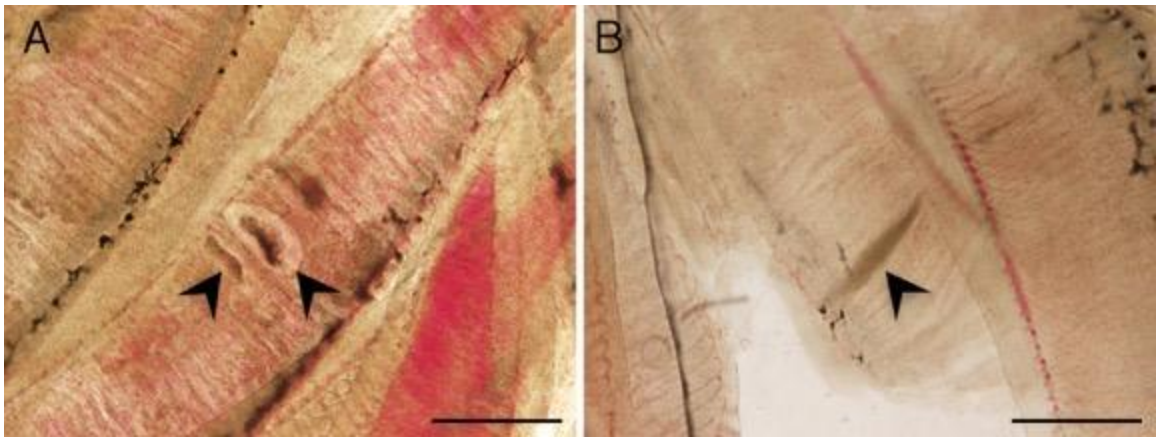

Figure 8.14 Gill clip wet mounts from channel catfish at Week 12 and Week 14 post exposure for Trial 2.

Photomicrographs of channel catfish gill filaments with *Henneguya* pseudocysts (arrowheads) at Week 12 (A) and Week 14 (B) post exposure. Bar (A–B) = 500 µm.

Histologically, evidence of PGD infection at Week 1 was characterized by mild to moderate mononuclear, multifocal, and proliferative branchitis, with only a single (17%; 1/6) channel catfish having developing plasmodia. Similarly, two hybrid catfish (33%; 2/6) had lesions resembling PGD, but no developing plasmodia were evident. Although minor nonspecific inflammatory changes were noted in the gills of some fish, no developing plasmodia or pseudocysts were observed at Week 2. At Week 3 evidence of recovery (callus formation) was seen in two channel catfish, but no plasmodia or developing pseudocysts were evident. Changes were unremarkable from Week 4–5, but starting at Week 6 *Henneguya* pseudocysts were seen in a single channel catfish (Figure 8.15), consistent with wet-mount observations. Additionally, callus formation was noted in two other channel catfish at Week 6. At Week 7, no pseudocysts were observed in any fish examined. By Week 8, 25% (3/12) of the channel catfish examined histologically possessed developing pseudocysts in the gills (Figure 8.21), which were larger and more

numerous than previous weeks, in line with wet-mount observations. Pseudocysts were interlamellar epithelial type, consistent with those observed in earlier weeks. By Week 9, *Henneguya* pseudocysts were evident in 50% (6/12) of channel catfish examined (Figure 8.16), a trend which continued at Week 10 and Week 11, where pseudocysts were seen in 42% (5/12) and 58% (7/12) of channel catfish examined, respectively (Figure 8.17). While fewer channel catfish (25%; 3/12) had developing pseudocysts at Week 12 (Figure 8.23), at Week 13, 50% of channel catfish examined had pseudocysts present in the gill tissue (Figure 8.18) suggesting the decline in pseudocyst prevalence at Week 12 may simply be a function of sectioning. At Week 14, at least one pseudocyst was observed in 92% (11/12) of channel catfish examined histologically. Week 14, Trial 2 was the first time any pseudocysts were observed in hybrid catfish (17%; 2/12) (Figure 8.19). Week 14 was also characterized by the highest number of channel catfish with pseudocysts as well as the most pseudocysts per fish. While pseudocysts were observed in two hybrid catfish at Week 14, these pseudocysts were considerably smaller and fewer in number compared to channel catfish. No plasmodia or pseudocysts were observed in any of the blue catfish or control catfish.

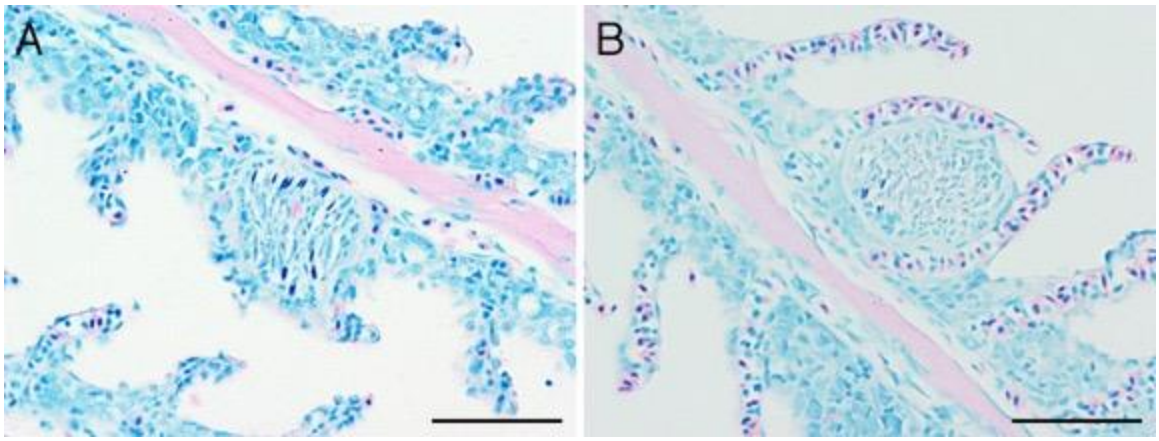

Figure 8.15 Gill histopathological changes at Week 6 post exposure for Trial 2

Photomicrographs of channel catfish gill tissue demonstrating *Henneguya* pseudocysts at Week 6 (A & B) post exposure. Giemsa stain. Bars (A–B) = 50 µm.

