PATHOLOGY, PATHOGENESIS, AND METAGENOMIC CHARACTERIZATION OF MYXOZOAN INFECTIONS IN CATFISH AQUACULTURE

By

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(Under the Direction of Alvin Camus & Matt Griffin)

ABSTRACT

Catfish aquaculture is a significant economic driver in the southeastern United States. The industry is heavily impacted by infectious disease, including myxozoan parasitism. The most significant myxozoan, *Henneguya ictaluri*, is cited as the cause of proliferative gill disease (PGD) in channel (*Ictalurus punctatus*) and channel × blue (*I. furcatus*) hybrid catfish, resulting in high mortality in severe outbreaks. Although susceptible to infection and presporogonic development, there is reduced incidence of PGD in hybrid systems. Previous research implies incomplete sporogenesis in hybrids, suggesting hybrid catfish may be a dead-end host in the *H. ictaluri* life cycle. This dissertation used a polyphasic approach to investigate myxozoan community dynamics and pathology in channel and hybrid catfish systems. Metagenomic analysis of industry PGD cases (Chapter 2) revealed mixed infections, with *H. ictaluri* always present but not always the most abundant myxozoan. Myxozoan communities in channel and hybrid catfish were incongruous, composed of numerous known and unknown myxozoan taxa, with reduced abundance of *H. ictaluri* in hybrids. *In situ* hybridization (ISH) assays applied to

laboratory-induced and natural infections (Chapter 3) confirmed *H. ictaluri* presporogonic stages were associated with PGD lesions in channel and hybrid catfish. However, mature *H. ictaluri* plasmodia failed to develop in hybrids, even as late as 20-weeks post-challenge, supporting assertions that hybrids are an aberrant, dead-end host to *H. ictaluri*. ISH assays were consistent with metagenomics findings, revealing mixed infections in PGD cases, with non-*H. ictaluri* myxozoans contributing secondary pathology, including rare PGD-like lesions. Histological examination and molecular confirmation of *H. adiposa* infections by laser capture microdissection (LCM) (Chapter 4) illustrate how non-*H. ictaluri* myxozoans contribute subclinical pathology and demonstrate antemortem release into the environment. Lastly, massive interlamellar *H. exilis* infections in hybrid catfish were defined by pathologic description and LCM (Chapter 5), as well as metagenomic analysis and ISH assays (Chapter 6). Collectively, these findings reveal fish host susceptibility dictates myxozoan community dynamics, which could be manipulated to reduce incidence of PGD on catfish farms. Optimized crop rotation strategies alternating channel and hybrid catfish monocultures can potentially prevent or mitigate PGD outbreaks in catfish aquaculture, significantly improving industry productivity and profitability.

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DEDICATION

This dissertation is dedicated to the catfish and the farmers who grow them with the hopes that the information provided herein might help them become a more sustainable, environmentally friendly source of seafood for tomorrow.

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CHAPTER 1

INTRODUCTION

Myxozoan Evolution, Morphology, and Taxonomy

The myxozoans represent a diverse group of obligate, metazoan parasites that infect a wide range of teleost and elasmobranch species as well as a handful of amphibians, reptiles, birds, and small mammals (Smothers et al. 1994, Atkinson et al. 2018). Derived from the cnidarians, their morphology reflects their evolutionary origins, consisting of 1–13 polar capsules, 1–15 shell valves with or without caudal processes, and a sporoplasm with 1–2 nuclei (Whipps et al. 2003, Atkinson et al. 2018). Polar capsules are frequently compared and considered homologous to cnidarian nematocysts (Cannon and Wagner 2003). Despite this evolutionary history, the composition and physiology of these parasites are poorly understood.

Of the few described life cycles of myxozoans, most are indirect involving an actinospore stage produced in an oligochaete, polychaete, or bryozoan definitive host and a myxospore stage produced in a fish intermediate host (Okamura et al. 2015). Their speciation and taxonomic classification are based on a combination of morphologic description of the myxospore life stage, molecular characterization focusing predominately on the 18S small subunit rRNA gene, host species, tissue tropisms, and geographic locales (Atkinson et al. 2015). Myxozoans often exhibit tissue tropism and localize within specific sites (microhabitats) in those tissues while developing (Molnár 2002, Molnár 2007, Molnár and Eszterbauer 2015). Sequence data is lacking for many myxozoans whose descriptions predate the advent of molecular techniques, making it difficult to

distinguish between morphologically similar species infecting the same host or tissues (Eszterbauer 2002, Rosser et al. 2016, Stilwell et al. 2019c). For those with molecular data, myxozoans often form distinct phylogenetic clades separated into those with oligochaete or polychaete hosts, occurrence in freshwater versus marine environments, by fish host family, or by site of infection within the fish (Fiala et al. 2015, Atkinson et al. 2018, Liu et al. 2019). Next generation sequencing (NGS) techniques are now being utilized to further characterize myxozoan genomics and diversity (Yang et al. 2014, Hartikainen et al. 2016, Yahalomi et al. 2017, Yahalomi et al. 2020).

Detection of Myxozoans in Fish Hosts and the Environment

While many myxozoan infections do not cause clinical disease, some, like *Henneguya ictaluri*, manifest disease through characteristic and destructive pathologic changes. Others, like *Myxobolus koi*, induce little tissue damage but act by forming excessive and debilitating parasite burdens (Wise et al. 2008, Griffin et al. 2010, Camus and Griffin 2010). Wet mount tissue preparations viewed by light microscopy may allow for detection of free myxospores, intact plasmodia, or characteristic lesions (Wise et al. 2004, Rosser et al. 2015). Plasmodia may even be grossly visible or disfiguring to the fish host (Griffin et al. 2009a, Camus and Griffin 2010, Rosser et al. 2014b, Rosser et al. 2016c). Histopathology is routinely used to detect myxozoans, especially in subclinical, microscopic infections (Gardiner et al. 1988). Histochemical stains, particularly the Giemsa stain, can highlight myxospores in histologic sections by staining structural components of the spore, often the polar capsules (Gardiner et al. 1988, Smith et al. 2018). While myxospore morphology on wet mounts or histopathology allows for identification to the genus level, molecular techniques are required for species level differentiation, especially

with myxozoans that share similar fish hosts, tissue sites, and morphologic characteristics (Eszterbauer 2002, Rosser et al. 2016c).

A variety of molecular and antibody-based techniques have been developed to detect and characterize myxozoans. Numerous published protocols using the polymerase chain reaction (PCR) test for myxozoan detection are available, and often broadly target the 18S small subunit rRNA gene (Barta et al. 1997, Kent et al. 2000, Hallett and Diamant 2001, Hanson et al. 2001, Griffin et al. 2008a). These assays range from detecting specific myxozoan species to eukaryotic-wide 18S rRNA sequences (Barta et al. 1997, Pote et al. 2000, Hanson et al. 2001, Griffin et al. 2008a). Quantitative PCR (qPCR) assays for rapid detection and quantification of myxozoan infections also exist for a number of commercially important species (Caventer et al. 2004, Hallett and Bartholomew 2006, Griffin et al. 2008b). These assays have been used to screen tissues and environmental DNA (eDNA) from water and sediment samples for myxozoans (Hallet and Bartholomew 2006, Griffin et al. 2009b, Richley et al. 2020). Antibody-based techniques, including indirect fluorescent antibody test and ELISA, exist for some myxozoans but are typically only utilized for research purposes (Belem and Pote 2001, Sitjà-Bobadilla et al. 2004). Species-specific *in situ* hybridization protocols are available for several myxozoans, including *Ceratonova shasta*, *Myxobolus cerebralis*, and *Tetracapsuloides bryosalmonae* (Antonio et al. 1998, Morris et al. 2000, Bjork and Bartholomew 2010). Additionally, *in situ* hybridization has been utilized to identify mixed infections of distantly related myxozoans (Holzer et al. 2010). Although these methods detect myxozoan infections, methods allowing for *in situ* detection provide the context of disease pathology and represent a powerful research tool while *ex vivo* methods generally offer more rapid, economical, and less labor intensive means of detection for diagnostic purposes.

Myxozoans Impacting Finfish Aquaculture, Fisheries, and Aquariums

Several myxozoans are associated with substantial fish mortality and significant economic losses in global aquaculture and fisheries. The myxozoan *Myxobolus cerebralis* causes whirling disease in various salmonid species, resulting in significant losses in wild fish along with aquaculture and hatchery settings (Blaylock and Bullard 2014). Arguably the most well known and best-studied myxozoan, *M. cerebralis* infects the cartilage of developing bones in the skull and vertebrae of fingerling salmonids (Hallett and Bartholomew 2012). Cartilage lysis and granulomatous inflammation incited by the parasite weakens cartilage and bone predisposing them to fracture and skeletal abnormalities (Hallett and Bartholomew 2012). Extension of inflammation into the meningeal space and pathologic fractures subsequently result in compression of the cerebellum and brainstem, hyperexcitability, and the characteristic whirling behavior noted clinically (Hallett and Bartholomew 2012). Inflammation and fracture of vertebrae similarly results in compression of the distal spinal cord, which interrupts neural suppression and control of dermal melanophores leading to hyperpigmentation of the caudal peduncle (Hallett and Bartholomew 2012). These pathologic lesions are irreversible and ultimately result in death of the fish.

Ceratomyxosis and proliferative kidney disease caused by *Ceratonova shasta* and *Tetracapsuloides bryosalmonae*, respectively, each cause significant mortality in salmonid hatcheries and wild fisheries (Kent et al. 1994). *Ceratonova shasta* targets the gastrointestinal tract, developing within and progressively invading through the intestinal wall to the liver and coelomic viscera (Hallett and Bartholomew, 2012). Granulomatous enteritis and tissue necrosis resulting from infection increases permeability of the intestinal wall predisposing secondary bacterial infection and impaired nutrient absorption (Hallett and Bartholomew 2012).

Proliferative kidney disease results in multifocal tissue necrosis, granulomatous inflammation, and hemorrhage within the kidney, ultimately impairing renal excretory and hematopoietic function (Kent et al. 1994).

The global spread of *Enteromyxum leei* has led to significant losses impacting sharpsnout seabream aquaculture in the Mediterranean and olive flounder and Malabar grouper aquaculture in Japan. Further, *E. leei* has been associated with multispecies mortality events in aquarium settings (Montero et al. 2007; China et al. 2013; Sekiya et al. 2016; Hyatt et al. 2018). *Enteromyxum leei* is relatively unique among myxozoans in that it has demonstrated direct, horizontal transmission and exhibits a wide host range among marine and freshwater fish, making it a notable threat and biosecurity priority to global fish production (Diamant 1997; Diamant et al. 2006; Golomazou et al. 2006; Picard-Sanchez et al. 2020).

In contrast to the above examples, some myxozoans have economic impacts without causing fish mortalities. While not associated with clinically significant disease, various *Kudoa* spp. infect the skeletal muscle of commercially important marine fishes such as salmonids, spotted wolffish, and spotted sea trout. Infections cause postmortem myoliquefaction, or "soft flesh syndrome," which lowers the fish's market value (Kent et al. 1994, Kristmundsson and Freeman 2014).

Channel Catfish Aquaculture in the United States

The commercial catfish aquaculture industry, based predominantly in the southeastern United States, represents the largest sector of foodfish aquaculture in the country, generating over $350 million dollars in annual revenue (USDA-NASS, 2020). Catfish aquaculture utilizes broodstock ponds, hatcheries, and intensively stocked, pond-based growout systems of various

types ranging from 8-50 acres (Boyd 2004). The industry cultivates channel catfish, *Ictalurus punctatus*, and hybrid catfish bred by crossing female channel catfish with male blue catfish, *Ictalurus furcatus* (Hargreaves and Tucker 2004). Channel catfish are grown in multiple-batch ponds with producers harvesting by seine netting, size grading, and restocking with more fish as needed to ensure a year-round market supply (Tucker et al. 2004). Hybrid catfish are typically grown in all-in/all-out, single batch culture as they are not conducive to grading (Tucker et al. 2004). Hybrid catfish have improved growth performance compared to channel catfish and resistance to several common channel catfish diseases (Wolters et al. 1996, Arias et al. 2012, Hargreaves and Tucker 2004, Griffin et al. 2010, Rosser et al. 2019). The catfish industry faces several challenges including rising feed and energy costs, competition from cheaper, imported fish, and disease outbreaks. Infectious and non-infectious diseases both result in fish mortality, lost growth performance and production days, and additional financial investment in treatment (Peterman and Posada 2019). Preventing and mitigating disease outbreaks through proper husbandry, biosecurity, and management practices are crucial to maintaining profitability and success in the industry. This requires a sound understanding of a disease's pathogenesis, epidemiologic factors, and causative agent(s).

Proliferative Gill Disease in Catfish Aquaculture

Henneguya ictaluri, the cause of proliferative gill disease (PGD) in channel (Pote et al. 2000) and hybrid catfish (Bosworth et al. 2003), is among the best-studied myxozoan species. PGD is endemic in the catfish aquaculture industry and, historically, is the most common parasitic disease and third most common infectious disease diagnosed in catfish submitted to the Aquatic Research and Diagnostic Laboratory at the Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS (Khoo et al. 2013). Although still among the most

important infectious diseases affecting the industry, PGD case submissions have declined in recent years likely due to the rise of commercial hybrid catfish culture (Figure 1.1) (Rosser et al. 2017). Because of the characteristic gross lesions associated with PGD, farmers often diagnose the disease pondside, which may also result in an underestimation of true disease prevalence (Wise et al. 2004). PGD is colloquially known as "hamburger gill disease" due to a characteristic "ground-meat" appearance of infected gills, which are mottled red and white, swollen, fragile, and bleed easily (Hawke and Khoo 2004). PGD occurs seasonally at temperature ranges of 14-26°C (optimum 16-20°C). Large outbreaks occur in the spring with less frequent, smaller outbreaks in the fall (Hawke and Khoo 2004). Fish with subclinical lesions and disease have also been observed in the winter months (Hawke and Khoo 2004). PGD predominantly affects fingerling to stocker-size catfish, but will occasionally cause mortality events in market-size catfish (Wise et al. 2004). Outbreaks are characterized by high morbidity and mortality due to respiratory compromise (Pote et al. 2000, Wise et al. 2008). Affected fish seek high oxygen concentrations by piping at the water-air interface, gathering near water inflows, and congregating around aerators (Wise et al. 2004). Differential diagnoses for respiratory distress and PGD include low environmental dissolved oxygen, anemia, and brown blood disease (methemoglobinemia) caused by elevated nitrite levels (Wise et al. 2004).

Proliferative gill disease has significantly impacted the catfish aquaculture industry since the 1970s (McCaren et al. 1975). Early research focused on the morphological description of myxospores from cultured catfish, transmission experiments, and characterization of PGD-associated gill pathology, which attributed lesions to various myxozoans, including *Sphaerospora* spp., *Henneguya* spp. or *Henneguya exilis* (McCaren et al. 1975, Minchew 1977, Bowser et al. 1985, Duhamel et al. 1986, MacMillan et al. 1989, Hedrick et al. 1990, Styer et al.

1994). The first experimental challenges demonstrated disease transmission via mud and water collected from ponds during PGD outbreaks, but not directly from fish to fish (MacMillan et al. 1989). Subsequently, the pelagic actinospore life stage, an Aurantiactinomyxon type actinospore, was identified in the oligochaete worm, *Dero digitata*, in mud from ponds experiencing PGD outbreaks (Burtle et al. 1991, Styer et al. 1991, Pote and Waterstat 1993, Bellerud et al. 1995). Experimental challenges by Styer et al. (1991) illustrated that only fish exposed to *D. digitata* and no other *Dero*. spp or macroinvertebrates developed PGD. Early pathologic descriptions predated the advent of molecular techniques and reported mixed stages of developing myxospores, potentially representing multiple species, and applied differing terminology, leading to contrasting (and at times conflicting) characterization of the disease (McCaren et al. 1975, Bowser et al. 1985, Duhamel et al. 1986). Presporogonic stages form within the gill filament and incite a granulomatous inflammatory response, causing lysis of the filament cartilage (Wise et al. 2008, Griffin et al. 2010, Lovy et al. 2011). Cartilage fractures, combined with epithelial hypertrophy and hyperplasia, lamellar fusion, and hemorrhage, result in irregular blunting and clubbing of gill filaments create the "meaty" gross appearance typical of the disease and severely impair respiratory function (Wise et al. 2008, Griffin et al. 2010). In outbreak survivors, damaged cartilage undergoes dysplastic proliferation in an attempt to stabilize the filament and bridge defects (Hawke and Khoo 2004).

Impacts to respiratory function manifest as decreases in oxygen partial pressures (hypoxia) and increases in carbon dioxide partial pressures in the blood of affected catfish despite sufficient dissolved oxygen levels in the water (Hawke and Khoo 2004, Beecham et al. 2010). Once infected and clinical signs manifest, fish must be provided oxygen support or harvested, as there is no curative treatment for the disease (Wise et al. 2004). A presumptive

diagnosis of PGD is made based on the gross appearance of the gills and presence of cartilage lysis observed in gill filaments during wet mount examination. Definitive diagnosis requires histologic detection of presporogonic stages of *H. ictaluri* within lesions and/or molecular confirmation using PCR, qPCR, and indirect fluorescent antibody techniques (Pote et al. 2000, Hanson et al. 2001, Whitaker et al. 2001, Belem and Pote 2001, Wise et al. 2004, Griffin et al. 2008b).

Multiple factors contribute to the severity of disease in catfish production ponds. The definitive host, *D. digitata*, is ubiquitous and the most numerous oligochaete worm found in the sediments of ponds experiencing PGD outbreaks. Densities of the oligochaete are significantly increased during outbreaks compared to non-PGD ponds, frequently contain *Aurantiactinomyxon* sp. actinospores on microscopic examination, and correlate with the severity of disease (Burtle et al. 1991, Styer et al. 1994, Bellerud et al. 1995, Pote et al. 2012). The static environment of the ponds facilitates continuous exposure between fish and oligochaete hosts, promoting transmission of the parasite. A critical actinospore abundance threshold must be exceeded to cause disease, which varies depending on the age and size of the fish host (Wise et al. 2004). The greatest risk for outbreaks occurs when naïve, fingerlings are stocked into ponds where large numbers of actinospores are being shed by the oligochaete host (Wise et al. 2004). In experimental challenges, fish removed after initial exposure to infective actinospores had significantly lower severity of disease compared to those subjected to continuous exposure (Wise et al. 2008). In the field, movement of diseased fish from a PGD affected pond to a non-PGD affected pond or a system supplied by well water can result in resolution of clinical signs and lesions, and is occasionally used to limit losses in commercial ponds (Styer et al. 1994, Wise et al. 2004). The influence of temperature optima on PGD outbreaks is unknown, whether ideal

conditions are created for *D. digitata* proliferation and *H. ictaluri* actinospore development, if the immune systems of catfish remain impaired following winter suppression, or if a combination of factors are involved (Hawke and Khoo 2004, Wise et al. 2004).