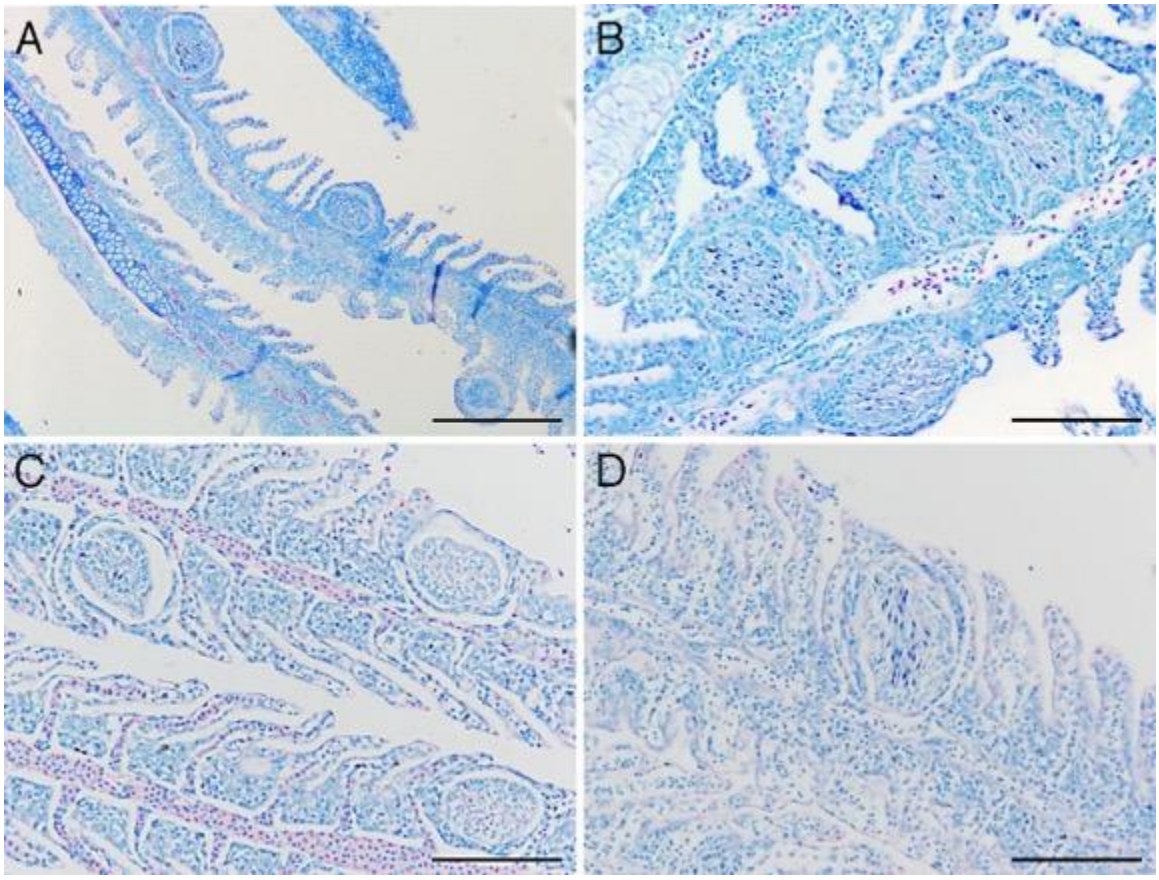

Figure 8.16 Gill histopathological changes at Week 8 and Week 9 post exposure for Trial 2.

Photomicrographs of channel catfish gill tissue demonstrating *Henneguya* pseudocysts at Week 8 (A & B) and Week 9 (C & D) post exposure. Giemsa stain. Bars (A)= 200 µm. Bars (B–D) = 100 µm.

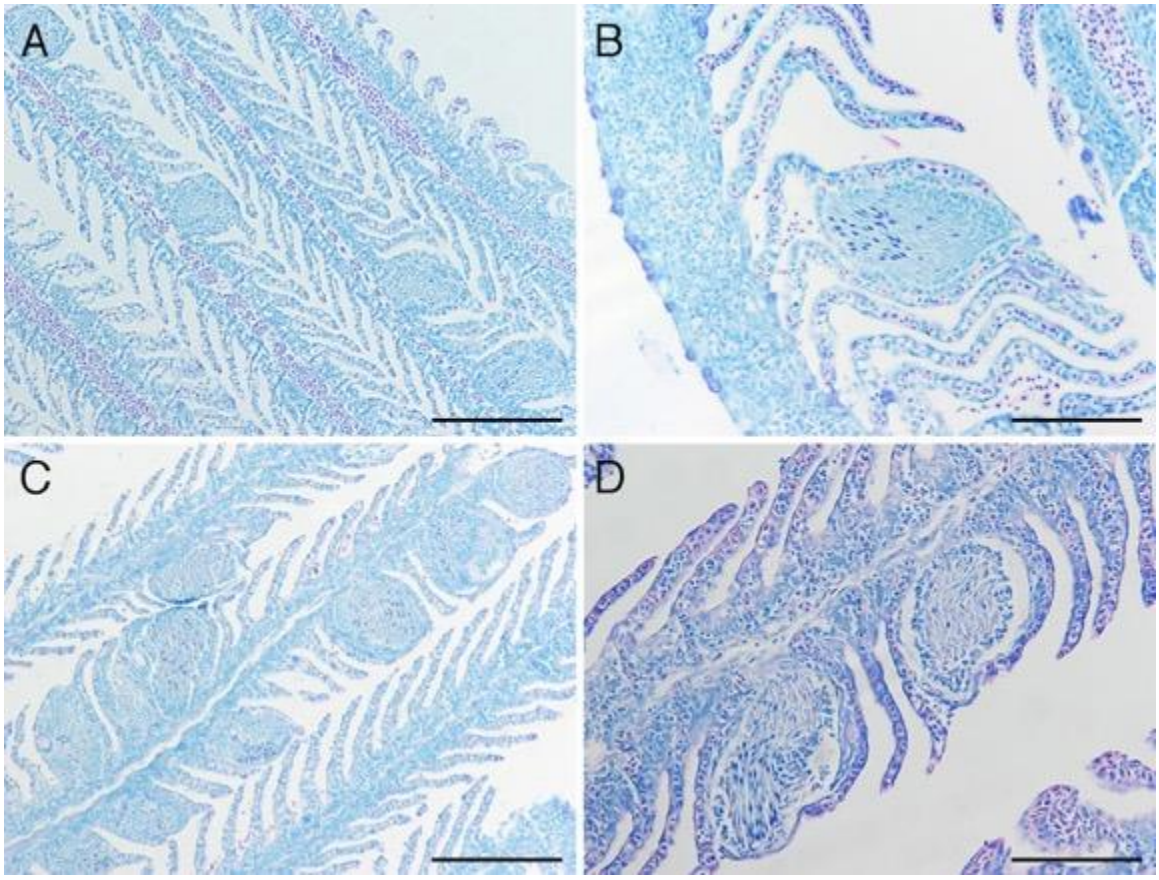

Figure 8.17 Gill histopathological changes at Week 10 and Week 11 post exposure for Trial 2.

Photomicrographs of channel catfish gill tissue demonstrating *Henneguya* pseudocysts at Week 12 (A & B) and Week 13 (C & D) post exposure. Giemsa stain. Bars (A & C) = 200 µm. Bars (B & D) = 100 µm.

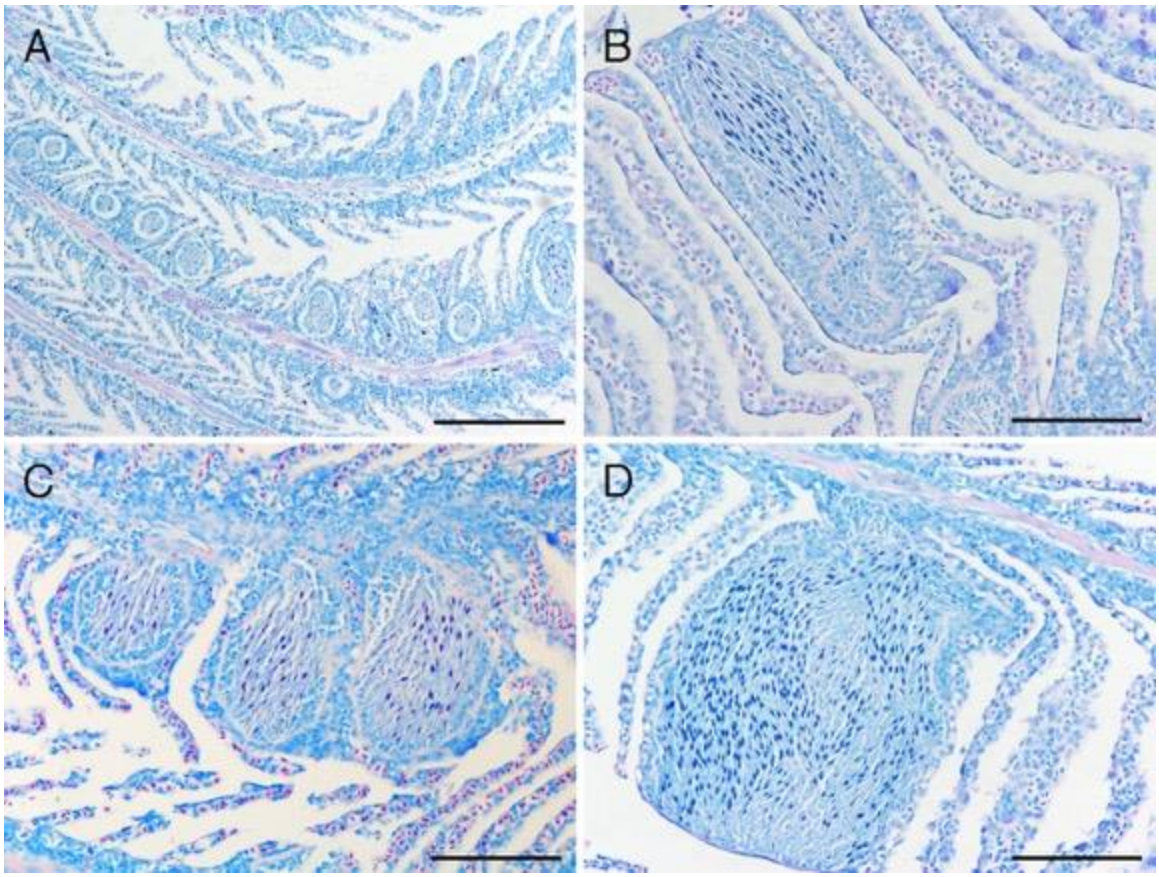

Figure 8.18 Gill histological changes at Week 12 and Week 13 post exposure for Trial 2.