Controlling Proliferative Gill Disease

PGD prevention and mitigation are important objectives and motivators of previous and ongoing research efforts as no chemotherapeutic agents or other curative treatment measures exist for the condition. Much work has focused on controlling populations of the oligochaete definitive host, *D. digitata*, through chemical and biological methods. In laboratory and pond settings, several chemical agents have shown promise for controlling *D. digitata* populations, although most were either cost prohibitive or the high doses required to penetrate the pond sediment were toxic to fish (Mischke et al. 2001, Mischke et al. 2014). Attempted biological methods include introducing fathead minnows, *Pimapheles promelas*, to ponds to predate upon *D. digitata*. However, supplying enough minnows to be effective proved difficult, as catfish consumed them in large numbers (Burtle 1998). Similarly, stocking smallmouth buffalo, *Ictiobus bubalus*, into ponds as predators of *D. digitata* and as dead-end hosts for catfish myxozoans had no effect on catfish production, PGD incidence, or parasite density between ponds with or without buffalo (Mischke et al. 2016). Infections of smallmouth buffalo gills by multiple *Myxobolus* spp. potentially introduced additional myxozoan diversity and influenced myxozoan community structures within pond environments (Rosser et al. 2016b). Finally, stocking mosquitofish, *Gambusia* sp., into experimental tanks at 25% and 50% of the catfish biomass decreased parasite burdens in catfish during the initial stages of infection, when compared to catfish in tanks without *Gambusia* sp. (Griffin et al. 2020a). However, in a pond study,

Gambusia affinis did not have a measurable impact on disease prevalence in catfish (Mischke et al. 2013).

Many myxobolid myxozoan species and strains are highly fish host specific (Hedrick et al. 1999, Hedrick et al. 2003, Bosworth et al. 2003, Griffin et al. 2010, Rosser et al. 2019). For example, the blue catfish is largely resistant to PGD with significantly reduced gill damage compared to channel and hybrid catfish in experimental infectivity trials (Bosworth et al. 2003, Griffin et al. 2010, Beecham et al. 2010). Large numbers of actinospores and prolonged exposure periods are required to overcome resistance mechanisms of blue catfish and produce mild PGD lesions (Griffin et al. 2010). Additionally, plasmodia apparently fail to develop and are not histologically detectable in gill tissue (Griffin et al. 2010). Unfortunately, the growth characteristics of blue catfish are not ideal for large-scale aquaculture production and are not a viable alternative for the channel catfish (Pote et al. 2012). Hybrid catfish bred from crossing male blue catfish and female channel catfish show some resistance to certain aspects of the disease, but suffer similar lesions during the acute, clinical stages of disease (Griffin et al. 2010). Repeated experimental infection challenges in combination with qPCR and histopathologic analysis suggest *H. ictaluri* within hybrid catfish gills exhibit decreased rates of infection and may undergo arrested development, precluding sporogony (Griffin et al. 2010, Rosser et al. 2019). While these findings suggest the production of hybrid catfish may decrease PGD infections in the catfish industry, the underlying mechanisms involved in fish host susceptibility to *H. ictaluri* are unknown (Griffin et al. 2010, Rosser et al. 2019). PGD continues to be diagnosed within hybrid catfish ponds in the industry, though *H. ictaluri* abundance and PGD severity was shown to decline in experimental hybrid catfish ponds over time (Griffin et al. 2020b).

Several management strategies exist to prevent and mitigate PGD outbreaks. An established scoring system exists for assessing the risk of PGD outbreaks in ponds using sentinel catfish (Wise et al. 2004, Wise et al. 2008). The catfish are held in mesh cages in ponds of interest for 7 days before being collected and their gills evaluated in wet mount preparations of approximately 40-80 filaments. Cartilage lysis in 1-5%, 6-15%, and >15% of filaments indicates mild, moderate, and severe infections with corresponding low, medium, and high risk of mortality for introduced fish (Wise et al. 2004, Wise et al. 2008).The scoring system provides an indirect estimation of the pond's parasite burden. An alternative, more quantitative and rapid method of screening ponds involves qPCR to amplify and detect *H. ictaluri* DNA from environmental DNA in the pond water prior to stocking fish (Griffin et al. 2009b). The latter method is limited as it detects all *H. ictaluri* DNA in water regardless of actinospore viability and by the abundance of plankton that inhibits filtration of water for actinospores (Griffin et al. 2009b).

Management of PGD outbreaks involves providing supportive care to mitigate the effects of respiratory distress. Paddle-wheel aerators provide supplemental oxygen during the mid-morning hours when oxygen levels are lowest and adding salt to the water provides osmoregulatory support (Wise et al. 2004). Affected fish may recover if transferred to ponds without PGD as this eliminates exposure to actinospores; however, this may also introduce *H. ictaluri* into a non-colonized pond (Wise et al. 2004). While effective, these strategies add further financial and labor costs on top of the lost productivity and mortality from the disease itself. Preventive screening and the use of resistant hybrid catfish offer the most promising methods of disease control, though more work is needed to determine the influence of channel versus hybrid catfish monoculture on overall myxozoan community structure and the incidence

of PGD outbreaks in ponds, as well as optimal cultivation, or crop rotation, strategies to exploit this disease resistance.

Overview of the Genus *Henneguya*

There are approximately 200 described *Henneguya* myxozoan species that infect various freshwater and marine finfish species (Eiras 2002, Eiras and Adriano 2012). The morphologic features of a typical *Henneguya* myxospore include a round, pyriform, or spindyloid spore body encased by two shell valves with long, tapering caudal processes. The spore body contains two elongate polar capsules with tightly coiled polar filaments, a binucleated sporoplasm, and occasionally a posterior vacuole (Eiras and Adriano 2012). *Henneguya* spp. are polyphyletic and most species group with other freshwater, histozoic myxobolids (Lom and Dyková 2006, Liu et al. 2019). The family Myxobolidae contains eight distinct subclades with *Henneguya* spp. often mixed with *Myxobolus* spp., illustrating that myxospore morphology is a poor indicator of phylogenetic relatedness (Liu et al. 2019).

The life cycles of four *Henneguya* spp. have been identified, three of which utilize the oligochaete worm *Dero digitata* as their definitive host and the channel catfish, *Ictalurus punctatus*, as their fish intermediate host (Lin et al. 1999, Pote et al. 2000, Kallert et al. 2005, Rosser et al. 2015). Following release into water from an oligochaete or polychaete definitive host, contact with fish mucus triggers actinospores to release their polar filaments facilitating attachment to the fish (Burtle et al. 1991, Cannon and Wagner 2003, Atkinson et al. 2018). The sporoplasm is released into epithelial surfaces of the skin, gill, and gastrointestinal tract through which it migrates to enter the vascular system and travel hematogenously to its preferred site of development (Belem and Pote 2001, Griffin et al. 2010). During dissemination, the sporoplasm beings replicating, forming presporogonic stages. The presporogonic stages then encyst, forming

plasmodia, and begin dividing and replicating into pansporoblast stages. As cells divide, they differentiate into several types, each with their own specific function (Current and Janovy Jr. 1977, Current 1979). For *H. adiposa* and *H. exilis*, the earliest stage of sporogenesis involves 2 generative cells, a sporont and a structural enveloping cell (Current et al. 1976, Current and Janovy Jr. 1977, 1978, Current 1979). During the pansporoblastic stage, the sporont divides and forms 10 sporont progeny cells that are compartmentalized within the enveloping cell (Current and Janovy Jr. 1977, Current 1979). Sporont progeny cells differentiate into 4 capsulogenic cells, 2 binucleated sporoplasms, and 4 valvogenic cells that surround the other cell types, producing two individual myxospores (Current and Janovy Jr. 1977, Current 1979). The capsulogenic and valvogenic cells develop into the polar capsules and shell valves of the myxospore, respectively (Current and Janovy Jr. 1977, Current 1979).

Similar to other myxobolid myxozoans, *Henneguya* spp. exhibit tissue tropisms for development and are reported from a variety of tissues in different fish hosts (Eiras 2002, Eiras and Adriano 2012). In most *Henneguya* spp. infections, plasmodia containing mature myxospores incite little to no inflammation or other host responses (Rosser et al. 2014b, Rosser et al. 2015, Stilwell et al. 2019c). However, developing stages and mature myxospores free in the tissues may incite a localized inflammatory response consisting predominately of macrophages, neutrophils, and lymphocytes (Duhamel et al. 1986, Wise et al. 2008, Lovy et al. 2011). Plasmodial rupture and the release of myxospores of histozoic species typically occurs upon death of the fish intermediate host (Fiala et al. 2015). Myxospores released from the fish host sink to the substrate, are ingested by the oligochaete definitive host, infect the digestive tract, and ultimately produce actinospores to complete the life cycle (Okamura et al. 2015, Fiala et al 2015, Atkinson et al. 2018).

Other *Henneguya* spp. Infecting Catfish

In the channel catfish, multiple *Henneguya* spp. infect one or more tissues including the gills, skin, adipose fin, and gall bladder (Table 1.1). *Henneguya exilis* infects both the skin and gills of channel catfish, one of the few *Henneguya* spp. that infects multiple tissue sites within its fish host (Duhamel et al. 1986, Lin et al. 1999, Rosser et al. 2016c). While less clinically significant than *H. ictaluri*, heavy burdens of *H. exilis* are rarely reported to produce grossly disfiguring plasmodial pseudocysts in the skin and lamellar fusion in the gills of channel catfish (Duhamel et al. 1986, Lin et al. 1999, Rosser et al. 2016c). *H. mississippiensis* forms large plasmodia within the tips of gill filaments while *H. bulbosus* forms prominent, grossly visible cysts in the gill epithelium (Rosser et al. 2014b, Rosser et al. 2015). *Henneguya adiposa*, which is closely related to *H. ictaluri* based on 18S rRNA sequencing, infects the adipose fin of channel catfish in aquaculture settings (Minchew 1977, Griffin et al. 2009c). It is not associated with clinical disease, though detailed histologic examination of lesions is lacking in the literature (Griffin et al. 2009c). *Henneguya sutherlandi* and *H. pellis* infect the epidermis of the channel and blue catfish, respectively (Griffin et al. 2008a, Griffin et al. 2009a). Historical morphologic descriptions of several additional *Henneguya* spp. from channel catfish exist, though they lack further molecular and detailed histological characterization (Minchew 1977). Investigation into the actinospore diversity within catfish production ponds suggests the presence of additional undiscovered *Henneguya* spp. potentially infective to catfish and other fish species occurring incidentally in ponds (Hanson et al. 2001, Rosser et al. 2014a).

Because several *Henneguya* spp. are known to infect the gills of channel catfish, there is a distinct possibility for mixed infections. Descriptions of *Henneguya* spp. within the gills of channel catfish often report plasmodia in multiple components of the gill including the filament

connective tissues, lamellar capillaries, lamellar epithelium, and epithelium of the interlamellar troughs (Minchew 1977, Bowser et al. 1985, Duhamel et al. 1986, Wise et al. 2008). The majority of these reports predate molecular characterization, making species identification questionable given the same host, tissue predilection, and similar morphology (Eszterbauer 2002, Rosser et al. 2016c). While the cause of PGD is attributed to *H. ictaluri*, potential contributions of other gill-infecting *Henneguya* species, particularly *H. exilis* and *H. mississippiensis*, to PGD-like lesions and exacerbation of disease signs due to mixed species infections have not been studied. To date, species identification of developing, particularly characteristic presporogonic stages, and mature plasmodia in histologic sections of gill tissue from PGD outbreaks has not been attempted. Questions remain as to whether other *Henneguya* spp. may cause subclinical pathology, PGD-like lesions, or contribute secondary pathology and clinical disease during PGD outbreaks. The dissertation seeks to examine the presence and roles of various *Henneguya* spp. myxozoans in causing clinical and subclinical pathology during PGD outbreaks (Chapters 2 and 3) and independently (Chapters 4, 5, and 6).

Figure

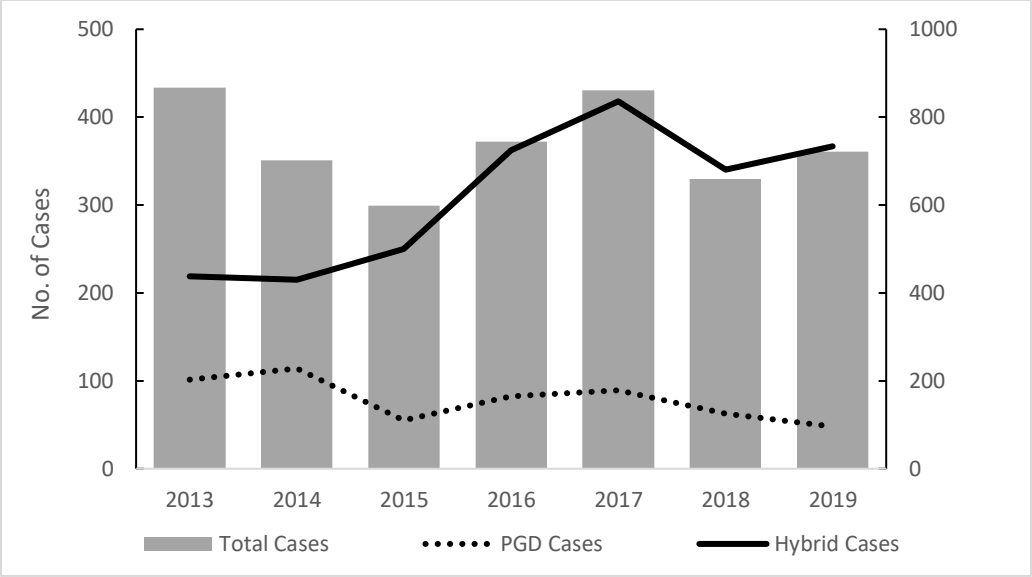

Figure 1.1. Summary of diagnostic submissions to the Aquatic Research and Diagnostic Laboratory of the Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS. The number of hybrid case submissions and total PGD cases are represented on the primary y-axis. Total case submissions are reported on the secondary y-axis.

Table

Table 1.1: *Henneguya* spp. that infect the channel catfish, *Ictalurus punctatus*

Henneguya spp.	Fish host	Oligochaete Host	Tissue Tropism	Reference
H. adiposa	*Ictalurus punctatus*	UNK	Adipose fin	Current 1979
H. bulbosus	*I. punctatus*	UNK	Gills	Rosser et al. 2014
H. diversis	*I. punctatus*	UNK	Barbels, kidney, liver, pectoral fins	Minchew 1977
H. exilis	*I. punctatus*	*Dero digitata*	Gills, skin	Kudo 1929; Rosser et al. 2016
H. ictaluri	*I. punctatus*	*D. digitata*	Gills	Pote et al. 2000
H. limatula	*I. punctatus*	UNK	Gall bladder	Minchew 1977
H. longicauda	*I. punctatus*	UNK	Gill	Minchew 1977
H. mississippiensis	*I. punctatus*	*D. digitata*	Gill	Rosser et al. 2015
H. postexilis	*I. punctatus*	UNK	Gill	Minchew 1977
H. sutherlandi	*I. punctatus*	UNK	Skin	Griffin et al. 2008

UNK, unknown

CHAPTER 2

MYXOZOAN COMMUNITY COMPOSITION AND DIVERSITY IN CLINICAL CASES OF PROLIFERATIVE GILL DISEASE IN CATFISH AQUACULTURE [1]

[1] Stilwell, J.M., M.J. Griffin, G.C. Waldbieser, J.B. Stanton, C. Ware, J.H. Leary, L.H. Khoo, D.J. Wise, & A.C. Camus. To be submitted to *Journal of Parasitology*.

Abstract

An abundance of morphologically variable *Henneguya* species complicate the understanding of disease relationships between ictalurid catfish and myxozoan (Phylum: Cnidaria) parasites on North American aquaculture operations. *Henneguya ictaluri*, the cause of proliferative gill disease (PGD) in channel and hybrid catfish, is the most important parasite of commercial catfish aquaculture in the southeastern United States. While research indicates arrested development and limited sporogenesis of *H. ictaluri* in channel (*Ictalurus punctatus*) × blue (*Ictalurus furcatus*) hybrid catfish, incidents of PGD persist in hybrid production systems. This work investigated the influence of fish host on myxozoan community composition and diversity within naturally infected gill tissues from diagnostic case submissions to the Aquatic Research and Diagnostic Laboratory in Stoneville, MS, from 2017 to 2019. Gills collected from farm-raised catfish with clinical PGD were subjected to metagenomic analysis and targeted amplicon, next-generation sequencing with Illumina MiSeq, targeting a hypervariable region of the myxozoan 18S rRNA gene. Myxozoan community composition significantly differed between channel and hybrid catfish PGD cases. Channel catfish gills had greater relative abundance of *H. ictaluri* in 2017 and 2019 and unclassified taxa in 2018 compared to hybrid catfish. *H. ictaluri* was present in all channel and hybrid catfish PGD cases, although in nearly half of these cases *H. ictaluri* was not the most abundant myxozoan. The detection of numerous known and unclassified myxozoan sequences provides evidence that PGD may involve mixed species infections. Additionally, numerous unclassified myxozoan sequences present in gill samples indicate the number of described species vastly underestimates the true myxozoan diversity present within the varied microcosms associated with catfish aquaculture.

Introduction

Myxozoan parasitism causes substantial mortality and economic losses in wild and cultured fish populations globally (Okamura et al. 2015). Important examples include *Enteromyxum leei, Ceratonova shasta, Kudoa thyrsites, Myxobolus cerebralis, Parvicapsula minicornis, Sphaerospora truttae* and *Tetracapsuolides bryosalmonae* (Kent et al. 2001, Lom and Dyková 2006). The myxozoans represent a morphologically diverse group of metazoan parasites with over 2,200 described species in 64 genera and 17 families that infect fish, amphibians, reptiles, birds, and small mammals (Fiala et al. 2015). Traditional classification schemes based on highly plastic myxospore morphology are increasingly contradicted by molecular phylogenies constructed primarily from small subunit (SSU) ribosomal DNA gene sequencing (Woodyard et al. 2020). Variable regions of the SSU rDNA provide information on diversification and are highly substituted, indicating rapid evolution within the myxozoans (Fiala et al. 2015).

Most myxozoans possess an indirect life cycle involving a pelagic actinospore stage released by either an annelid worm or bryozoan definitive host, and a myxospore stage in the fish intermediate host. Myxospore morphology is simplified compared to their distant relatives within the phylum Cnidaria and consists of 1–13 polar capsules, 2–13 shell valves with or without caudal processes, a nucleated sporoplasm, and one or more vacuoles (Kent et al. 2001, Lom and Dyková 2006). The genus *Henneguya* alone contains over 200 species characterized by pyriform, ellipsoidal, spindyloid, and rounded myxospores with two polar capsules and two shell valves, each possessing a caudal process. Their polysporic plasmodia are often large and cyst-like (Lom and Dyková 2006). As parasites, myxozoans exhibit host and tissue tropisms, exploiting specific sites of development within host tissues, which may represent adaptation to particular

microhabitats (Molnár and Eszterbauer 2015, Stilwell et al. 2020). Because of this phenomenon, closely related myxozoan species infecting the same hosts or tissues may vary in their pathogenicity, clinical presentation, and level of host mortality (Wise et al. 2008, Rosser et al. 2015, Stilwell et al. 2019a, Stilwell et al. 2019b). For example, at least 10 morphologically similar *Henneguya* spp. infect gill and other tissue sites in North American ictalurid catfish (Minchew 1977, Pote et al. 2000, Griffin et al. 2008a, Iwanowicz et al. 2008, Griffin et al. 2009b, Rosser et al. 2014b, Rosser et al. 2015). While most are innocuous parasites, *H. ictaluri* and *H. exilis* have both been associated with morbidity and mortality in farm-raised catfish (Duhamel et al. 1986, Pote et al. 2000, Wise et al. 2004; Stilwell et al. 2019a).