Photomicrographs of channel catfish gill tissue demonstrating *Henneguya* pseudocysts at Week 12 (A & B) and Week 13 (C & D) post exposure. Giemsa stain. Bar (A) = 500 µm. Bars (B–D) = 100 µm.

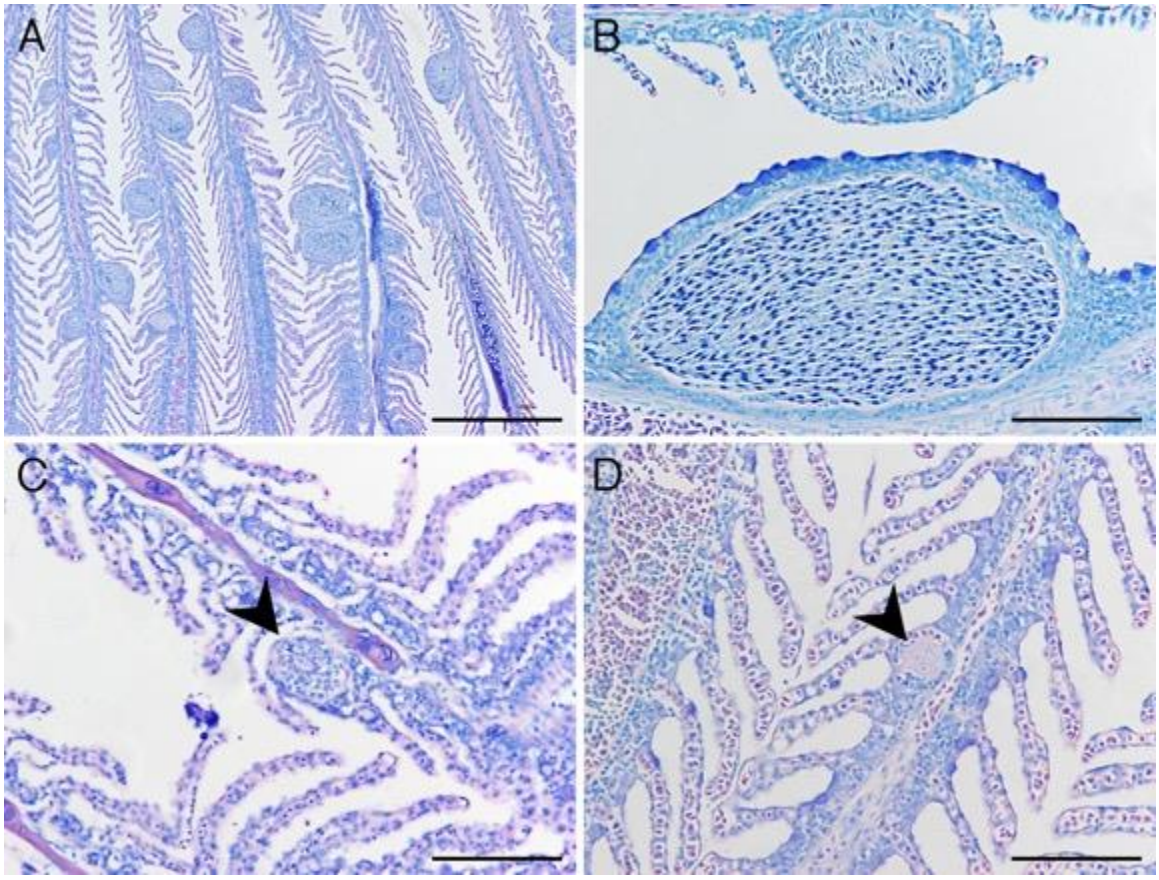

Figure 8.19 Gill histological changes at Week 14 post exposure for Trial 2.

Photomicrographs of channel catfish gill tissue demonstrating *Henneguya* pseudocysts (arrows) (A & B) and hybrid catfish (C & D) at Week 14 post exposure. Giemsa stain. Bar (A) = 500 µm. Bars (B–D) = 100 µm.

8.5 Discussion

The development of *Henneguya ictaluri* in channel catfish, blue catfish, and their hybrid cross was investigated in two separate infectivity trials, wherein parasite development was carried to completion in the fish host. In Trial 1, early developmental stages were observed in both channel and hybrid catfish exposed to infectious pond water containing *H. ictaluri* actinospores. Acute stages of infection were similar among channel catfish and hybrid catfish, with developing plasmodia detectable in the gills of

both channel and hybrid catfish at 7 days post-challenge (Week 1). Moreover, callus formation was observed in both fish groups in later weeks. In Trial 1, channel catfish consistently carried significantly higher amounts of detectable *H. ictaluri* DNA in most tissues examined during the first two weeks. In line with previous studies, gill tissue was the predominant predilection site for *H. ictaluri* DNA and myxozoan life stages as detected by qPCR and microscopic observation.

Results from Trial 1 and 2 are congruous with previous studies focusing on the acute stages of PGD in channel, blue, and hybrid catfish where blue catfish incurred fewer PGD related lesions and *H. ictaluri* DNA was less prevalent in blue and hybrid catfish gill tissues compared to channel catfish (Bosworth et al. 2003; Beecham et al. 2010; Griffin et al. 2010). Throughout Trial 1 and Trial 2, blue catfish had no lesions associated with PGD or detectable levels of *H. ictaluri* DNA by qPCR. Previous studies where *H. ictaluri* has been detected microscopically or by qPCR in blue catfish have involved catfish housed in net cages in ponds with active PGD outbreaks in the resident fish population resulting in significantly higher exposure doses than were achieved in this study (Bosworth et al. 2003; Beecham et al. 2010; Griffin et al. 2010).