Farm-raised catfish is the largest aquaculture industry in the United States, wherein proliferative gill disease (PGD) is a leading cause of economic losses. The disease, caused by *Henneguya ictaluri* (Pote et al. 2000), results in a devastating parasitic infection in channel (*Ictalurus punctatus*) and hybrid (*I. punctatus* ♀ x *I. furcatus* ♂) catfish (Bosworth et al. 2003; Wise et al. 2004; Griffin et al. 2010). While the true economic impact of PGD in catfish aquaculture is unknown, it is estimated to cost the industry millions of dollars annually in the form of lost feed days and mortality. Initial actinospore penetration and proliferation of the presporogonic stage incites locally extensive granulomatous inflammation, cartilage lysis and filament fracture leading to respiratory compromise and, in severe cases, death (Wise et al. 2008; Lovy et al. 2011). While comparably susceptible to *H. ictaluri* infection and presporogonic development, hybrid catfish have shown reduced parasite transmission, limited sporogenesis, and arrested parasite development when compared to channel catfish cohorts (Griffin et al. 2010; Rosser et al. 2019). Further, continuous production of hybrid catfish has been shown to reduce *H. ictaluri* abundance in experimental pond studies (Griffin et al. 2020b). This is in line with

anecdotal reports from industry that suggest reduced incidence and severity of PGD in hybrid catfish production systems. However, despite these findings, outbreaks of PGD still occur in hybrid catfish, with gross and histologic lesions compatible with *H. ictaluri* infection in channel catfish (Griffin et al. 2010; Rosser et al. 2019; Griffin et al. 2020b).

The purpose of this work was shed light on the contradiction between laboratory evidence and industry reports by investigating the differences in myxozoan community composition and diversity in clinically diagnosed proliferative gill disease between channel and hybrid catfish and assess the role of other myxozoans in clinical presentations of PGD in channel and hybrid catfish. Our null hypothesis assumed PGD involved only H. ictaluri while our alternative hypotheses assumed PGD involved mixed species infection with H. ictaluri always present and either similar or differing myxozoan communities between channel and hybrid catfish PGD cases.

Materials & Methods

Gill sampling

The myxozoan communities associated with clinical PGD cases were determined from diagnostic case submissions to the Mississippi State University Aquatic Research and Diagnostic Laboratory, Stoneville, MS, USA, during the spring and fall of 2017, 2018, and 2019. The number of fish for each case varied, ranging from 1 to 4 individuals. For each case, gill holobranchs from catfish diagnosed with clinical PGD (Pote et al. 2003) were pooled. After initial sampling for wet mount diagnosis, the holobranchs were equally divided into 10% neutral buffered formalin for histological assessment and 70% ethanol for molecular analysis. Presumptive diagnoses were based on microscopic examination and observation of filament

cartilage lysis typical of PGD (Wise et al. 2008). Formalin-fixed gills were routinely processed, embedded in paraffin, and sectioned at 4 µm for histologic examination to confirm myxozoan infection (Spencer and Bancroft 2013). DNA from corresponding ethanol-fixed gill samples was extracted using a Qiagen DNA Mini Kit (Hilden, Germany) according to the manufacturer's protocol. Fish host species was confirmed by duplex-PCR following methods outlined by Waldbeisser and Bosworth (2008).

Amplicon sequencing

Genomic DNA extracted from gill tissues were transferred to individual wells of 96 well plates and submitted to the Georgia Genomics and Bioinformatics Core, University of Georgia, Athens, GA, USA for library preparation and targeted amplicon next-generation sequencing. Sequencing was performed using Illumina MiSeq for 600 cycles, producing paired, 300 bp reads. Custom, general myxozoan primers were used to amplify an approximately 432 bp amplicon of the myxozoan 18S rRNA gene. The forward primer (H9, 5'-TTACCTGGTCCGGACATCAA-3') developed by Hanson et al. (2001) targets a highly conserved region of the myxozoan 18S rRNA gene (Griffin et al. 2008, 2009a, 2009b; Rosser et al. 2014a, 2014b; Stilwell et al. 2019a, 2019b, 2019c; 2020b). The reverse primer (1862R, 5'-ATTGTAGCGCGCGTGCAG-3') targets a conserved region 400–500 bp upstream of the H9 primer. The primers surround a diagnostic variable region (DVR3) used for species identification in myxobolids (Iwanowicz et al. 2008; Griffin et al. 2009a, b). The 1862R primer design was based on alignments of 18S rRNA sequences of ten myxozoan species endemic to catfish aquaculture ponds in Mississippi and other closely related myxozoans available in the National Center for Biotechnology's (NCBI) nucleotide database (GenBank). These locus-specific primers each target regions conserved

among the catfish myxozoa, as well as other myxozoans that infect non-ictalurids, to better target myxozoans that have yet to be molecularly characterized.

Metagenomic analysis

Raw, paired end reads were merged (10 maximum differences), trimmed to remove primer sequences, and filtered by quality (maximum expected error rate of 1.0) and sequence length (370–490 bp) using USEARCH v10.0.240 (Edgar, 2010). Unique reads were clustered and binned (0.95 minimal fractional identity) into operational taxonomic units (OTUs) using USEARCH, then identified through a Blastn search of the NCBI non-redundant nucleotide (nr/nt) database (accessed January 7, 2020). A sequence identity matrix of the 432 bp region using all previously sequenced *Henneguya* species infecting catfish yielded a maximum nucleotide similarity of 92%. Therefore, a cutoff point of 95% nucleotide similarity and above was used for species identification. OTUs below 95% similarity with a myxozoan as the best match were considered unique, unknown taxa and included for phylogenetic analysis. Analyses from 2019 were split into two separate analyses due to data limitations of the USEARCH software, resulting in two sets of OTUs which were merged together for downstream analyses. All unique, known and unknown myxozoan OTUs produced from merged alignments containing greater than 10 reads were aligned with the corresponding region of the *T. bryosalmonae* 18S rRNA gene using MAFFT online server using default parameters (Katoh et al. 2019). The alignment was used to generate maximum likelihood phylogenetic trees employing the General Time Reversible model, determined as the best-fit model in MEGA X (Kumar et al. 2018).

Statistical analysis

All analyses were performed with RStudio software version 1.2.5033 (RStudio Team, 2019) to determine OTU counts, proportions, or relative (percent) abundance. For each year, myxozoan community composition was compared between channel and hybrid catfish using PERMANOVA analysis of dissimilarity (ADONIS) with the vegan package (Oksanen et al. 2019) and Generalized Dirichlet Multinomial modeling (GDM) with the MGLM package (Kim et al. 2018). Nonmetric multidimensional scaling (NMDS) plots were generated with the vegan package to visualize community differences in beta diversity. Similarly, the Shannon diversity index was calculated with the vegan package, while species evenness was calculated manually. Two sample, independent T-tests and one-way ANOVA were used to compare individual taxa relative abundance, Shannon diversity, and species evenness between fish species each year and year-over-year trends within each fish species, respectively. To account for multiple testing, p-values were corrected using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) at a false error rate of 20%. PGD cases were excluded from analysis if there were too few myxozoan reads in a sample (<100 per gill sample, total of 3 gill samples from 1 case) or mixed catfish species detected (n=0).

Results

Metagenomics from diagnostic case submissions

Four Illumina MiSeq runs generated a total of 38,166,384 paired reads, averaging 12,722,128 paired reads per year (Table 2.1). Three hundred and forty-two gill samples from 35 channel catfish PGD cases (2017, n=6; 2018, n=11; 2019, n=18) and 30 hybrid catfish PGD cases (2017, n=10; 2018, n=15; 2019, n=5) were included in the analyses. While PGD cases showed some overlap in myxozoan community composition in NMDS ordination plots (Figure 2.1), channel and hybrid catfish cases of PGD showed significantly different myxozoan

community composition in all three years examined ($p < 0.01$ for all PERMANOVA and GDM analyses). PGD cases in both channel catfish and hybrid catfish always contained mixed species of myxozoans, although *H. ictaluri* was always present at varying levels of relative abundance. Similarly, other described *Henneguya* spp. and sequences from actinospores collected from commercial catfish ponds were frequently detected at varying levels, including *H. mississippiensis* (n=65/65 cases, 100%), Raabeia type TGR 2014 (n=61/65 cases, 93.8%), *H. exilis* (n=59/65 cases, 90.7%), *H. adiposa* (n =46/65 cases, 70.7%), and *H. bulbosus* (n=28/65 cases, 43.1%). A summary of these findings by catfish type is presented in Table 2.2. *Henneguya ictaluri* had the greatest relative abundance compared to other myxozoans in 54% and 50% of channel and hybrid catfish PGD cases, respectively, but varied by year (Figure 2.2).

In comparing individual taxa prevalence, the relative abundance of *H. ictaluri* was greater in channel catfish PGD cases in 2017 and 2019 compared to hybrid catfish cases (Figure 2.3, Table 2.3). In 2018, unclassified taxa relative abundance was greater in channel catfish cases of PGD compared to hybrid catfish (Figure 2.3, Table 2.3). Channel catfish cases exhibited year-over-year increases in *H. mississippiensis* relative abundance, while hybrid catfish cases showed decreases in *H. ictaluri* relative abundance from 2017 and 2018 to 2019 (Figure 2.3, Table 2.4). Shannon diversity and species evenness showed no significant differences between channel and hybrid catfish or between years within channel or hybrid catfish (Table 2.5 & 2.6).

Phylogenetic analyses

Thirty-seven unique myxozoan OTUs were identified in the diagnostic gill samples, which clustered with known catfish-infecting myxozoan species with high bootstrap (>80) support (Figure 2.4). Of these, 31 (84%) OTUs did not have any direct matches to myxozoan sequences deposited in NCBI's GenBank, potentially representing undescribed myxozoan taxa.

Eighteen OTUs (49%) were present in all three years examined. Unidentified OTUs with 100% nucleotide sequence homology present in multiple years were relabeled as “unclassified taxa” followed by a numerical indicator (Table 2.7).

Discussion

Previous research has demonstrated *H. ictaluri* sporogenesis is arrested in hybrid catfish (Rosser et al. 2019) and *H. ictaluri* is suppressed in hybrid catfish monoculture systems (Griffin et al. 2020b). Despite these findings, PGD outbreaks still occur in hybrid production ponds. It has been hypothesized that other myxozoan species contribute to PGD in hybrids. Metagenomic examination of industry PGD cases revealed that diseased channel and hybrid catfish gills were infected by multiple myxozoan species. While *H. ictaluri* was always present in clinical PGD cases, it was not always the most abundant myxozoan and there were several cases where *H. ictaluri* relative abundance was negligible. Prevalent taxa in PGD cases aside from *H. ictaluri* included *H. exilis*, *H. mississipiensis*, and several other unclassified taxa, particularly Unclassified Myxozoan 1, which was detected in 100% of PGD cases at varying relative abundances. With the exception of *H. ictaluri* and *H. exilis*, none of the myxozoans detected have been previously associated with PGD outbreaks. As such, the significance of year-over-year increases in *H. mississippiensis* relative abundance in channel catfish PGD cases and its involvement in the disease process is unknown. These observations suggest myxozoans other than *H. ictaluri* may be involved in the disease process or at least contribute to the morbidity, mortality, and underlying pathology. Although historical myxozoan identification relied on morphologic rather than molecular documentation, mixed species infections potentially explain conflicting historic descriptions of PGD pathology in the literature (McCraren et al. 1975,

Bowser and Conroy 1985, Duhamel et al. 1986, MacMillan et al. 1989) and the continued detection of PGD cases in hybrid catfish in the industry (Rosser et al. 2019).

While the relative abundance of *H. ictaluri* in hybrid catfish was less than that observed for channel catfish, *H. ictaluri* was still present in all hybrid catfish diagnostic submissions of PGD. This indicates that *H. ictaluri* is being introduced to these systems despite evidence hybrid catfish are likely a dead-end host for the parasite (Rosser et al. 2019, Griffin et al. 2020b). While speculative, it is hypothesized myxospores deposited in pond sediment from channel catfish could persist in the environment, resulting in PGD outbreaks when hybrids are subsequently stocked into the same system. While the longevity of *H. ictaluri* myxospores is unknown, viability studies with *M. cerebralis* myxospores revealed the long-term-viability to be <1 year at 5°C (Nehring et al. 2015), suggesting that carryover of myxospores deposited in the sediments may occur from one year to the next, but persistence across multiple years or multiple catfish production cycles is less certain. Alternatively, there is evidence that *M. cerebralis* myxospores survive passage through piscine and avian piscivores (El-Matbouli et al. 1991, Koel et al. 2010). Given the abundance of piscivorous birds endemic on catfish aquaculture farms, avian transmission of myxozoans in catfish aquaculture seems a plausible route of myxozoan dissemination, though further study is needed.

The unexpected detection of *H. adiposa* in branchial tissue suggests the gills may provide a portal of entry for this organism, although it completes plasmodial development at tissue sites distant from the gills (Stilwell et al. 2019b). Infrequent occurrences of massive interlamellar *H. exilis* infections have been reported, particularly in hybrid catfish (Stilwell et al. 2019a). In contrast to PGD, disease associated with *H. exilis* occurs due to mature plasmodia which form within the lamellar epithelium and obstruct the interlamellar space. Massive burdens can result in

morbidity with signs of respiratory distress and anorexia similar to PGD, albeit with limited mortality (Stilwell et al. 2019a). Other *Henneguya* spp. that infect various sites within the gill tissue occasionally incite inflammation but are not believed to be associated with significant morbidity or mortality (Minchew 1977; Rosser et al. 2014b; Rosser et al. 2015). The impacts these other myxozoans have on catfish production are presently unknown and warrant further study.

The abundance of potentially novel, catfish-associated myxozoan sequences may reflect overlap in myxospore morphometrics and/or tissue tropism, resulting in confusion between novel, undescribed species and those previously reported. This illustrates the importance of molecular confirmation when investigating morphologically ambiguous species. Further, diagnostic examinations of catfish gills for PGD focus primarily on characteristic lesions without in-depth investigations of myxospore morphology, as few other ictalurid infecting *Henneguya* spp. have been associated with clinical disease (Stilwell et al. 2019a). Moreover, routine diagnostic assessments of channel and hybrid catfish typically focus on characteristic lesions for endemic pathogens and rarely involve the scrutiny required to uncover rare myxozoans in peripheral tissues. As a result, most myxozoan descriptions in catfish aquaculture have been limited to those commonly encountered or those that result in noticeable burdens or grossly visible lesions. As such, unknown myxozoan sequences identified in these datasets could represent presporogonic stages of myxozoans that develop in other tissues that are not routinely inspected on diagnostic examinations. Alternatively, they could represent taxa that are able to infect the fish host, but do not undergo sporogony (i.e., *H. ictaluri* in hybrid catfish).

Uncontrollable factors that potentially biased the results included fish age, pond age, time of year, possibility of mixed fish species in ponds, previous myxozoan disease history, stage of

myxozoan development/disease progression, and sample selection bias by farmers. For example, older fish with previous myxozoan exposures may have different susceptibilities to infection, morbidity, and mortality compared to fingerling catfish. Ponds accidentally stocked with both channel and hybrid catfish would allow for completion of myxozoan life cycles and potentially negate selective pressures. While these diagnostic submissions were comprised of fish diagnosed with clinical PGD based on gross gill lesions and histological assessment, the myxozoan communities could potentially differ between early/acute stages of disease, peak outbreak, or late stage, recovering fish. Finally, farmers may choose not to submit infected fish due to pathognomic lesions of PGD, thus underestimating the true number and diversity of cases. Potential biases were limited by preemptively screening samples for PGD lesions and confirming fish species by PCR.

Beta diversity measures differences in the composition of species communities between disparate environments such as channel versus hybrid catfish pond systems or microhabitats such as gill tissues. Myxozoan beta diversity in clinical PGD cases significantly differed ($p<0.01$) between channel and hybrid catfish in all three years examined, reflecting differences in the species present between the two catfish types. Unsurprisingly, *H. ictaluri* was present in all cases, with variable relative abundance. Despite differences in myxozoan community composition between channel and hybrid catfish, there were no differences in Shannon diversity indices, which is a measure of alpha diversity that reflects a combination of myxozoan species richness (i.e., the number of species present) and species evenness (the relative or proportional distribution of species present) within a sample. Similarly, myxozoan species evenness alone was similar between channel and hybrid catfish. In short, while there were differences in the myxozoan communities and relative abundances of individual taxa, the number of myxozoan

species and proportional distribution of myxozoans within samples was consistent between channel and hybrid catfish with PGD. This could be due to restricting sampling to only PGD cases, which would presumptively have similar myxozoans comprising infections based upon clinical pathology and the presence of myxozoan life stages. Examination of all myxozoan infections occurring in diagnostic samples or pond environments at various stages of an outbreak could provide more accurate illustrations of myxozoan community dynamics in the industry and differences in alpha diversity between channel and hybrid catfish systems.

Metagenomic analysis of PGD cases from the commercial catfish aquaculture industry revealed differences in myxozoan community composition between channel and hybrid catfish PGD cases as well as mixed species infections and abundant, previously unknown myxozoan diversity. Lower *H. ictaluri* relative abundance in hybrid catfish PGD cases supports anecdotal industry reports of less severe disease in hybrid catfish along with experimental evidence demonstrating decreased rates of infection, arrested parasite development, and suppression of *H. ictaluri* in hybrid catfish monoculture systems (Rosser et al. 2019; Griffin et al. 2020b). Furthermore, these data suggest *H. ictaluri* may not be the sole etiological agent of PGD and that other myxozoans may contribute to the disease process. Future research should focus on the longevity of *H. ictaluri* myxospores in pond systems, mechanisms of parasite dissemination on catfish aquaculture farms, particularly in reference to hybrid systems, and the role of other *Henneguya* spp. in PGD lesion development and myxozoan-induced gill disease.

Figures

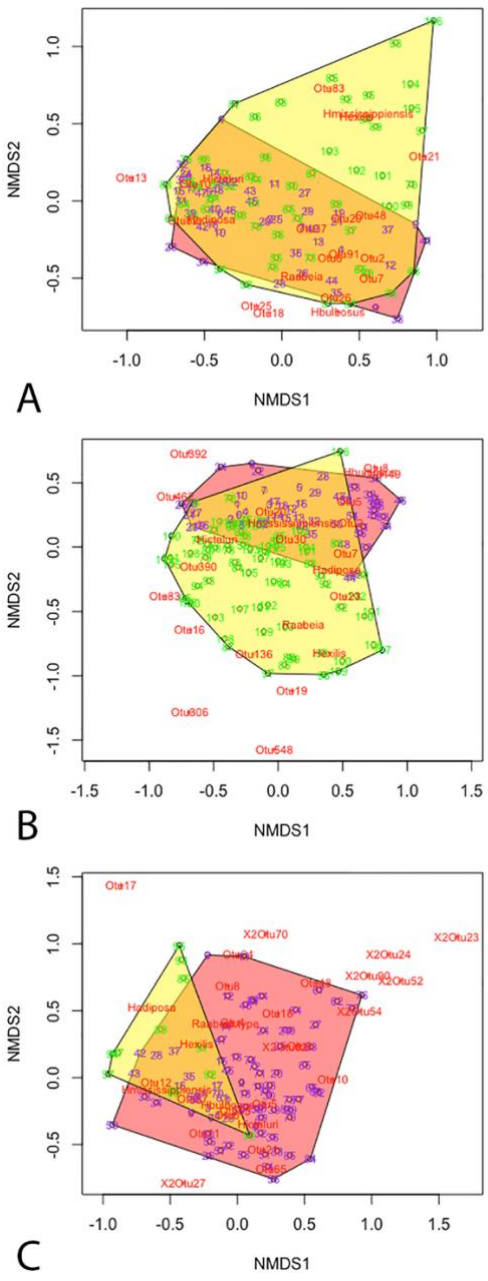

Figure 2.1. Visualization of beta diversity among cases of PGD in channel and hybrid catfish using Nonmetric Multidimensional Scaling plots. Cases of PGD from 2017 (A), 2018 (B), and 2019 (C). Purple numbers in red regions represent channel gill samples, green numbers in yellow

regions represent hybrid gill samples. The region of overlapping similarity (containing purple and green numbers) is orange. Red labels represent myxozoan species.

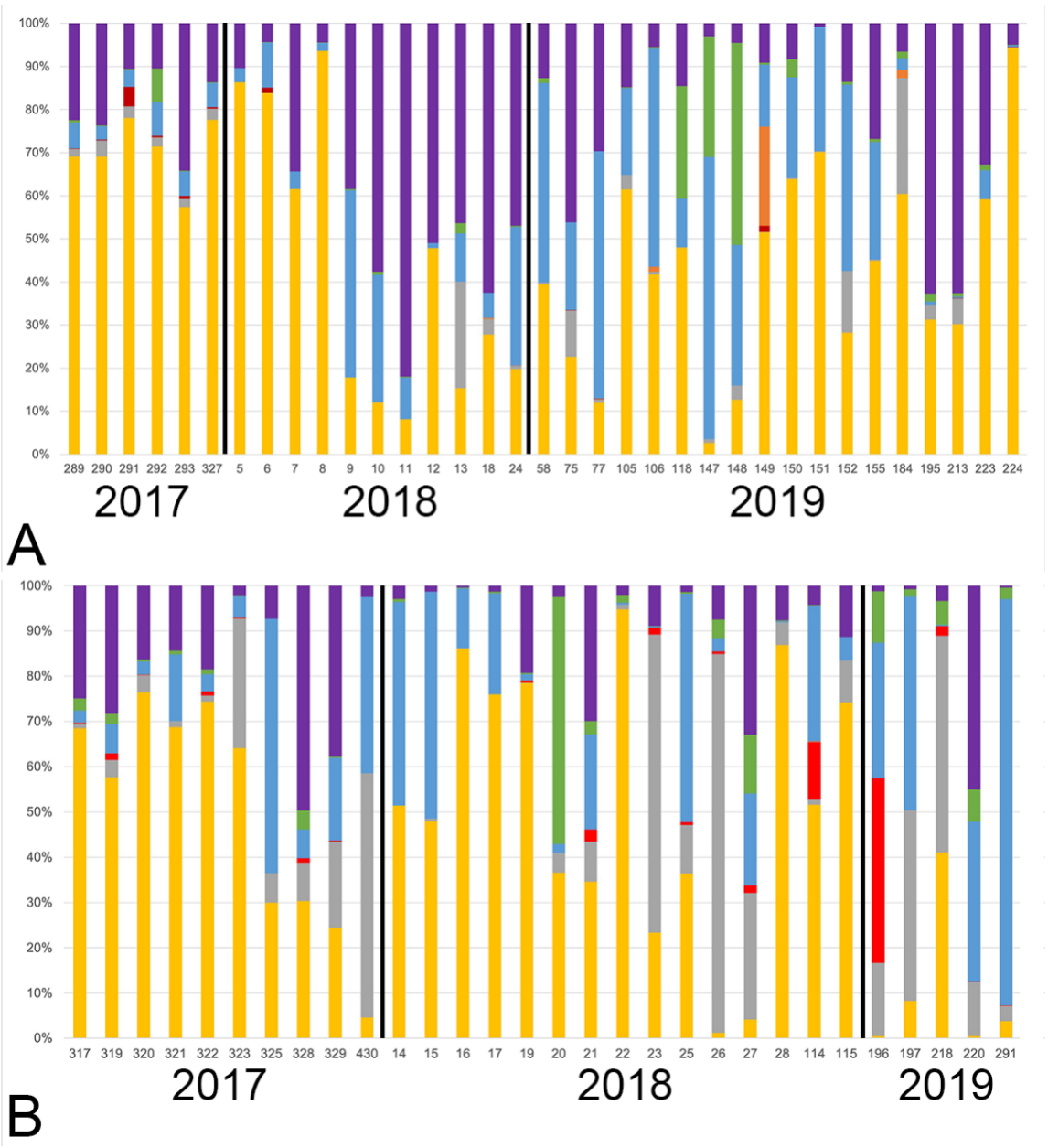

Figure 2.2. Relative abundances of myxozoan taxa in channel and hybrid catfish cases of PGD. PGD cases represent mixed infections in both channel (A) and hybrid (B) catfish. The y-axis represents myxozoan relative abundance as stacked percentages while the x-axis lists case numbers divided by years. Taxa are color-coded as follows: all unclassified taxa, purple; Raabeia-type, green; *H. mississippiensis*, blue; *H. bulbosus*, orange; *H. adiposa*, red; *H. exilis*, gray; *H. ictaluri*, yellow.

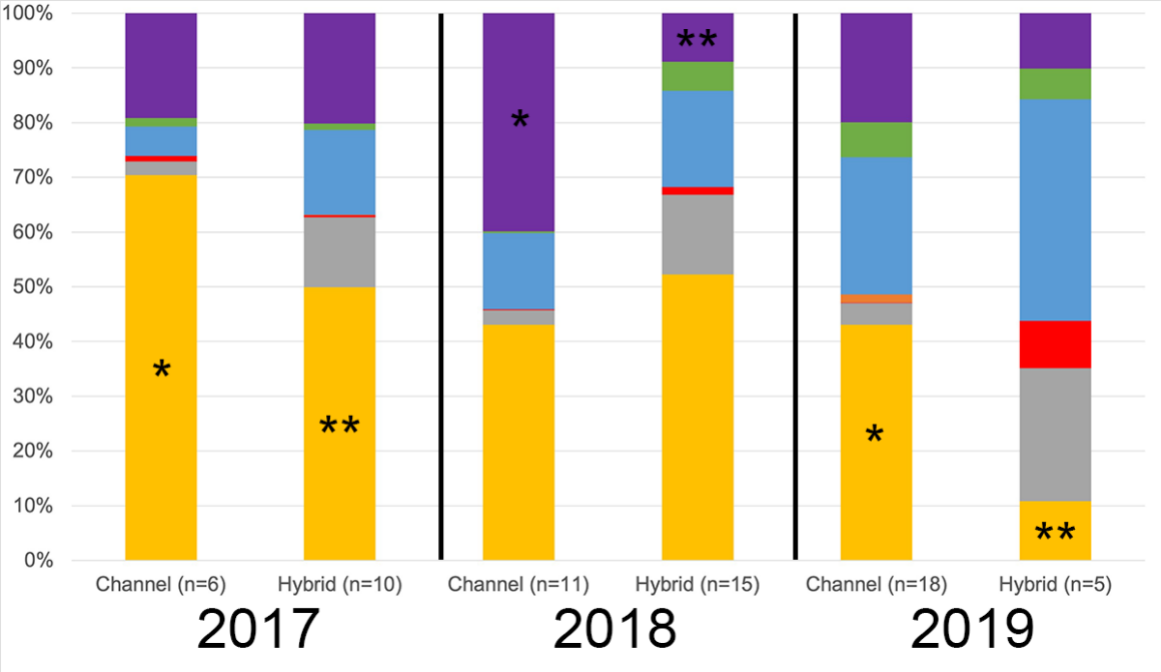

Figure 2.3. Average yearly relative abundance of myxozoan taxa in channel and hybrid catfish cases of proliferative gill disease. The y-axis represents relative abundance as stacked percentages while the x-axis is divided into channel and hybrid catfish with the number of cases in each year. Taxa are color-coded as follows: all unclassified taxa, purple; Raabeia-type, green; *H. mississippiensis*, blue; *H. bulbosus*, orange; *H. adiposa*, red; *H. exilis*, gray; *H. ictaluri*, yellow. Asterisks indicate statistically significant differences in average relative abundance for a particular myxozoan between channel (*) and hybrid catfish (**) in a year.

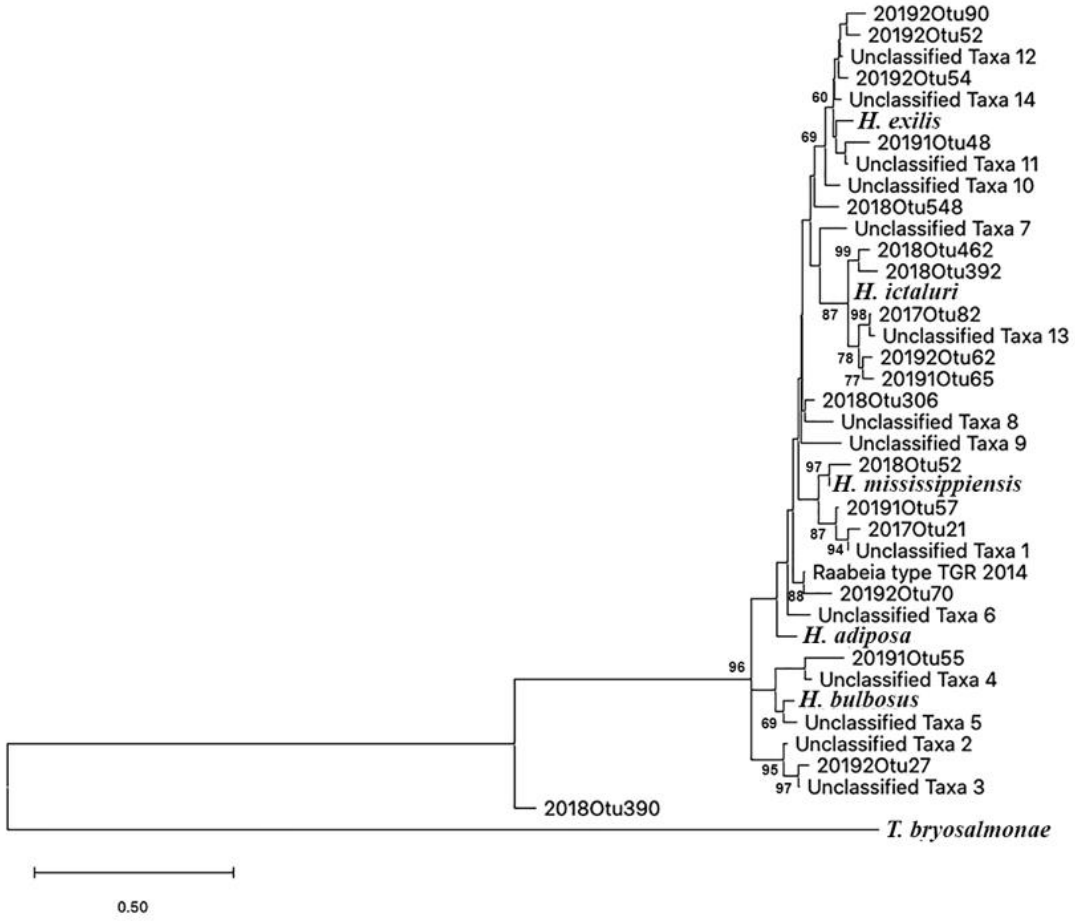

Figure 2.4. Phylogenetic tree constructed from 18S rRNA gene sequences of detected, unique, known and unknown myxozoan sequences in the PGD samples. Branch tip labels consist of myxozoan OTUs or species names. Branch values represent maximum likelihood analysis using the General Time Reversible model and a discrete Gamma distribution with bootstrap confidence values obtained from 1,000 pseudoreplicates. The tree with the highest log likelihood (-5325.60) is shown. Branch values below 60% not shown. Scale-bar: number of nucleotide substitutions per site.

Table 2.1 Combined Illumina MiSeq sequencing metadata from industry PGD cases

Sample type	Total # of Paired Reads (average per year)	Total # of Merged Reads	Total # of Myxozoan Reads	Excluded Gill Samples	Average QC>30
Gill tissue (n=345)	38,166,384 (12,722,128)	8,028,939 (2,676,313)	7,517,528 (2,505,842)	3 (TFR)	82.0%

TFR, too few reads

Table 2.2 Prevalence of myxozoans in industry PGD cases by catfish host type

Myxozoan Species	Channel Catfish (n=35)	Hybrid Catfish (n=30)
H. adiposa	68.6% (24/35)	73.3% (22/30)
H. bulbosus	54.3% (19/35)	30% (9/30)
H. exilis	85.7% (30/35)	96.7% (29/30)
H. ictaluri	100% (35/35)	100% (30/30)
H. mississippiensis	100% (35/35)	100% (30/30)
Raabeia type TGR 2014	94.3% (33/35)	93.3% (28/30)

Table 2.3 Statistical comparisons of individual taxa relative abundance within gill tissues between channel and hybrid catfish cases

Taxon & Year (n=cases)	Channel Mean (%) +/- SD	Hybrid Mean (%) +/- SD	df	T statistic	Original p-value	*Corrected p-value
2017 (n=16)						

H. adiposa	1.02 +/-1.72	0.39 +/- 0.51	5.533	0.86	0.4229	0.143
H. bulbosus	0.004 +/- 0.005	0.011 +/- 0.023	10.481	-0.86	0.4081	0.114
H. exilis	2.49 +/-0.71	12.80 +/- 17.03	9.053	-1.91	0.0881	0.057
H. ictaluri	70.43 +/- 7.56	49.91 +/- 25.29	11.423	2.39	**0.0349**	**0.028**
H. mississippiensis	5.36 +/-1.66	15.52 +/- 18.13	9.250	-1.75	0.1115	0.085
Raabeia type TGR 2014	1.51 +/-3.09	1.18 +/- 1.39	6.248	0.25	0.8086	0.171
Unclassified taxa	19.18 +/- 9.35	20.18 +/- 15.35	13.963	-0.16	0.8730	0.200
2018 (n=28)						
H. adiposa	0.11 +/-0.37	1.35 +/- 3.23	14.492	-1.47	0.1623	0.085
H. bulbosus	0.04 +/-0.08	0.01 +/- 0.04	15.220	0.85	0.4060	0.143
H. exilis	2.64 +/-7.39	14.60 +/- 25.73	17.025	-1.71	0.1060	0.057
H. ictaluri	43.10 +/- 32.89	52.23 +/- 29.84	20.407	-0.73	0.4754	0.200
H. mississippiensis	13.88 +/- 14.37	17.65 +/- 18.67	23.910	-0.58	0.5672	0.171
Raabeia type TGR 2014	0.36 +/-0.69	5.24 +/- 14.06	14.092	-1.34	0.2012	0.114
Unclassified taxa	39.85 +/- 24.98	8.91 +/- 10.42	12.571	3.87	**0.0020**	**0.028**
2019 (n=28)						
H. adiposa	0.09 +/-0.33	8.63 +/- 18.04	4.001	-1.06	0.3496	0.114

H. bulbosus	1.45 +/-5.41	0.0006 +/- 0.0014	17.000	1.14	0.2699	0.085
H. exilis	3.98 +/-6.98	24.32 +/- 19.52	4.288	-2.29	0.0795	0.057
H. ictaluri	43.05 +/- 23.36	10.79 +/- 17.23	8.600	3.41	**0.0083**	**0.028**
H. mississippiensis	25.12 +/- 20.58	40.52 +/- 32.56	4.923	-1.00	0.3624	0.171
Raabeia type TGR 2014	6.36 +/- 13.23	5.59 +/- 3.88	20.718	0.22	0.8319	0.200
Unclassified taxa	19.94 +/- 19.62	10.14 +/- 19.52	6.440	0.99	0.357	0.143

*Benjamini-Hochberg method for p-value correction for multiple tests. Estimated false error rate of 20%.

Table 2.4 Statistical comparisons of year-over-year changes in individual myxozoan relative abundance within gill tissues of channel and hybrid catfish PGD cases

Taxa & Fish Host	2017 Mean (%)*	2018 Mean (%)*	2019 Mean (%)*	F value	Original p-value	Corrected p-value**
Channel						
H. adiposa	1.02	0.11	0.09	4.842	0.035	0.057
H. bulbosus	0.004	0.04	1.45	0.962	0.334	0.143
H. exilis	2.49	2.64	3.98	0.349	0.559	0.200
H. ictaluri	70.43	43.10	43.05	3.737	0.062	0.085
H. mississippiensis	5.36	13.88	25.12	7.254	**0.011**	**0.028**
Raabeia type TGR 2014	1.51	0.36	6.36	2.023	0.164	0.114

Unclassified taxa	19.18	39.85	19.94	0.492	0.488	0.171
Hybrids						
H. adiposa	0.39	1.35	8.63	3.308	0.080	0.085
H. bulbosus	0.011	0.01	0.0006	0.143	0.708	0.200
H. exilis	12.80	14.60	24.32	0.751	0.394	0.171
H. ictaluri	49.91	52.23	10.79	4.289	**0.048**	**0.028**
H. mississippiensis	15.52	17.65	40.52	3.519	0.071	0.057
Raabeia type TGR 2014	1.18	5.24	5.59	0.887	0.354	0.143
Unclassified taxa	20.18	8.91	10.14	2.733	0.109	0.114

*Means generated from same data as Supp Table 2 but reorganized to examine differences between years within catfish species.

** Benjamini-Hochberg method for p-value correction for multiple tests. Estimated false error rate of 20%.

Table 2.5 Shannon diversity index and species evenness in PGD cases between channel and hybrid catfish.

Year	Shannon Diversity index			Species Evenness		
	Channel Catfish Mean	Hybrid Catfish Mean	p-value	Channel Catfish Mean	Hybrid Catfish Mean	p-value
2017	1.119	1.221	0.4214	0.378	0.454	0.1209

2018	1.203	0.917	0.1415	0.538	0.378	0.0834
2019	1.186	1.052	0.4773	0.448	0.429	0.7981

Table 2.6 Intraspecies comparison in year-over-year trends for Shannon diversity index and species evenness

	Shannon Diversity Index*		Species Evenness*	
	Channel Catfish	Hybrid Catfish	Channel Catfish	Hybrid Catfish
F-value	0.085	1.383	0.085	0.344
p-value	0.773	0.25	0.773	0.562

*Same data as Supp Table 4 but reorganized to examine differences between years within catfish species.

Table 2.7 Unclassified taxa and corresponding myxozoan OTU sequences detected in PGD cases from each year. Within each unclassified taxa, sequences share 100% nucleotide similarity.