Trials 1 and 2 had conflicting results in regards to the development of pseudocysts in hybrid catfish. In Trial 1 no *H. ictaluri* DNA was detectable in hybrid catfish past Week 8 and no evidence of pseudocyst development was observed in any hybrid catfish by gill clip wet mounts or histology. However, in Trial 2 at Week 14 post exposure, 2/12 hybrid catfish examined had a single developing pseudocyst observed histologically and 1/6 hybrid catfish was positive by qPCR. Although *H. ictaluri* DNA was detected, verification of these gross stages as *H. ictaluri* requires further study since it is likely

infectious pond water also contained myxozoan actinospores other than *H. ictaluri*. Similarly, the identity of the pseudocysts observed histologically is inconclusive as other myxozoan actinospore stages could likely have been introduced during the exposure. However, molecular evidence, based on qPCR amplification of *H. ictaluri* DNA, points to the possible presence of *H. ictaluri* pseudocysts in at least one hybrid catfish at Week 14 in Trial 2. Development of in situ hybridization protocols specific to *H. ictaluri* and other known myxozoans from catfish aquaculture would likely clarify this point.

During early stages of infection, *H. ictaluri* appears to be systemic throughout numerous organs with detectable levels of *H. ictaluri* DNA by qPCR in the brain, heart, anterior and posterior kidneys, spleen, liver, and stomach. However, only the posterior kidney and liver had visible developing myxozoan-like stages by histology. The confirmation of these as *H. ictaluri* remains in question. A previous study provides evidence of *H. ictaluri* stages in multiple organ systems of channel catfish as detected by indirect fluorescent antibody tests using polyclonal antibodies against the actinospore stage of *H. ictaluri* (Belem and Pote 2001). In addition to the gills, Belem and Pote (2001) observed fluorescent inclusions in the stomach, heart, liver, spleen, anterior kidney, posterior kidney, and intestine of channel catfish at 24 hours post exposure to pure *H. ictaluri* actinospores collected from *Dero digitata*. By 72 hours post-exposure, a marked decrease in fluorescence was observed in all tissues except the gills, which at this point possessed distinct nuclei visible in developing multinucleated plasmodia (Belem and Pote 2001). Their results coincide with the qPCR data herein, suggesting the systemic nature of the acute stages of infection, especially during the first week of development. For most tissues, besides gill tissue, no *H. ictaluri* DNA was detected past

Week 4, however, anterior kidney, posterior kidney, spleen, and liver were positive for *H. ictaluri* DNA in channel catfish tissue extracts at Week 11. The significance of this is unclear, but could suggest development of *H. ictaluri*, but no putative myxozoan stages were seen in these tissues at Week 11.

In regards to suspected myxozoan development in posterior kidney and liver tissues at Week 2, other *Henneguya* spp. have been reported from these tissues and could likely have been introduced during exposure to infectious pond water and explain the developing stages observed in these tissue during Trial 1. *Henneguya diversis* was reported from the kidney, liver, skin, and fins of channel catfish collected from a commercial catfish farm in Alabama (Minchew 1977). Additionally, *Henneguya exilis* has been reported developing in the posterior kidney of farm raised channel catfish in Mississippi (Matt Griffin, personal communication). However, no pseudocysts or myxospores were observed in any organ other than the gills in Trial 1.

Pseudocysts of *H. ictaluri* have previously been studied in experimentally infected channel catfish (Pote et al. 2000). Interlamellar pseudocysts were observed in the gills of a single channel catfish at 3 months post-exposure (Pote et al. 2000). Gill pseudocysts observed in Trial 1 and Trial 2 of this study were similar in size, shape and location as described by Pote et al. (2000). In Trial 2, interlamellar pseudocysts were observed in the gills of channel catfish as early as Week 6 (42 days) post exposure and were present in 36% (58/162) of channels from Week 6–14. Comparatively, *Henneguya* spp. pseudocysts were observed in only two hybrid catfish at Week 14 (98 days post-exposure).

The propagation of a refractory fish species to minimize losses to myxozoan infections in aquaculture and fisheries management is not a novel concept. In salmonid fisheries in the Pacific Northwest, management of *Ceratonova* (*Ceratomyxa*) *shasta* through breeding of resistant salmonid species is a widely accepted practice (Bartholomew 1998). In a controlled study of host susceptibility to *C. shasta*, Zinn et al. (1977) found varying degrees of susceptibility among the nine salmonid species investigated, with brook trout *Salvelinus fontinalis*, cutthroat trout *Oncorhynchus clarkii*, rainbow trout *Oncorhynchus mykiss*, and fall Chinook salmon *Oncorhynchus tshawytscha* being most susceptible. Coho salmon *Oncorhynchus kistuch* were considered moderately resistant and exhibited lower percent mortality. Brown trout *Salmo trutta*, Atlantic salmon *Salmo salar*, sockeye salmon *Oncorhynchus nerka* and spring Chinook salmon had the lowest measured mortalities (Zinn et al. 1977). Bjork and Bartholomew compared the dose effects of *C. shasta* to susceptible rainbow trout and more resistant Chinook and coho salmon (2009). In the highly susceptible rainbow trout, a single actinospore was capable of causing death of the host (Bjork and Bartholomew 2009). In addition to varying degrees of inherent resistance based on the fish host, there is evidence for multiple genotypes of *C. shasta* resulting in varied degrees of mortality (Hurst and Bartholomew 2012). Investigation into the diversity of *H. ictaluri* genotypes existing in catfish aquaculture in the Southeastern United States has not been examined.