Tree Name	2017 OTUs	2018 OTUs	2019 OTUs
Unclassified Taxa 1	OTU2	OTU2	1OTU5, 2OTU7
Unclassified Taxa 2	OTU83	OTU136	1OTU18, 2OTU20
Unclassified Taxa 3	OTU26	OTU30	1OTU11, 2OTU10
Unclassified Taxa 4	OTU37	OTU20	1OTU6, 2OTU16
Unclassified Taxa 5	OTU18	OTU83	1OTU24
Unclassified Taxa 6	OTU20	ND	2OTU23
Unclassified Taxa 7	OTU48	OTU8	1OTU8, 2OTU13
Unclassified Taxa 8	OTU10	OTU16	1OTU12, 2OTU14
Unclassified Taxa 9	OTU25	OTU23	1OTU10, 2OTU11
Unclassified Taxa 10	OTU13	OTU19	1OTU17, 2OTU21
Unclassified Taxa 11	OTU7	OTU7	1OTU4, 2OTU6
Unclassified Taxa 12	OTU6	OTU5	1OTU9, 2OTU5
Unclassified Taxa 13	ND	ND	1OTU21, 2OTU19
Unclassified Taxa 14	OTU91	OTU149	2OTU24

ND, not detected

CHAPTER 3

PARTIAL VALIDATION AND APPLICATION OF HENNEGUYA SPECIES SPECIFIC IN SITU HYBRIDIZATION ILLUSTRATES MIXED SPECIES INFECTIONS IN PROLIFERATIVE GILL DISEASE OF CATFISH AQUACULTURE [1]

[1] Stilwell, J.M., A.C. Camus, E.T. Woodyard, C. Ware, T.G. Rosser, M. Gunn, A. Lopez-Porras, D. Wise, & M.J. Griffin. To be submitted to *Journal of Aquatic Animal Health*.

Abstract

The myxozoan *Henneguya ictaluri* is cited as the causative agent of proliferative gill disease (PGD) in channel (*Ictalurus punctatus)* and channel × blue (*I. furcatus*) catfish. Despite evidence of decreased *H. ictaluri* infection rates and impaired parasite development in hybrid catfish, PGD still occurs in hybrid production systems. In a previous metagenomic analysis of clinical PGD cases, numerous other myxozoans were detected within affected gill tissues in addition to *H. ictaluri*. The objective of this work was to develop and partially validate *Henneguya* species-specific *in situ* hybridization assays using RNAscope® technology and to investigate the development and pathologic contributions of *H. ictaluri* and other myxozoans in natural and experimentally induced PGD infections. Natural PGD infections were sourced from diagnostic case submissions in 2019. Experimental challenges involved channel and hybrid catfish exposed to water from a pond with an active PGD outbreak and were sampled at 1, 7, 10, 12, 14, 16, 18 and 20 weeks post-challenge. Nine, unique ISH probes were designed targeting a diagnostic variable region of the 18S rRNA gene of select myxozoan taxa identified in previous research to be present in clinical PGD cases. Partial validation using tissues from pure infections of *H. ictaluri, H. adiposa, H. postexilis* and *H. exilis* illustrated species-specific labelling and no cross reactivity between myxozoans or with the catfish host tissues. In the experimental challenge, plasmodia of *H. ictaluri* and *H. postexilis* formed in channel catfish but no mature plasmodia formed in hybrid catfish, suggesting impaired development of multiple myxozoans. ISH investigations of both experimental and naturally infected tissues confirmed the presence of mixed infections with characteristic PGD lesions associated with *H. ictaluri.* Developmental stages of other myxozoans were associated with secondary lesions and, rarely, PGD-like lesions.

These findings suggest other *Henneguya* spp. may contribute to PGD lesions and supports previous research implying hybrid catfish are a dead-end host in the *H. ictaluri* life cycle.

Introduction

Farm-raised catfish represents the largest sector of foodfish aquaculture in the United States. For decades the industry has relied on the channel catfish, *Ictalurus punctatus*, produced largely in multiple-batch (repeated stocking and harvesting), continuous, pond culture systems. As production has intensified, the industry has suffered significant losses to infectious diseases, including proliferative gill disease (PGD), a seasonal condition with potentially devastating impacts on pond aquaculture operations (Hawke and Khoo 2004). Beginning in the early 2000s, the industry began to shift towards the production of channel catfish × blue catfish, *Ictalurus furcatus*, hybrids in single batch (all-in/all-out) culture (USDA-NAHMS 1998, 2003, 2010, Tucker et al. 2004). Hybrid catfish have improved growth performance and increased resistance to many diseases that typically affect channel catfish (Wolters et al. 1996, Bosworth et al. 2003, Arias et al. 2012, Rosser et al. 2019).

Proliferative gill disease is attributed to *Henneguya ictaluri*, a myxozoan that infects the gill filaments (Pote et al. 2000). In common with other myxozoan parasites, *H. ictaluri* has a complex developmental cycle that includes presporogonic circulating and sporogonic plasmodial stages in its catfish host (Pote et al. 2000). For poorly understood reasons, presporogonic stages of *H. ictaluri* incite pathognomonic cartilage lysis and dense infiltrates of neutrophils and macrophages (Wise et al. 2008, Lovy et al. 2011). Destabilized filaments are subject to breakage with resultant tissue collapse, hemorrhage, and lamellar epithelial hyperplasia (Wise et al. 2008). Lesions cumulatively induce respiratory distress and high mortality during outbreaks. In fish that survive, regenerating cartilage becomes dysplastic and proliferative as it attempts to bridge gaps in damaged filaments (Hawke and Khoo 2004). The negative impacts of PGD on commercial catfish production have promoted extensive research into the etiologic agent, its disease

pathogenesis, predisposing factors, and mitigation strategies (McCraren et al. 1975, Bellerud et al. 1995, Pote et al. 2000, Wise et al. 2004, 2008, Griffin et al. 2010, Mischke et al. 2014, Mischke et al. 2016, Rosser et al. 2019, Griffin et al. 2020b).

In addition to *H. ictaluri*, several other *Henneguya* spp. infect and develop within the gills of catfish (Minchew 1977, Lin et al. 1999, Rosser et al. 2015). Some of these species share the same definitive oligochaete host, *Dero digitata*, and intermediate host, the channel catfish, with *H. ictaluri*, suggesting similar factors determine host susceptibility and predispose to infections (Lin et al., 1999, Pote et al. 2000, Rosser et al. 2015). One species, *H. exilis*, causes massive infections of the branchial interlamellar epithelium, resulting in morbidity similar to PGD (Stilwell et al. 2019a). In contrast to *H. ictaluri*, massive interlamellar *H. exilis* is rarely reported in channel catfish but is a putative emerging disease of hybrids (Duhamel et al. 1986, Stilwell et al. 2019a). These observed differences in disease incidence indicate varied susceptibilities among catfish intermediate hosts and these closely related myxozoan species. Furthermore, variable susceptibilities to one myxozoan over another in a given catfish species may produce selective pressures on overall myxozoan diversity in pond monoculture systems (Griffin et al. 2010, Rosser et al. 2019, Stilwell et al. 2019a, Griffin et al. 2020b, Chapter 2).

While the other *Henneguya* spp. described in the gills of farm-raised catfish have not been associated with specific disease processes, the presence of multiple, genetically related and morphologically similar organisms in the same fish host has confounded the understanding of disease etiopathogenesis. Among the related myxozoans that share general tropism for the gill, each localizes within a specific predilection site in the complex branchial architecture, sometimes overlapping with other myxozoans (Molnár 2002). Presporogonic stages of *H. ictaluri* initially infect connective tissues of the filament, while plasmodia with mature

myxospores develop within epithelium at the bottom of the interlamellar troughs (Pote et al. 2000, Wise et al. 2008, Griffin et al. 2010). *H. exilis* and *H. mississippiensis* plasmodia develop within the lamellar epithelium and epithelium at the filament tips, respectively (Lin et al. 1999, Rosser et al. 2015). These tissue tropisms were elucidated based on the presence of plasmodia containing mature myxospores identified by morphologic and molecular methods or by experimental infection with isolates of pure actinospores (Pote et al. 2000, Rosser et al. 2015, Stilwell et al., 2019a). However, because PGD is associated with the early presporogonic stages, definitive species identification within histologic lesions has not been possible. Furthermore, during PGD outbreaks the myxozoan community composition within infected tissues and pond water is highly variable. While *H. ictaluri* is always present, dozens of known and unknown myxozoan sequences have been detected in ponds and PGD cases, suggesting additional myxozoan species could be involved in the pathologic changes and pathogenesis of PGD (Chapter 2, Stilwell unpublished data).

In situ hybridization (ISH) technology offers a potential means to investigate the intricate relationships that exist between myxozoan parasites and their development within host fish tissues. ISH techniques detect RNA and DNA gene targets in histologic sections using chromogen or fluorescent labeled probes (Jensen 2014). Limited ISH assays exist for specific, economically important myxozoans, particularly *Myxobolus cerebralis*, *Ceratonova shasta*, and *Tetracapsuloides bryosalmonae* (Antonio et al. 1998, Morris et al. 2000, Bjork and Bartholomew 2010). These assays are typically used in research settings to study portals of entry into and migration within fish hosts to sites of development (Morris et al. 2000, Holzer et al. 2003, Bjork and Bartholomew 2010). Interactions of two distantly related myxozoans involved in

coinfections in the kidneys of Atlantic cod, *Gadus morhua*, were similarly studied using ISH assays (Holzer et al. 2010).

While notable exceptions exist, myxozoan parasites generally exhibit a high degree of specificity for individual or closely related fish hosts (Okamura et al. 2015). Investigation into the host fish specificity of *H. ictaluri* revealed blue catfish to be refractory to infection, while hybrid catfish were susceptible to infection and development of presporogonic stages associated with PGD (Bosworth et al. 2003, Griffin et al. 2010). In combination with molecular techniques, challenge studies illustrated decreased rates of infection and arrested parasite development of *H. ictaluri* in hybrid catfish in comparison to channel catfish (Griffin et al. 2010, Rosser et al. 2019). These challenges utilized water from ponds with active PGD outbreaks, potentially containing a mixed inoculum of myxozoans (Griffin et al. 2010, Rosser et al. 2019, Chapter 2). Thus, while *H. ictaluri* DNA could be detected by PCR methods, species level identification of presporogonic stages and developing plasmodia within lesions could not be confirmed (Rosser et al. 2019).

The objectives of this work were to develop and partially validate *Henneguya* sp. specific *in situ* hybridization (ISH) techniques to identify the presporogonic and mature myxospore stages associated with PGD. The probes were then used to identify myxozoan species in naturally and experimentally infected gills of channel and hybrid catfish collected during PGD outbreaks. Our null hypothesis assumed characteristic presporogonic stages associated with PGD lesions were *H. ictaluri* is all case and only *H. ictaluri* contributed to disease pathology while the alternative hypothesis assumed other myxozoans were capable of producing PGD-like lesions and contributing secondary pathology in PGD outbreaks.

Materials & Methods

Experimental challenge – Henneguya ictaluri pure challenge

To obtain gill tissues for ISH validation, *Henneguya ictaluri* actinospores were collected from *Dero digitata* following previously established procedures (Pote et al. 1994; Belem and Pote 2001; Griffin et al. 2009b; Rosser et al. 2014a). Five channel catfish fingerlings (5-10 cm) were placed in 7.6 L containers holding 2 L of well water and supplied with supplemental aeration. The container was inoculated with 50,000 actinospores (10,000 actinospores/fish) and challenged for 4 hours with stochastic, intermittent agitation. Every 30 minutes, the container was agitated by swirling to create a current and encourage contact between fish and actinospores. After 4 hours, fish were transferred to 115 L aquaria supplied with aerated, flow through well water (25-26°C) at a rate of 1 L/min. Five additional fish were treated similarly but not exposed to actinospores and served as negative controls. Fish were fed *ad libitum* and monitored daily for morbidity and mortality. After 7 days, fish were euthanized by an overdose of tricaine methanesulfonate (MS-222 at 300 mg/L; Syndel, Ferndale, WA) and gill arches fixed in 10% neutral buffered formalin. Formalin-fixed gills were processed routinely according to standard histologic methods, sectioned at 4 µm, stained with hematoxylin and eosin (H&E) (Spencer and Bancroft 2013).

In situ hybridization design, protocol, and validation

The ISH assays used in this study were based on RNAscope® technology (Advanced Cell Diagnostics Inc., Hayward, CA) (Wang et al. 2012). Individual ZZ probes (RNAscope 2.5, Advanced Cell Diagnostics Inc., Hayward, CA) were designed to specifically target 5 known *Henneguya* species, an actinospore sequence phylogenetically related to other catfish *Henneguya*

species, and 3 presumptive novel myxozoan taxa identified in Chapter 2 (Table 3.1). Probes targeted a hypervariable region of the myxozoan 18S small subunit rRNA gene, which contained enough dissimilarity to prevent cross reactivity between discrete myxozoan and the catfish host (Table 1). Probes were diluted by half concentration using equal volumes of stock probe solution and RNAscope® probe diluent (Catalog No. 300041, Advanced Cell Diagnostics Inc.). Singleplex (RNAscope® 2.5 HD Detection Kit (RED) User Manual, Part 2, Catalog No. 322360-USM, Advanced Cell Diagnostics Inc.) and duplex (RNAscope® 2.5 HD Duplex Detection Kit User Manual, Part 2, Catalog No. 322500-USM, Advanced Cell Diagnostics Inc.) assays were performed according to the manufacturer's protocols. Briefly, unstained histologic sections prepared on charged slides were deparaffinized, dried, treated with hydrogen peroxide, rinsed with distilled water, and immersed in target retrieval solution at 100°C for 25 minutes using an Oster® steamer (Model 5715, Sunbeam Products Inc., Boca Raton, FL). Slides were dried overnight and subjected to protease for 30 minutes at 40°C, probe hybridization for 2 hours at 40°C, and a series of signal amplification and detection steps using a RNAscope® HybEZ oven (Advanced Cell Diagnostics Inc.). Slides were counterstained with hematoxylin and TBS (bluing reagent), dried, and coverslipped using EcoMount (Biocare Medical LLC, Concord, CA). One observer (Stilwell) recorded presence or absence of staining within tissues. Superficial and faint ISH labelling was considered artifact.

Initial validation of ISH assays were performed with histologic sections of channel catfish gills experimentally infected with *H. ictaluri.* Additional samples included archived tissues from previous studies documenting natural infections of *H. adiposa* in the channel catfish adipose fin (Stilwell et al. 2019a), *H. postexilis* in channel catfish gills (Woodyard unpub data) and *H. exilis* from hybrid tissues (Stilwell et al. 2019b). Each respective tissue was tested with its

respective probe as well as other non-target probes to assess potential cross-reactivity of specific ISH probes and non-target myxozoans and host tissue.

Experimental challenge – Parasite development

Fish challenges were performed according to previously published methods (Griffin et al. 2010; Rosser et al. 2019). One hundred-eighty laboratory reared, channel and hybrid catfish fingerlings (7-13 cm) were first vaccinated against the endemic bacterial pathogen *Edwardsiella ictaluri* according to previous methods (Wise et al. 2015). Thirty days later, fish were arbitrarily distributed into six replicate exposure tanks and three control tanks (20 fish/tank) for each group (channels and hybrids). Each 115 L tank was supplied with aerated, flow-through well water (25-26°C) at a rate of 1 L/min. During pond water exposures, water supply was suspended and tank water was lowered to a depth of 4" (~10 L). Tanks were then filled (~100 L) with water from a catfish pond experiencing an outbreak of PGD. Water flow was stopped during exposure and resumed after 4-6 hours. The procedure was repeated for four consecutive days. Actinospore concentrations (exposure dose) in the inoculum were determined following the methods of Griffin et al. (2009) and averaged 106 spores/L (range 23-265).

Twelve fish from each exposed group and six fish per control group (two fish per tank) were sampled on Day 10 post-challenge to confirm successful exposures. Previous work by Rosser et al. (2019) reported limited plasmodia development in channel and hybrid catfish less than 8 weeks post-challenge and negligible sporogenesis in hybrid catfish up to 14 weeks post-challenge. Building upon these findings, fish in the experimental challenge were sampled at weeks 7, 10, 12, 14, 16, 18, and 20 post-exposure. Sampled fish were euthanized by an overdose of tricaine methanesulfonate (MS-222 at 300 mg/L; Syndel, Ferndale, WA) and whole gill arches were removed. Half of the excised gill arches were preserved in 10% neutral buffered formalin

and half in 70% ethanol. Formalin-fixed gills were processed routinely according to standard histologic methods, sectioned at 4 µm, stained with hematoxylin and eosin (H&E) (Spencer and Bancroft 2013), and examined by light microscopy for the presence of PGD lesions and myxozoan life stages. Differences between channel and hybrid catfish in the presence of myxozoan plasmodia at later stages (Week 7 through Week 20) were assessed by Pearson Chi-Square with Yates' continuity of correction in GraphPad Prism9 (GraphPad Software, San Diego, CA). A subset of gill tissues identified by histology to contain developing myxozoans were assessed by *in situ* hybridization (ISH) using probes targeting *H. ictaluri, H. exilis* and other ictalurid infecting myxozoan species suspected to be associated with PGD (Table 3.1). From each exposure group, tissues of four fish from the Day 10 sampling and two fish from each subsequent sampling were probed using *H. ictaluri* specific ISH. Subsequent serial sections were used to investigate the identities of *H. ictaluri* ISH-negative plasmodia in channel catfish gills using probes targeting *H. exilis*, *H. mississippiensis*, *H. postexilis,* the Raabeia type myxozoan as well as an additional taxon identified from metagenomic analysis of PGD cases (Myxo 12) (Chapter 2). As no mature plasmodia were observed by H&E in hybrid tissues, no further testing beyond *H. ictaluri* ISH was performed.

Fish collection and histopathology from natural PGD outbreaks

Throughout the spring of 2019, channel and hybrid catfish submitted to the Aquatic Research and Diagnostic Laboratory at the Thad Cochran National Warmwater Aquaculture Center for diagnostic assessment were screened for PGD. Presumptive diagnoses were made based on the presence of cartilage breaks visualized in gill clip wet mounts (Pote et al. 2003; Wise et al. 2008). Right and left gill arches from fish diagnosed with clinical PGD were fixed in 10% neutral buffered formalin and 70% ethanol, respectively. Ethanol-fixed gills were reserved

for metagenomic analysis (Chapter 2). Formalin-fixed gill arches were processed routinely for histopathology as described above, stained with H&E, and examined by light microscopy for the presence of myxozoans and PGD lesions. Three blocks each from seven cases (channel, n=6; hybrid, n=1) were used for light microscopic examination and further ISH analyses. Four serial tissue sections of each block were placed on charged slides and each subjected to one of four duplex ISH assays using 8 different myxozoan specific probes (Table 3.1). A PGD case characterized previously by metagenomic analysis exhibited high relative abundances of *H. adiposa* in hybrid catfish (Chapter 2). Three slides from this case were arbitrarily selected for *H. adiposa* singleplex ISH assay to investigate its role in disease pathology.

Results

Validation

Partial validation of these ISH methods were performed with histologic sections of channel catfish gills experimentally infected with *H. ictaluri*. Additional archived tissues involved natural infections of *H. adiposa* in the channel catfish adipose fin (Stilwell et al. 2019a), *H. postexilis* in channel catfish gills (Woodyard unpub data) and hybrid catfish gills naturally infected with *H. exilis* (Stilwell et al. 2019b). Probes designed for these myxozoans were specific to their respective targets and showed no cross reactivity between other myxozoan species or host tissues (Figure 3.1). Similarly, probes targeting *H. mississippiensis*, the Raabeia type TGR 2014 actinospore, and other unknown myxozoan taxa did not react with *H. ictaluri, H. adiposa, H. exilis, H. postexilis* life stages or host tissues.

Experimental challenge

Channel (9/12; 75%) and hybrid (8/12; 66.7%) catfish had PGD lesions with associated presporogonic stages at the Day 10 sampling. All multinucleated presporogonic stages associated with PGD lesions were identified as *H. ictaluri* by ISH including both channel (4 of 4 fish) and hybrid catfish (4 of 4 fish) (Figure 3.2). Further, numerous *H. ictaluri* mononuclear presporogonic stages were randomly disseminated throughout the gill tissue, often in close proximity to multinucleated presporogonic stages (Figure 3.2). At Day 10, presporogonic stages of *H. exilis* were also identified by ISH in 4/4 channel catfish but were absent in subsequent weeks.