Similar levels of host specificity have been demonstrated for *M. cerebralis*, the causative agent of whirling disease in salmonids. El-Matbouli et al. (1999) exposed a panel of non-salmonid fish, such as goldfish *Carassius auratus*, carp *Cyprinus carpio,* common nase *Chondrostoma nasus*, medaka *Oryzias latipes*, guppy *Poecilia reticulate*,

the amphibian tadpole *Rana pipiens* and rainbow trout to *M. cerebralis* triactinomyxons (TAMs). Their findings indicated a specificity for salmonids. Moreover, Hedrick et al. (1999a) demonstrated discrete differences between susceptibility of rainbow trout and brown trout *Salmo salar* to *M. cerebralis* infection. The prevalence of infection, myxospore numbers, and severity of *M. cerebralis* associated lesions were dramatically reduced in brown trout compared to rainbow trout. More importantly, exposing rainbow trout to as few as 10 *M. cerebralis* TAMs initiated infection, while only exposure doses exceeding 100 TAMs were successful in establishing infection in brown trout. Similar work identified Chinook salmon, westslope cutthroat trout *O. clarki lewisi*, Yellowstone cutthroat trout *O. clarki bouvieri*, and bull trout *Salvelinus confluentus* all demonstrate varying degrees of susceptibility to *M. cerebralis* infections (Hedrick et al. 1999b; 2001), and even resistant salmonid strains have been identified (Schisler et al. 2006).

In addition to the variations in vulnerability to myxozoan infections among closely related fish hosts, there is evidence of variations in susceptibility among different genetic lineages of the oligochaetes. In one study, *Tubifex tubifex* populations from 2 different sites consisted of 4 genetically distinct lineages that varied with respect to their susceptibility to experimental exposure with *Myxobolus cerebralis*. Two genetic lineages were highly susceptible, while others were largely refractory (Beauchamp et al. 2002). Similarly, Arsan et al. (2007) found variation in susceptibility to *Myxobolus cerebralis* infection in 4 different genetic lineages of *Tubifex tubifex* worms from Alaska. These studies suggest certain habitats are conducive to large and more homogenous populations of various oligochaete lineages, which could have dramatic impacts on myxozoan infections in native fish species. There has been little investigation into the possibility of

multiple genetic lineages of the oligochaete *D. digitata* or if varying susceptibility to infection exists, but similar studies are likely warranted.

From an aquaculture management perspective, the importance of the work described herein cannot be overstated. PGD related losses are a seasonal occurrence primarily during the spring when water temperatures are ideal for the propagation of the oligochaete host *D digitata*. In some cases, outbreaks can result in tramatic mortality events, sometimes reaching 100% (Bowser and Conroy 1985; Wise et al. 2004). Moreover, research has demonstrated that *H. ictaluri* is present at some level in nearly all catfish ponds from March through May (Wise et al. 2004). While the direct economic impact is difficult to determine, it is estimated to be in the millions of dollars on account of lost feed days and mortality. In addition to these losses, the impact PGD has on increasing susceptibility to secondary infection with other important pathogens, mainly *E. ictaluri* which often overlaps with the PGD season, has not been carefully examined and warrants further study. In the absence of economically viable control or chemotherapeutic methods, PGD is still an annual problem for producers.

Based on two controlled infection studies, hybrid catfish appear to be less suitable hosts for *H. ictaluri*. No pseudocysts were detected by twelve weeks post-exposure in Trial 1, while only two pseudocysts, each in separate fish, were detected at fourteen weeks post-exposure in Trial 2. Meanwhile robust numbers of myxozoan pseudocysts were evident in channel catfish cohorts. Moreover, limited *H. ictaluri* DNA was detectable in hybrid catfish when compared to channel catfish during late stages of infection (Weeks 8–14). Based on growing experimental evidence from these and other studies, the production of hybrid catfish has the potential to dramatically reduce the

economic burden of PGD on commercial catfish production in the southeastern United States. It is hypothesized that continuous production of hybrid catfish, or at the least occasional crop rotations, would reduce *H. ictaluri* in production systems to tolerable levels, or at a minimum, to levels below that which cause catastrophic losses or parasite induced inappetence.

This work supports anecdotal reports from the catfish industry supporting this hypothesis suggesting that PGD is limited in ponds devoted to hybrid production, and the number of PGD related diagnostic case submissions to the Aquatic Research and Diagnostic Laboratory in Stoneville, MS, suggests that incidence and severity of PGD on catfish operations raising hybrids is greatly reduced. That said, field studies investigating this aspect of catfish aquaculture are needed to verify these claims.

8.6 References

Arsan, E. L., S. L. Hallett, and J. L. Bartholomew. 2007. *Tubifex tubifex* from Alaska and their susceptibility to *Myxobolus cerebralis*. Journal of Parasitology 93:1332–1342.

Bartholomew, J. L. 1998. Host resistance to infection by the myxosporean parasite *Ceratomyxa shasta*: A review. Journal of Aquatic Animal Health 10:112–120.

Beauchamp, K. A., M. Gay, G. O. Kelley, M. El-Matbouli, R. D. Kathman, R. B. Nehring, and R. P. Hedrick. 2002. Prevalence and susceptibility of infection to *Myxobolus cerebralis* and genetic differences among populations of *Tubifex tubifex*. Diseases of Aquatic Organisms 51:113–121.

Beecham, R. V, M. J. Griffin, S. B. LaBarre, D. Wise, M. Mauel, L. M. W. Pote, and C. D. Minchew. 2010. The effects of proliferative gill disease on the blood physiology of channel catfish, blue catfish, and channel catfish × blue catfish hybrid fingerlings. North American Journal of Aquaculture 72:213–218.

Belem, A. M. G., and L. M. Pote. 2001. Portals of entry and systemic localization of proliferative gill disease organisms in channel catfish *Ictalurus punctatus*. Diseases of Aquatic Organisms 48:37–42.

Bjork, S. J., and J. L. Bartholomew. 2009. Effects of *Ceratomyxa shasta* dose on a susceptible strain of rainbow trout and comparatively resistant Chinook and coho salmon.

Bosworth, B. G., D. J. Wise, J. S. Terhune, and W. R. Wolters. 2003. Family and genetic group effects for resistance to proliferative gill disease in channel catfish, blue catfish and channel catfish × blue catfish backcross hybrids. Aquaculture Research 34:569–573.

Bowser, P. R., and J. D. Conroy. 1985. Histopathology of gill lesions in channel catfish associated with *Henneguya*. Journal of Wildlife Diseases 21:177–179.

Brown, T. W., J. A. Chappell, and C. E. Boyd. 2011. A commercial-scale, in-pond raceway system for ictalurid catfish production. Aquacultural Engineering 44:72–79.

Dunham, R. A., and M. J. Masser. 2012. Production of hybrid catfish. Southern Regional Aquaculture Center, Publication 190, Stoneville, Mississippi, USA.

El-Matbouli, M., R. W. Hoffmann, H. Schoel, T. S. McDowell, and R. P. Hedrick. 1999. Whirling disease: host specificity and interaction between the actinosporean stage of *Myxobolus cerebralis* and rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 35:1-12.

Griffin, M. J., L. M. Pote, A. C. Camus, M. J. Mauel, T. E. Greenway, and D. J. Wise. 2009. Application of a real-time PCR assay for the detection of *Henneguya ictaluri* in commercial channel catfish ponds. Diseases of Aquatic Organisms 86:223–233.

Griffin, M. J., D. J. Wise, A. C. Camus, M. J. Mauel, T. E. Greenway, and L. M. Pote. 2008. A real-time polymerase chain reaction assay for the detection of the myxozoan parasite *Henneguya ictaluri* in channel catfish. Journal of Veterinary Diagnostic Investigation 20:559–566.

Griffin, M. J., A. C. Camus, D. J. Wise, T. E. Greenway, M. J. Mauel, and L. M. Pote. 2010. Variation in susceptibility to *Henneguya ictaluri* infection by two species of catfish and their hybrid cross. Journal of Aquatic Animal Health 22:21–25.

Hanson, T. and D. Sites. 2015. 2014 U. S. Catfish Database. USDA National Agricultural Statistics Service (NASS), Mississippi Agricultural Statistics Service (MASS).

Hawke, J. P., and L. H. Khoo. 2004. Health Management. *In* Biology and culture of the channel catfish, ed. Tucker C. S., Hargreaves J. A., Elsevier B.V., Amsterdam, The Netherlands. pp. 387–443.

Hedrick, R. P., T. S. McDowell, M. Gay, G. D. Marty, M. P. Georgiadis, and E. MacConnell. 1999. Comparative susceptibility of rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta* to *Myxobolus cerebralis*, the cause of salmonid whirling disease. Diseases of Aquatic Organisms 37:173–183.

Hedrick, R. P., T. S. McDowell, K. Mukkatira, M. P. Georgiadis, and E. MacConnell. 1999. Susceptibility of selected inland salmonids to experimentally induced infections with *Myxobolus cerebralis*, the causative agent of whirling disease. Journal of Aquatic Animal Health 11:330–339.

Hedrick, R. P., T. S. McDowell, K. Mukkatira, M. P. Georgiadis, and E. MacConnell. 2001. Susceptibility of three species of anadromous salmonids to experimentally induced infections with *Myxobolus cerebralis*, the causative agent of whirling disease. Journal of Aquatic Animal Health 13:43–50.

Hurst, C. N., and J. L. Bartholomew. 2012. *Ceratomyxa shasta* genotypes cause differential mortality in their salmonid hosts. Journal of Fish Diseases 35:725–732.

Kent, M. L., K. B. Andree, J. L. Bartholomew, M. El-Matbouli, S. S. Desser, R. H. Devlin, S. W. Feist, R. P. Hedrick, R. W. Hoffmann, J. Khattra, S. L. Hallett, R. J. Lester, M. Longshaw, O. Palenzeula, M. E. Siddall, and C. Xiao. 2001. Recent advances in our knowledge of the Myxozoa. Journal of Eukaryotic Microbiology 48:395–413.

Minchew, C. D. 1977. Five new species of *Henneguya* (Protozoa: Myxosoprida) from ictalurid fishes. Journal of Protozoology 24:213–220.

Molnár, K. 2002. Site preference of fish myxosporeans in the gill. Diseases of Aquatic Organisms 48:197–207.

Pote, L. M., L. A. Hanson, and R. Shivaji. 2000. Small subunit ribosomal RNA sequences link the cause of proliferative gill disease in channel catfish to *Henneguya* n. sp. (Myxozoa: Myxosporea). Journal of Aquatic Animal Health 12:230–240.

Pote, L. M. W., L. Khoo, and M. Griffin. 2012. *Henneguya ictaluri*. *In* Fish parasites pathobiology and protection, P. T. K. Woo and K. Buchmann (eds.). CAB International, Oxfordshire, U. K., p. 177–192.