Observations made from H&E stained tissue sections revealed PGD lesions in both channel and hybrid catfish groups had resolved by Week 7 post challenge, with channel and hybrid catfish exhibiting significant differences (X^2 [1, N= 167] = 20.7927, $p < 0.00001$) in myxozoan burdens from Week 7 through Week 20. In channel catfish, 33.7% of fish (28 of 83) exhibited the presence of myxozoan plasmodia after Day 10, with 3-5 fish in at each sampling interval containing low to moderate numbers of unidentified plasmodia in various stages of development and sites within gill tissue (Table 3.2). In contrast, myxozoan plasmodia were observed in 4.7% (4 of 84) of hybrid catfish from Week 7 through Week 20, with only 1-2, multinucleated, early sporogonic plasmodia observed in hybrid gill lamellae and no mature plasmodia identified (Table 3.2). Mononuclear presporogonic stages of *H. ictaluri* were rarely detected by ISH within the gill epithelium of channel catfish in weeks 7 and 12, while sporogonic plasmodia of *H. ictaluri* in various stages of maturity were identified in channel catfish gills in weeks 14, 16, 18, and 20 (Figure 3.2). In addition, developing to mature *H. postexilis* plasmodia were observed in channel catfish gills in weeks 7, 10, 12, 14 and 16 (Figure 3.3). No *H. ictaluri* life stages were detected by ISH in hybrid catfish after week 1 (Table 3.2).

As no mature plasmodia were identified in hybrid catfish by H&E, no further ISH testing was performed. No developing presporogonic stages or sporogonic plasmodia were identified in control channel or hybrid catfish.

Clinical PGD cases

Each case of PGD in channel catfish represented a mixed species infection containing 5 to 7 different myxozoan species per case (Table 3.3). *H. ictaluri* was detected most frequently and in all cases with presporogonic stages directly associated with typical PGD lesions similar to the experimentally challenged fish. Presporogonic stages and sporogonic plasmodia of *H. exilis*, *H. mississippiensis*, and *H. postexilis* were present, but rare, in more than half of the gill tissues examined. These taxa were found in association with inflammation, hemorrhage, and/or lamellar epithelial hyperplasia and resultant space occupying lesions (Table 3.3). Additionally, ISH revealed rare presporogonic stages, eliciting no host reaction, for 2 of the 3 undescribed myxozoan taxa tested (Myxo 11 & Myxo 12). Life stages of the third unknown myxozoan sequence probed by ISH (Myxo 7) were not identified in histologic sections.

Only three myxozoan species were identified in the hybrid catfish PGD case. These included presporogonic stages of *H. ictaluri* associated with characteristic PGD lesions, as well as rare presporogonic stages of *H. exilis* and the myxozoan associated with the Raabeia-type actinospore described by Rosser et al. (2014).

PGD-like lesions associated with other Henneguya spp.

Two clinical PGD cases were identified from a previous metagenomic study to harbor high relative abundances of *H. adiposa* and Raabeia type TGR 2014 (19-148). Both of these cases exhibited PGD-like cartilage and epithelial lesions with mixed inflammation. In the *H.*

adiposa case, numerous mononuclear presporogonic stages and rare, multinucleated presporogonic stages were located within and adjacent to cartilage supporting the tips of filaments in association with mild to moderate cartilage degeneration and lysis, mild inflammation, and edema but little to no cartilage proliferation (Figure 3.4). In the case carrying high abundance of the Raabeia type myxozoan (Raabeia type TGR 2014, 19-148), multinucleated, presporogonic stages and scattered mononuclear presporogonic stages were located among filament connective tissues adjacent to the cartilage or along basement membranes in the bottoms of lamellar troughs. These stages were associated with cartilage lysis and rare fractures, dysplastic proliferation of chondrocytes, severe mixed inflammation involving neutrophils, macrophages, and lymphocytes, and epithelial hyperplasia (Figure 3.5).

Discussion

The development and validation of *Henneguya* species specific ISH probes successfully identified myxozoans in known pure infections without cross reaction to other myxozoans or the catfish host. This was achieved using RNAscope® ISH technology and probe design targeting a diagnostic variable region (DVR3) of the 18S rRNA gene (Iwanowicz et al. 2008, Griffin et al. 2009a). Previous studies of myxozoan development in fish hosts using ISH techniques targeted single species or mixed infections by distantly related parasites (Morris et al. 2000, Holzer et al. 2003, Bjork and Bartholomew 2010, Holzer et al. 2010). The probes designed for this study allowed for the detection and discrimination of multiple, closely related species.

Previous studies by Rosser et al. (2019) showed similar findings with negligible *H. ictaluri* DNA after week 9 and rare developing plasmodia in hybrid catfish, suggesting arrested development or clearance of the parasite in hybrid catfish. Channel catfish showed progressively declining amounts of *H. ictaluri* DNA in weeks 8-12 consistent with limited ISH signal during

the same time period in this study (Rosser et al. 2019). This period could represent a transition between presporogonic proliferation and plasmodial development, though this is speculative. Plasmodia were seen as early as week 7 in this study in channel catfish; however, plasmodia were often identified as *H. postexilis* with the first detectable *H. ictaluri* plasmodia occurring in week 14. This is consistent with Rosser et al. (2019) which identified plasmodia in the gills at week 8, but with substantially decreased in *H. ictaluri* DNA until week 14, suggesting these early plasmodia were not *H. ictaluri*. These myxozoans shared similar sites of development within the gills, making differentiation by histologic examination extremely difficult without knowing their developmental timelines or the assistance of ISH techniques.

Results from the current study support the work of Rosser et al. (2019) and other works describing significant variation in fish host susceptibility and myxozoan development in channel and hybrid catfish (Griffin et al. 2010, Griffin et al. 2020b, Chapter 2). Various stages of *H. ictaluri* were confirmed by ISH in channel catfish from most samples for up to 20 weeks post-challenge, but were absent from all hybrid catfish sampled after Day 10. With the detection of other *Henneguya* spp. in channel catfish gills but not in hybrid catfish gills, these findings suggest differences in host susceptibility between channel and hybrid catfish may extend to other *Henneguya* spp. The studies performed by Rosser et al. 2019 were terminated at 14 weeks post-challenge. It was postulated the reason for negligible plasmodia present in hybrid catfish was due to arrested development of myxospores in hybrid catfish and the study may have been terminated before mature myxospores could be observed. In the present study, development was assessed out 20 weeks post-challenge, yielding similar results in regard to the absence of *H. ictaluri* after the acute stages of disease and negligible presence of non-*H. ictaluri* myxozoan plasmodia in hybrids. This is consistent with the decreased myxozoan diversity previously demonstrated in

hybrid compared to channel catfish ponds and supports observations that hybrid catfish monoculture may result in suppression of other myxozoan species in addition to *H. ictaluri* (Griffin et al. 2020b, Chapter 2, Stilwell unpublished data).

Previous metagenomic analyses of clinical PGD cases from catfish industry samples reveal that while *H. ictaluri* is always associated with outbreaks, numerous other known and unknown myxozoan sequences are also present, sometimes at greater relative abundances than those of *H. ictaluri* (Chapter 2). The ISH assays developed for this study revealed similar findings. In congruence with results from the experimental challenge, presporogonic stages *H. ictaluri* were identified in association with characteristic cartilage lysis and inflammation in all naturally occurring PGD samples from both channel and hybrid catfish. In channel catfish, naturally occurring PGD represented mixed species infections with 5 to 7 myxozoans identified by ISH in each case, including two currently undescribed myxozoan taxa identified by metagenomic analysis (Chapter 2). Additionally, multiple stages of *H. adiposa* and the myxozoan corresponding to the Raabeia type TGR 2014 actinospore rarely induced cartilage lysis and proliferation in the gill filaments of one catfish from two separate cases involving hybrid and channel catfish, respectively. Based on these findings, we hypothesize that *H. ictaluri* is the primary cause of PGD, but other myxozoans may contribute to disease pathology and respiratory compromise that exacerbate disease impacts by further altering normal gill morphology and inducing inflammation or simply by acting as space occupying lesions.

The detection of *H. adiposa* at comparatively high relative abundances in a few PGD cases was an unexpected finding from previous metagenomic analyses (Chapter 2). In this study, *H. adiposa* specific ISH confirmed these findings, demonstrating presporogonic stages clustering within the tips of gill filaments in association with cartilage lysis. While the contributions of *H.*

adiposa to gill lesions remains uncertain, the presence of presporogonic stages indicates the gill may serve as a portal of entry for infection and initial site for presporogonic replication, further illustrating the utility of ISH as a method to study the migration and development of myxozoans within tissues (Morris et al. 2000, Holzer et al. 2003, Stilwell et al. 2019b). Previous investigations of infection and portals of entry for myxozoans in catfish focused on *H. ictaluri* and used indirect fluorescent antibody testing to show the gills were the portal of entry for infection (Belem and Pote 2001). Like *H. adiposa*, other myxozoans that utilize the gills as portals of entry but complete development in distant tissue sites include *Ceratonova shasta*, *Sphaerospora truttae*, and *Tetracapsuloides bryosalmonae* (Morris et al. 2000, Holzer et al. 2003, Bjork and Bartholomew 2010).

Using species-specific ISH techniques, this work provides additional evidence supporting differential susceptibility to infection and development of *H. ictaluri* and other myxozoans exists between channel and hybrid catfish (Bosworth et al. 2003, Griffin et al. 2010, Rosser et al. 2019). This work also demonstrated that PGD cases often include a mixture of myxozoan species (Chapter 2). While *H. ictaluri* was always associated with typical PGD lesions in natural outbreaks, findings suggest additional *Henneguya* and other myxozoan species may contribute to pathologic changes and the overall severity of disease outbreaks. Elucidation of myxozoan life cycles involving commercially farmed catfish species and challenge experiments using pure actinospores of *H. ictaluri* and other myxozoans are needed to investigate their portals of entry, tissue migration, variability in catfish host susceptibilities, and, in the case of myxozoans aside from *H. ictaluri*, to determine whether presporogonic stages also cause PGD-like or other unique lesions.

No curative chemotherapies exist for PGD, leaving farmers with only supportive measures, such as increased aeration of ponds, or early marketing of fish to limit losses during outbreaks. Preventative management strategies include the use of physical and molecular screening of caged sentinel fish and pond water for evidence of PGD and *H. ictaluri* prior to stocking fish in ponds (Wise et al. 2004, Griffin et al. 2009b). Experimental challenges and ISH in this study confirm the arrested development of *H. ictaluri* in hybrid catfish, supporting metagenomic and other data showing potential mitigation of losses from PGD in hybrid catfish monoculture ponds (Rosser et al. 2019, Griffin et al. 2020b, Chapter 2). Despite these findings, PGD still occurs in hybrid catfish monoculture, raising questions as to how *H. ictaluri* persists within or is introduced to ponds. This work expands our understanding of the influence of host susceptibility on the disease process and may provide answers on how to control myxozoan populations and mitigate disease outbreaks using hybrid catfish (Griffin et al. 2010, Rosser et al. 2019, Griffin et al. 2020b, Chapter 2). These works suggest strategic crop rotations alternating between channel and hybrid catfish stocks could harness the influence of fish host susceptibility on myxozoan infections, precluding *H. ictaluri* and other myxozoans from reaching levels within ponds associated with catastrophic PGD outbreaks (Rosser et al. 2019, Griffin et al. 2020b). The feasibility of these measures require further investigation in the context of fish production and inventory management, but have the potential to significantly reduce the economic burden of PGD to the US catfish industry.

Figures

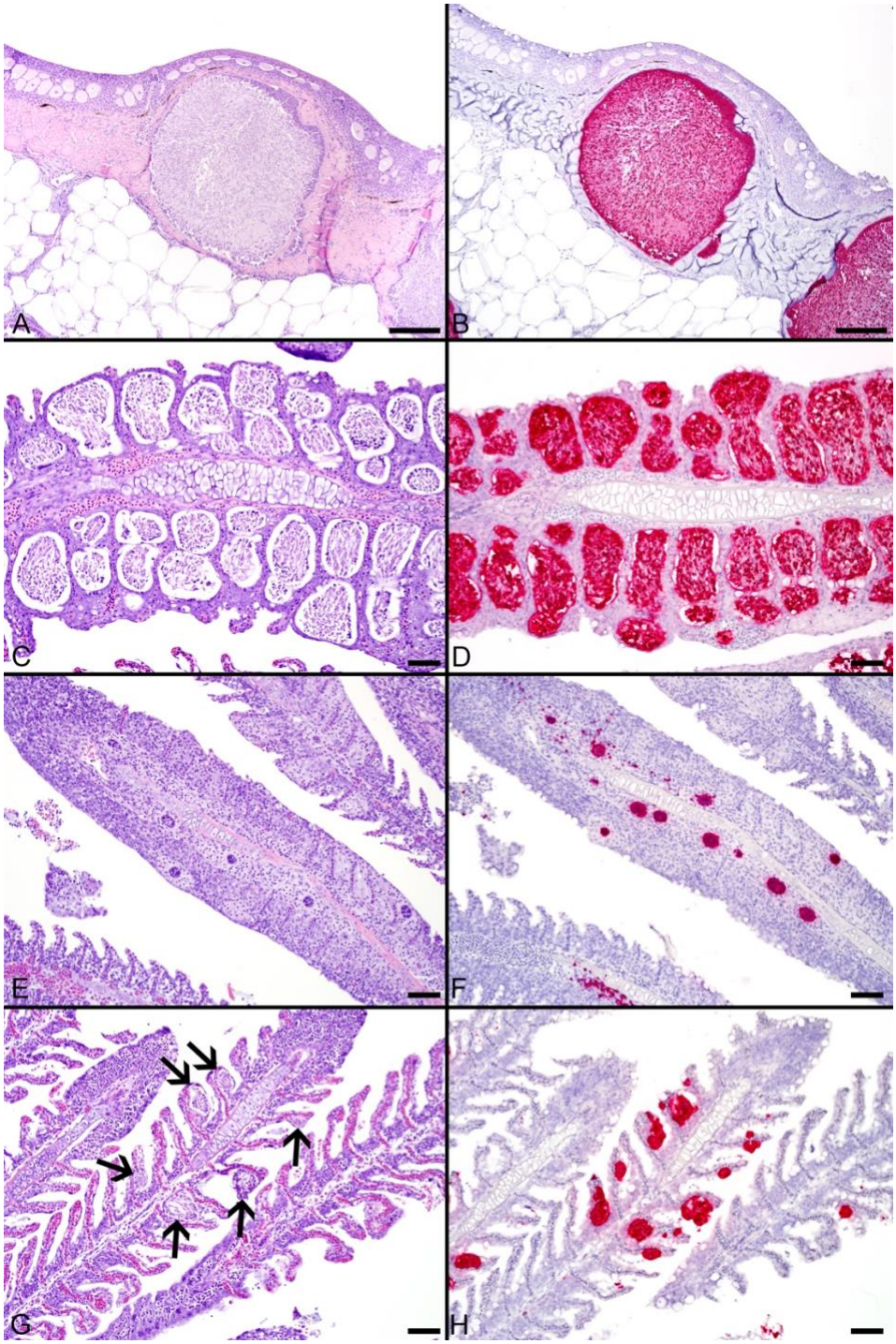

Figure 3.1 Partial validation of species specific RNAscope *in situ* hybridization (ISH) probes with corresponding H&E and ISH for known infections of *H. adiposa* (A&B; Bar = 100 µm), *H.*

exilis (C&D; Bar = 50 µm), *H. ictaluri* (E&F; Bar = 50 µm), and *H. postexilis* (G&H; Bar = 50 µm). All other known infection-probe combinations were ISH negative.

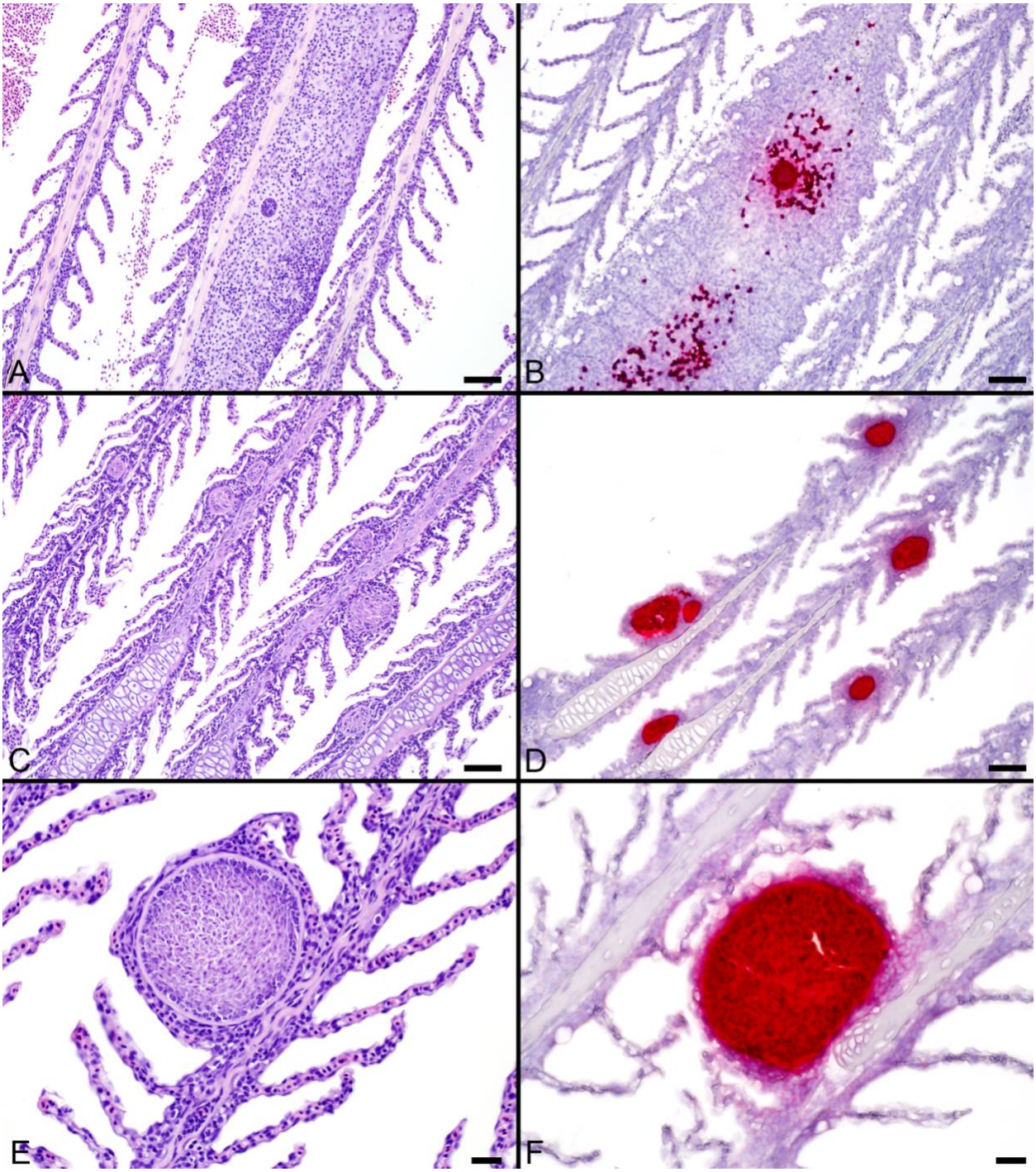

Figure 3.2 Gill lesions associated with *H. ictaluri* in channel and hybrid catfish after multiple exposures to water from a pond experiencing a PGD outbreak. A) Day 10 exposed hybrid catfish with a characteristic PGD multinucleated, presporogonic stage and associated inflammation (H&E, Bar = 50 µm). B) Corresponding slide to panel A highlighting presporogonic stages of *H.*

ictaluri not detected by H&E (*H. ictaluri* ISH, Bar = 50 µm). C) Week 14 exposed channel catfish with interlamellar plasmodia in various stages of development, several of which contain few myxospores (H&E, Bar = 50 µm). D) Corresponding slide to panel C highlighting the developing *H. ictaluri* plasmodia (*H. ictaluri* ISH, Bar = 50 µm). E) Week 20 exposed channel catfish still contain plasmodia within interlamellar spaces with more numerous myxospores (H&E, Bar = 20 µm). Corresponding slide to panel E highlighting *H. ictaluri* plasmodia (*H. ictaluri* ISH, Bar = 20 µm).

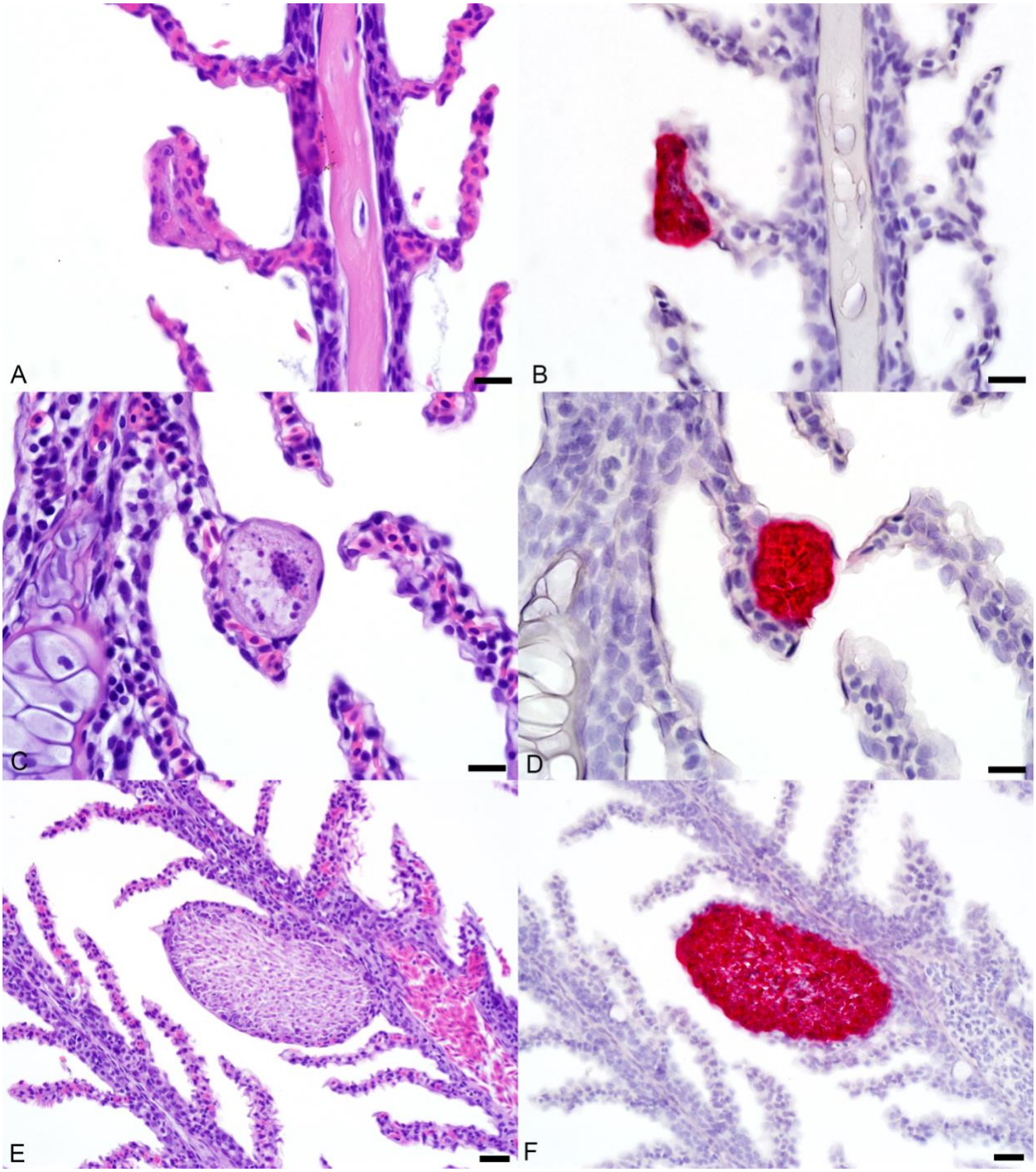

Figure 3.3 *H. postexilis* plasmodia development in the gills of channel catfish after multiple exposures to water from a pond experiencing a PGD outbreak. A) Week 7 exposed channel catfish with a developing, multinucleated plasmodia at the tip of the lamellae within the epithelium (H&E, Bar = 10 µm). B) Corresponding slide to panel A highlighting early-stage

plasmodia (*H. postexilis* ISH, Bar = 10 µm). C) Week 10 exposed channel catfish with enlarged plasmodia containing pansporoblasts and few mature myxospores (H&E, Bar = 10 µm). D) Corresponding slide to panel C highlighting developing *H. postexilis* plasmodia (*H. postexilis* ISH, Bar = 10 µm). E) Week 14 exposed channel catfish with mature plasmodia containing numerous myxospores, which filled the interlamellar space (H&E, Bar = 20 µm). F) Corresponding slide to panel E highlighting *H. postexilis* plasmodia and myxospores (*H. postexilis* ISH, Bar = 20 µm).

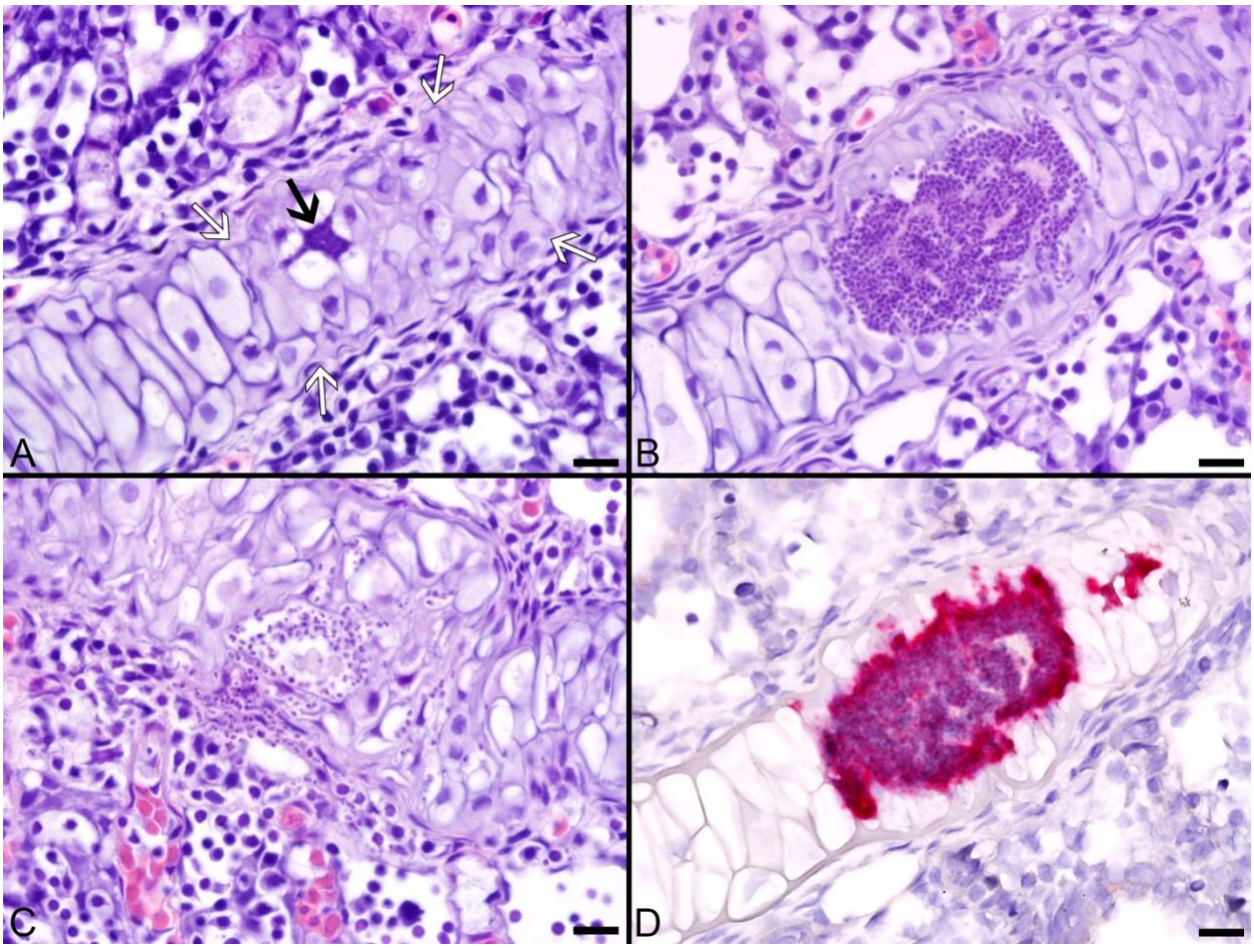

Figure 3.4 PGD-like lesions associated with *H. adiposa* in the gills of hybrid catfish. A) A multinucleated, presporogonic stage within the filament cartilage (solid arrow) is surrounded by degenerate chondrocytes and matrix (open arrows) (H&E, Bar = 10 µm). B) Numerous presporogonic stages expand the cartilage core (H&E, Bar = 10 µm). C) Cartilage lysis leads to release of presporogonic stages into adjacent connective tissues (H&E, Bar = 10 µm). D) Corresponding slide to panel B highlighting *H. adiposa* presporogonic stages in red within the cartilage (*H. adiposa* ISH, Bar = 10 µm).

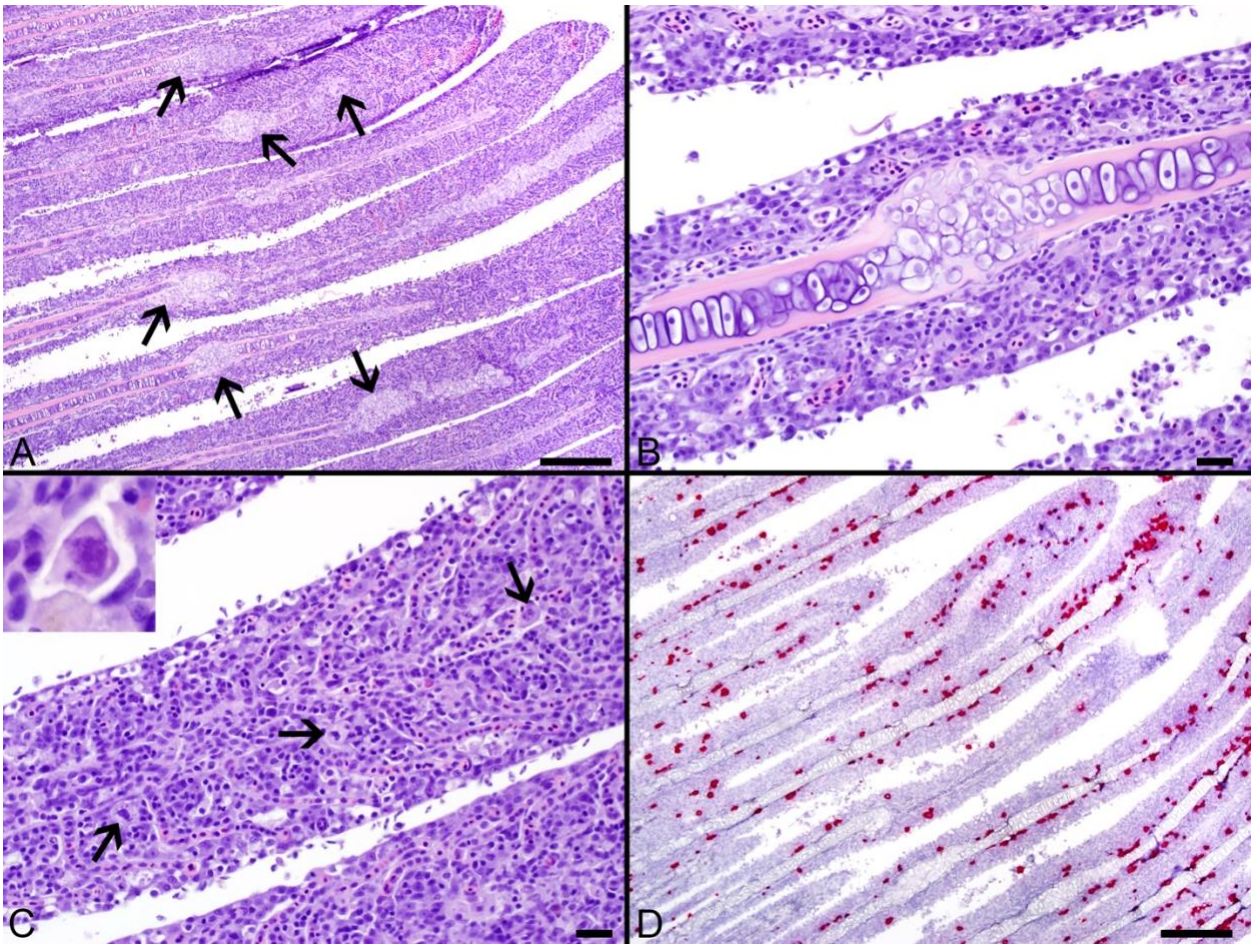

Figure 3.5 PGD-like lesions associated with myxozoan life stages of Raabeia type TGR 2014 actinospore in the gills of channel catfish. A) Diffuse epithelial hyperplasia fills interlamellar spaces along with inflammation and multifocal regions of dysplastic chondrocyte proliferation or fracture (arrows) (H&E, Bar = 100 µm). B) Uniform epithelial hyperplasia and infiltrating macrophages and lymphocytes with a focal region of dysplastic chondrocyte proliferation and no lysis or fracture. Note large numbers of *Ichthyobodo* sp. flagellates along the epithelial surface (H&E, Bar = 20 µm). C) Multinucleated, presporogonic stages of a myxozoan (arrows and inset) located within the filament connective tissue and along basement membrane in interlamellar troughs. Note large numbers of *Ichthyobodo* sp. flagellates along the epithelial surface (H&E, Bar = 20 µm; Bar does not apply to inset). D) Numerous, presporogonic stages identified

throughout the gills with sequence specific for the Raabeia type TGR 2014 actinospore, which are often associated with PGD-like lesions (Raabeia ISH, Bar = 100 µm).

Tables

Table 3.1 Probe name, myxozoan species, catalog number, targeted region of the 18S rRNA gene, and reference sequence accession number for *Henneguya* sp. specific RNAscope *in situ* hybridization probes

Myxozoan	Probe name	Catalog number	Targeted region of 18S rRNA	NCBI Reference Sequence
Henneguya adiposa	H.adiposa-18S	566921	1543-1581	EU492929.1
H. exilis	H.exilis-18S	566941	1540-1581	AF021881.1
H. ictaluri	H.ictaluri-18S	566951	1494-1543	AF195510.1
H. mississippiensis	H.miss-18S	566961	1574-1609	KP404438.1
H. postexilis	Myxo-Taxa1-18S	861561	N/A*	N/A*
Raabeia type TGR 2014	Raaebia.sp-18S	566971	1533-1571	KF263539.1
Myxo 7	Myxo-Taxa7-18S	861571	N/A*	N/A*
Myxo 11	Myxo-Taxa11-18S	861581	N/A*	N/A*
Myxo 12	Myxo-Taxa12-18S	861591	N/A*	N/A*

*Full length 18S rRNA sequences were not available (N/A) at the time of probe design for these myxozoans, but were designed against the same hypervariable region as the others using sequence derived from previous metagenomic analysis (Chapter 2).

Table 3.2 Presence of developing myxozoan life stages in the gills of catfish challenged with water from a PGD outbreak and positivity for *H. ictaluri* with *in situ* hybridization

	Channel Catfish							Hybrid Catfish	
Sampling Week	H&E	*H. ictaluri* ISH	*H. exilis* ISH	*H. mississippiensis* ISH	*H. postexilis* ISH	Raabeia type TGR 2014 ISH	Myxo 12 ISH	H&E	*H. ictaluri* ISH
Week 1	9/12	4/4	4/4	0/4	0/4	0/4	0/4	8/12	4/4
Week 7	4/12	2/2	0/2	0/2	2/2	0/2	0/2	0/12	0/2
Week 10	3/12	0/2	0/2	0/2	2/2	0/2	0/2	2/12	0/2
Week 12	4/12	1/2	0/2	0/2	2/2	0/2	0/2	0/12	0/2
Week 14	4/12	1/2	0/2	0/2	1/2	0/2	0/2	0/12	0/2
Week 16	5/12	1/2	0/2	0/2	2/2	0/2	0/2	1/12	0/2
Week 18	4/12	2/2	0/2	0/2	0/2	0/2	0/2	1/12	0/2
Week 20	4/11	2/2	0/2	0/2	0/2	0/2	0/2	0/12	0/2
Total	37/84	13/18	4/18	0/18	9/18	0/18	0/18	12/84	4/18

Table 3.3 Detection of myxozoan life stages in the gills of naturally infected channel and hybrid catfish by *in situ* hybridization

Case Number	Catfish Species	*H. ictaluri*	*H. exilis*	*H. mississippiensis*	*H. postexilis*	Raabeia type	Myxo 7	Myxo 11	Myxo 12	Number of species per case
19-58	Channel	3/3	3/3	3/3	3/3	3/3	0/3	3/3	3/3	7/8
19-75	Channel	3/3	2/3	2/3	3/3	0/3	0/3	2/3	1/3	6/8
19-105	Channel	3/3	2/3	3/3	2/3	2/3	0/3	0/3	0/3	5/8
19-148	Channel	3/3	3/3	0/3	1/3	3/3	0/3	2/3	0/3	5/8
19-155	Channel	3/3	0/3	1/3	1/3	3/3	0/3	2/3	0/3	5/8
19-213	Channel	2/3	3/3	0/3	3/3	2/3	0/3	2/3	0/3	5/8
19-218	Hybrid	2/3	3/3	0/3	0/3	1/3	0/3	0/3	0/3	3/8
Total		19/21	16/21	9/21	13/21	14/21	0/21	11/21	4/21	

CHAPTER 4

MOLECULAR CONFIRMATION OF HENNEGUYA ADIPOSA (CNIDARIA: MYXOZOA) AND ASSOCIATED HISTOLOGIC CHANGES IN ADIPOSE FINS OF CHANNEL CATFISH, ICTALURUS PUNCTATUS (TELEOST) [1]

[1] Stilwell, J.M., A.C. Camus, J.H. Leary, H.H. Mohammed, & M.J. Griffin. 2019. *Parasitology Research*. 118(5):1639-1645.