Schisler, G. J., K. A. Myklebust, and R. P. Hedrick. 2006. Inheritance of *Myxobolus cerebralis* resistance among F1-generation crosses of whirling disease resistant and susceptible rainbow trout strains. Journal of Aquatic Animal Health 18:109–11.

Taylor, S., M. Wakem, G. Dijkman, M. Alsarraj, and M. Nguyen. 2010. A practical approach to RT-qPCR–publishing data that conform to the MIQE guidelines. Method*s* 50:S1-5.

Whitaker, J. W., L. M. Pote, L. Khoo, R. Shivaji, and L. A. Hanson. 2001. The use of polymerase chain reaction assay to diagnose proliferative gill disease in channel catfish (*Ictalurus punctatus*). Journal of Veterinary Diagnostic Investigation 13:394–398.

Wise, D. J., M. J. Griffin, J. S. Terhune, L. M. Pote, and L. H. Khoo. 2008. Induction and evaluation of proliferative gill disease in channel catfish fingerlings. Journal of Aquatic Animal Health 20:236–244.

Wolf, K., and M. E. Markiw. 1984. Biology contravenes taxonomy in the Myxozoa: new discoveries show alternation of invertebrate and vertebrate hosts. Science 225:1449–1452.

Wolters, W. R., D. J. Wise, and P. H. Klesius. 1996. Survival and antibody response of channel catfish, blue catfish, and channel catfish female × blue catfish male hybrids after exposure to *Edwardsiella ictaluri*. Journal of Aquatic Animal Health 8:249–254.

Zinn, J. L., K. A. Johnson, J. E. Sanders, and J. L. Fryer. 1977. Susceptibility of salmonid species and hatchery strains of chinook salmon (*Oncorhynchus tshawytscha*) to infections by *Ceratomyxa shasta*. Journal of the Fisheries Research Board of Canada 34:933–936.

CHAPTER IX

CONCLUSION

Myxozoans are common parasites of fish species worldwide, but are especially problematic for fish intensively cultured, where levels of infection may reach clinical significance. Proliferative gill disease of channel and hybrid catfish caused by *Henneguya ictaluri* is a continuous burden to the catfish aquaculture industry primarily in the southeastern United States. With no effective method of control or treatment, management strategies aimed at reducing the impacts of PGD on production have targeted biological control measures to break the parasite life cycle. In addition to *H. ictaluri,* the presence of other species of myxozoans present within these closed aquaculture systems suggests that the impacts of myxozoan infections on catfish health may not be limited to a single species.

The survey of actinospore stages infecting the ubiquitous oligochaete host *Dero digitata* in catfish ponds demonstrated an unexpected diversity of myxozoan species, with at least four distinct collective groups of actinospore types representing six distinct species. Many of which represent currently undocumented life cycles. Supplementing the morphologic data of myxozoan actinospores with molecular data aids in the completion of parasite life cycles when the myxospore counterpart is discovered and characterized. More intriguingly, given that host family is a strong phylogenetic signal

for the Myxobolidae, on the occasion the fish host of a newly discovered actinospore is unknown, phylogenetic analysis can offer suggestions as to the likely fish host.

There is also tremendous diversity among myxozoan species in the catfish host, with three novel species being described throughout this work. One of which, *Henneguya mississippiensis*, was united with the aurantiactinomyxon actinospore stage of the life cycle and represents the fourth life-cycle of any *Henneguya* spp. worldwide. A second *Henneguya* sp., *Henneguya bulbosus*, was characterized in the gills of channel catfish and the first *Unicauda* sp. was described from the intestinal tract of channel catfish, *Unicauda fimbrethilae*. Moreover, this work demonstrated a surprising lack of tissue specificity for *Henneguya exilis*, traditionally a parasite of the gills, which was documented as the cause of tumor-like growths on the caudal peduncle of channel catfish.

Additionally, several non-ictalurid fish species inhabit catfish production ponds, either in the form of polyculture or simply through incidental introduction. Many of these have a unique myxozoan fauna unto themselves. Two novel *Myxobolus* spp., *Myxobolus ictiobus* and *Myxobolus minutus*, were described from the gills of smallmouth buffalo *Ictiobus bubalus* ineffectively stocked as biological control of the oligochaete *D. digitata*. Molecular characterization and phylogenetic inference of these *Henneguya* spp., *Myxobolus* spp. and *Unicauda* sp. support previous assertions that fish host family is an important phylogenetic signal in the Myxobolidae. The *Henneguya* spp. infecting ictalurid fish in the southeastern United States form a strongly supported clade, while the *Myxobolus* spp. described from smallmouth buffalo populate a growing clade of catostomid infecting *Myxobolus* spp.

Lastly, investigations into the host specificity and development of *H. ictaluri* in susceptible and nonsusceptible catfish hosts were performed. Results from these experimental studies confirm that blue catfish are not suitable hosts for *H. ictaluri*, as *H. ictaluri* was not detected molecularly or histologically in blue catfish in either study. Channel catfish were found to be susceptible and suitable hosts with pseudocysts containing myxospores developing as early as 6 weeks post exposure and persisting to the end of the trials at 12 weeks (Trial 1) and 14 weeks (Trial 2) post exposure. Hybrid catfish demonstrated an intermediate susceptibility with lower quantities of *H. ictaluri* DNA detectable in all tissues examined compared to channel catfish and no pseudocysts observed in hybrid catfish in Trial 1 and two pseudocysts at 14 weeks post exposure in Trial 2. These results hold promise for the use of hybrid catfish as a culture species to reduce the burden of *H. ictaluri* in catfish aquaculture.

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