Abstract

Henneguya adiposa is one of ten known, closely related myxozoan species that parasitize a variety of tissue sites in the channel catfish, *Ictalurus punctatus*. Reported to specifically target the adipose fin, *H. adiposa* is not associated with morbidity or mortality, although detailed descriptions of its associated histologic pathology are lacking. The objective of this work was to confirm the presence of *H. adiposa* within fin lesions of affected channel catfish using DNA sequenced from histologic sections obtained by laser capture microdissection, as well as to describe pathologic changes induced by infection. The parasite formed large, white, elongate, nodular plasmodia that caused localized tissue damage and incited a granulomatous inflammatory response within a deep connective tissue layer at the base of the adipose fin. Myxospores released from ruptured plasmodia into adjacent tissue were observed to migrate superficially in tracts through the skin, indicating a portal of exit for environmental dispersal. Defects in the connective tissue layer created by ruptured plasmodia were infiltrated by granulomatous inflammation and fibroplasia, suggesting lesion resolution by scar formation over time. Sequencing of the 18S rRNA gene amplified from excised myxospores confirmed the myxozoan's identity as *H. adiposa*, with 100% similarity to the reference sequence from previous published work.

Introduction

Myxozoans comprise a group of over 2000 endoparasitic metazoans belonging to the phylum Cnidaria. Degraded by the transition to a parasitic life cycle, these morphologically and functionally simplified organisms share two-host life cycles that most commonly utilize fish as intermediate and oligochaete worms as definitive hosts. Representatives infect virtually all classes of fish, in all aquatic environments, with remarkably variable tissue tropism. While most

infections are innocuous, some cause severe disease and significant economic losses in cultured and wild fish. Following infection, pre-sporogonic stages may proliferate as single cells before reaching final sites of sporogonic development, which occurs in either multinucleated plasmodia or unicellular pseudoplasmodia. Sporogony results in the production of a morphologically diverse array of myxospores unified by the presence of one or more polar capsules containing extrudable filaments. However, due to convergence in myxospore morphologies, features such as host preference, tissue specificity, developmental features, and increasingly, molecular sequence data are required for proper identification (Okamura et al. 2015).

Over 200 *Henneguya* species have been characterized to varying extents in freshwater and marine fishes (Eiras 2002, Eiras and Adriano 2012). Ten of these have been described in the channel catfish, *Ictalurus punctatus,* from commercial aquaculture operations in the southeastern USA, where static earthen ponds and the mixing of fish age classes provides an ideal environment for the perpetuation of myxozoan life cycles. The different *Henneguya* spp. infect a variety of tissues including the gills, skin, gall bladder, and adipose fin (Rosser et al. 2016b). Elongate myxospores with two shell valves, each possessing a caudal projection, and two apical polar capsules develop within histozoic polysporic plasmodia that are usually large and cyst-like (Eiras 2002, Lom and Dyková 2006, Fiala et al. 2015). Most significant among these is *Henneguya ictaluri,* the cause of proliferative gill disease (PGD) in catfish, which results in severe respiratory distress and significant economic losses in the form of lost feed days and direct mortality (Pote et al. 2000). Other species, including *Henneguya adiposa*, produce disfiguring lesions, but are thought to have little to no adverse effects on fish health (Griffin et al. 2009c, Rosser et al. 2016). While initial descriptions were based entirely on myxospore morphology, partial 18S small subunit rRNA gene sequence data are now available for most

documented species affecting channel catfish (Lin et al. 1999, Pote et al. 2000; Griffin et al. 2008a, 2009a, Rosser et al. 2014b, 2015). However, *in situ* studies linking sequence data from myxospores to specific tissue sites is not well documented and remains a matter of conjecture.

First described by Minchew (1977), *H. adiposa* infects the adipose fin of the channel catfish. The ultrastructure of its large plasmodia and asynchronous sporogenesis was reported shortly thereafter by Current (1979). Griffin et al. (2009c) further refined descriptions of myxospore morphology and reported its 18S small subunit rRNA gene sequence, which revealed a monophyletic clade for the *Henneguya* spp. infecting North American ictalurids. Despite investigations into actinospore diversity in catfish ponds, the definitive host and actinospore stages of *H. adiposa* have yet to be identified (Hanson et al. 2001, Rosser et al. 2014a). To supplement the existing literature concerning the morphology and molecular characteristics of *H. adiposa*, this report details histologic features of myxospores in situ and descriptions of pathologic changes induced in channel catfish. In addition, presence of the parasite within lesions was confirmed using laser capture microdissection, coupled with myxozoan-specific PCR and sequencing.

Materials & Methods

Channel catfish fingerlings reared in 264-L flow-through tanks supplied by pond water spontaneously developed lesions consistent with *H. adiposa* infection (Minchew 1977, Current 1979, Griffin et al. 2009c). Twenty-five adipose fins with gross lesions suggestive of *H. adiposa* infection were sampled from fingerlings and fixed in either 10% neutral buffered formalin (n = 10), 70% ethanol (n = 10), or 95% ethanol (n = 5). Prior to histologic processing, fins were sectioned transversely and placed into tissue cassettes. Formalin-fixed samples were processed routinely in a Sakura Tissue-Tek VIP 5 tissue processor (Sakura Finetek USA Inc., Torrance,

CA). Ethanol-fixed samples were prepared by programming the processor to avoid any contact with formalin, which might interfere with molecular analysis as a result of DNA crosslinking. Processed tissues were then embedded in paraffin wax, sectioned at 4 μm thick, and stained with hematoxylin and eosin (H&E), Giemsa, and a modified Brown-Hopps Gram stain for light microscopic observation.

Individual plasmodia were excised from ethanol-fixed, H&E stained sections using laser capture microdissection (LCM). Histologic sections were prepared on MMI nuclease-free membrane slides and the plasmodia collected using an MMI CellCut Plus® microdissection system (Molecular Machines & Industries, Eching, Munich, Germany). DNA was extracted from excised plasmodia using a Qiagen QIAamp DNA Mini kit according to the manufacturer's protocols (Qiagen, Hilden, Germany). Conventional PCR targeting the 18S small subunit rRNA gene was performed using previously published primers and methods to confirm the presence of *H. adiposa* in adipose fin sections (Griffin et al. 2008a, 2009a). The PCR products were purified by gel extraction (QIAquick Gel Purification Kit, Qiagen), sequenced commercially (Genewiz, South Plainfield, NJ), assembled and edited using Geneious® 10.1.3 (Kearse et al. 2012), and used in a Blastn search for somewhat similar sequences in the National Center for Biotechnology Information non-redundant nucleotide (nr/nt) database for myxozoan identification. Sequence data was deposited in GenBank (MK253077).

Results

Consistent with histologic descriptions by Grizzle and Rogers (1976), the base of the adipose fin is composed of a typical stratified epidermis and circumferential superficial dermis of irregular collagen bundles. Oriented perpendicular to the base is a deeper connective tissue layer

formed by distinct parallel bundles of dense regular collagen separated by areolar fibers and fibroblasts. The core of the fin is comprised of a mixture of adipose and elastic connective tissue.

Grossly, multifocal, spindyloid to nodular, white, plasmodia were most numerous at the fin base (Fig. 4.1). Microscopically, expansile plasmodia up to approximately 500 × 1000 μm were localized to the deep connective tissue layer where they displaced and effaced collagen bundles (Fig. 4.2a). Plasmodia were limited by 4–6 μm thick, pale eosinophilic walls with striations compatible with pinocytotic channels (Lom and Dyková 1992), and often further enveloped by thin, 2–3 cell thick, fibrous capsules (Fig. 4.2b, c). Myxospores demonstrated asynchronous development with immature, pansporoblastic stages located peripherally within plasmodia and mature myxospores located centrally (Fig. 4.2b, c). Intact plasmodia elicited minimal to mild granulomatous inflammation within the adjacent collagenous tissue that occasionally extended into the central adipose core of the fin (not shown). Ruptured plasmodia released myxospores into adjacent tissue where myxospore-filled tracts sometimes extended through the overlying dermis and epithelium (Fig. 4.2d). More intense granulomatous infiltrates, dominated by macrophages and lymphocytes, with infrequent multinucleated giant cells (not shown), surrounded ruptured plasmodia and free myxospores (Fig. 4.2e). In more advanced lesions, ruptured plasmodia and lytic collagen fibers were replaced by extensive granulomatous infiltrates and fibroplasia that effaced the normal tissue architecture (Fig. 4.2f). Myxospores were characteristic of the genus *Henneguya*, with two shell valves, two polar capsules containing coiled polar filaments, two long superimposed caudal processes, a central binucleated sporoplasm, and a clear posterior vacuole (Fig. 4.3a, b). Polar capsules stained magenta and deep purple with Giemsa and Gram stains, respectively (not shown). A 2058-bp segment of the 18S

small subunit rRNA gene was 100% similar to the 2022 bp reference sequence for *H. adiposa* (EU492929) at the nucleotide level, confirming the presence of *H. adiposa* within lesions.

Discussion

Relatively few myxozoan parasites, including the many *Henneguya* species, cause significant disease in fish. While previous work suggested *H. adiposa* did not have adverse health effects on catfish, detailed histopathologic evaluations were not performed (Griffin et al. 2009c). Among the fish examined in this study, lesions were often severe, but localized to the base of the adipose fin where plasmodia disrupted the normal tissue architecture, destroyed dermal and deep collagenous layers, and incited a granulomatous inflammatory response. While the granulomatous response induced by *H. adiposa* is similar to that described with other myxozoan diseases in fish (Sitjà-Bobadilla et al. 2015), this appeared to occur late in the course of infection when numerous large plasmodia were present and particularly when plasmodia were ruptured. In catfish suffering from *H. ictaluri* induced PGD, cartilage damage has been observed before a host inflammatory response develops. It has been hypothesized that collagen damage results initially from proteases released by trophozoite stages of the parasite (Lovy et al. 2011). In turn, collagen fragments incite a pronounced inflammatory response that further contributes to collagenolysis during later stages of disease as potentially seen here (Castillo-Briceno et al. 2009, Griffin et al. 2010, Lovy et al. 2011). The extensive presence of fibroplasia in some lesions suggests resolution may ultimately occur via fibrous scarring.

Mature myxospores of histozoic myxozoans may be either released into host tissue, discharged outside the host, encapsulated, or destroyed by the host's inflammatory response (Lom and Dyková 1992). Host death and decomposition are commonly required for the release of myxospores produced internally by many histozoic myxozoans, such as *Myxobolus cerebralis*

(Eszterbauer et al. 2015). Antemortem release of myxospores from ruptured plasmodia located immediately adjacent to epithelial surfaces of the gills and skin, including *Henneguya* and *Myxobolus* spp., is frequently assumed, although histopathologic documentation is limited (Walsh et al. 2012, Rosser et al. 2019). An unusual observation in these catfish involved the release of myxospores into tissue following plasmodial rupture, accompanied by the formation of subcutaneous tracts and migration of myxospores through multiple layers of collagen and epithelium to reach the skin surface. Findings suggest death of the host is not required for environmental dissemination of myxospores and perpetuation of the parasite's life cycle. Mechanisms involved in the release of *H. adiposa* are unknown but could include direct tissue compression and atrophy induced by enlarging plasmodia, damage induced by products of the cellular inflammatory response, or the secretion of an unknown protease by myxospores (Lom and Dyková 1992; Lovy et al. 2011; Sitjà-Bobadilla et al. 2015). Although the inflammatory response was intense, plasmodia were confined to deep connective tissue layers and did not result in atrophy of the overlying dermis or epithelium in the 25 fins examined. Although not observed microscopically, skin perforation associated with myxospore release could provide a portal of entry for opportunistic bacterial infection.

Most myxozoans infecting fish exhibit a high degree of host, tissue, and organ specificity. Tissue selection is a useful taxonomic indicator for histozoic myxozoans and occurs independently of its migration route to final sites of myxospore production (Molnár and Eszterbauer 2015). This is reflected in the remarkably specific localization of *H. adiposa* plasmodia to a distinct deep layer of connective tissue unique to the base of the adipose fin. Although unknown at present, it is presumed that *H. adiposa* utilizes similar portals of entry and migrates in a manner similar to that of the closely related *H. ictaluri*. Previous work

demonstrated pre-sporogonic stages of *H. ictaluri* in the skin, buccal cavity, gill, and gastric wall within 24 h of experimental challenge, with stages also detected later in the heart and vasculature of the liver, suggesting a hematogenous component to its dissemination (Belem and Pote 2001). With the exception of the gill, the site of *H. ictaluri* maturation, the organism could not be detected after 96 h (Belem and Pote 2001). Most myxozoans start development in blood vessels before reaching their final site of sporulation, although the mechanisms behind tissue homing and site specificity have not been determined for myxozoans (Molnár and Eszterbauer 2015, Okamura et al. 2015, Rosser et al. 2016c). Similar work has shown that pre-sporogonic stages of *Ceratonova shasta* and *Sphaerospora truttae* enter via the gills and migrate hematogenously before reaching the intestines and kidney, respectively (Holzer et al. 2003, Bjork and Bartholomew 2010).

Light microscopic features of *H. adiposa* myxospores were characterized originally by Minchew (1977) and supplemented with ultrastructural descriptions by Current (1979). More recent work refined their morphologic characterization, determined the 18S rRNA gene sequence, and investigated the phylogeny of *H. adiposa*, determining it to be most closely related to *H. ictaluri*, the cause of PGD (Pote et al. 2000, Griffin et al. 2009c). For unknown reasons, these morphologically and genetically similar myxozoans produce dissimilar changes in the channel catfish, with vastly different tissue tropism. Infection and maturation of *H. adiposa* results in subclinical cutaneous lesions in the adipose fin, while *H. ictaluri* is known to cause severe gill damage during entry and is the most significant parasitic disease affecting channel catfish aquaculture (Wise et al. 2008). Comparatively, tissue damage and immune response to encysted mature *H. ictaluri* myxospores in the gills are negligible (Pote et al. 2012). While descriptions of *H. adiposa* infection have been previously limited (Minchew 1977, Griffin et al.

2009c), this report confirmed *H. adiposa* within intact plasmodia by excision of myxospores using LCM and sequencing of the 18S rRNA gene, as well as details histopathologic changes associated with the presence of mature plasmodia. Unfortunately, early stages of infection and processes leading to the final maturation site were not observed and are unlikely to be revealed until the actinospore stage is identified and controlled developmental studies can be performed.

Figures

Figure 4.1 Gross image of nodular, white *Henneguya adiposa* plasmodia up to approximately 1 mm in length at the base of the adipose fin of a fingerling channel catfish

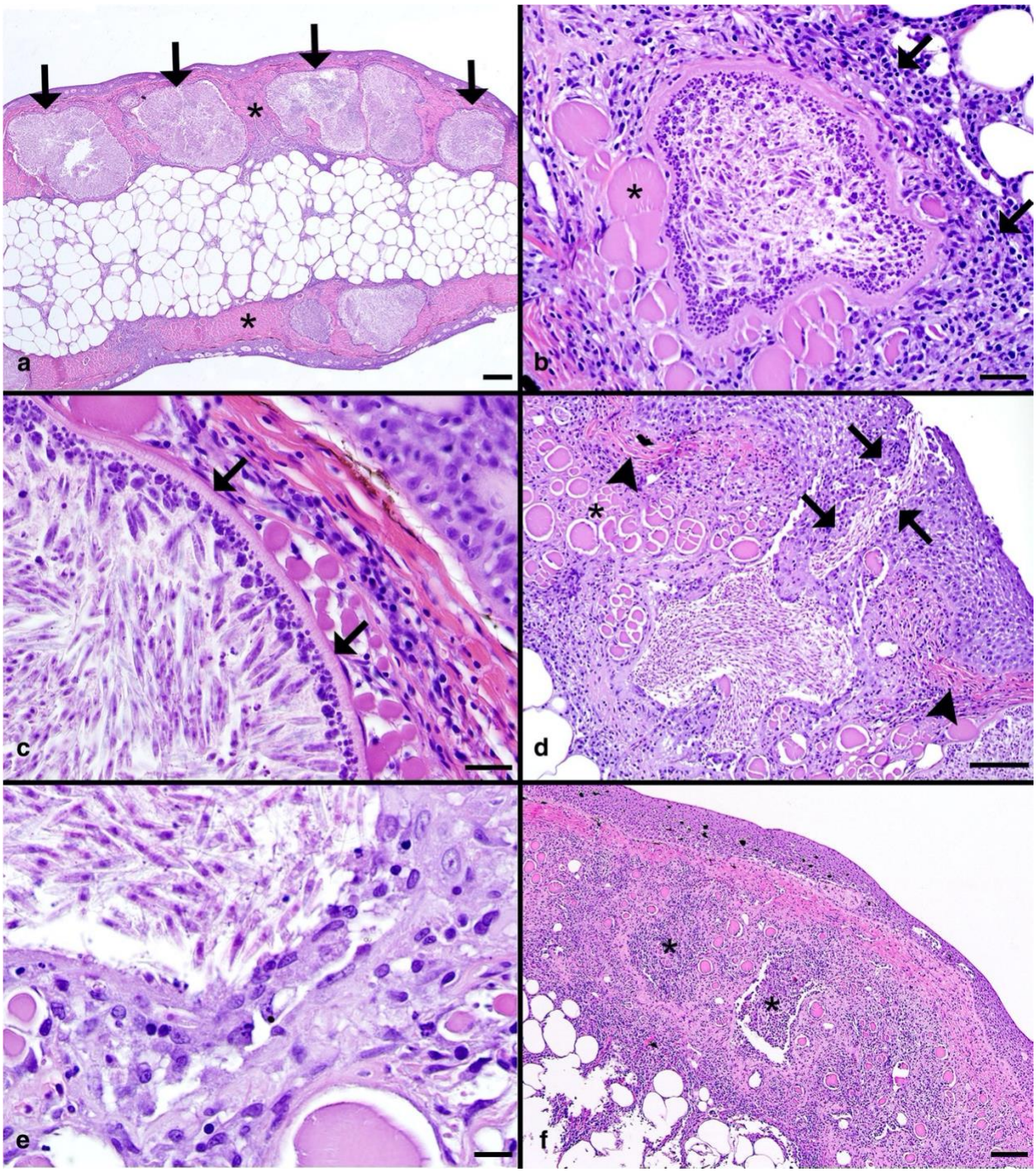

Figure 4.2 Histological lesions in the adipose fin of a fingerling channel catfish infected with *Henneguya adiposa*. **a** Plasmodia (arrows) multifocally expand and efface the deep dermal collagenous layer (asterisks) (H&E, bar = 200 μm). **b** Mild inflammation (arrows) disrupts the deep collagenous layer (asterisk) and surrounds an intact plasmodium (H&E, bar = 20 μm). **c**

Plasmodia consist of an eosinophilic wall (arrows) with peripheral immature and centrally located mature myxospores (H&E, bar = 20 μm). **d** A ruptured, collapsed plasmodium incites a granulomatous inflammatory response, with free myxospores forming a tract (arrows) through the dermis and epidermis. Note the severe disruption of the dermis (arrowhead) and deep collagenous layer (asterisk) by the intense inflammation (H&E, bar = 50 μm). **e** Free myxospores are surrounded by chronic inflammation (H&E, bar = 10 μm). **f** Ruptured plasmodia in the deep collagenous layer are replaced by granulomatous inflammation (asterisks) and fibroplasia in more advanced lesions that isolate the few remaining collagen fibers (H&E, bar = 100 μm)

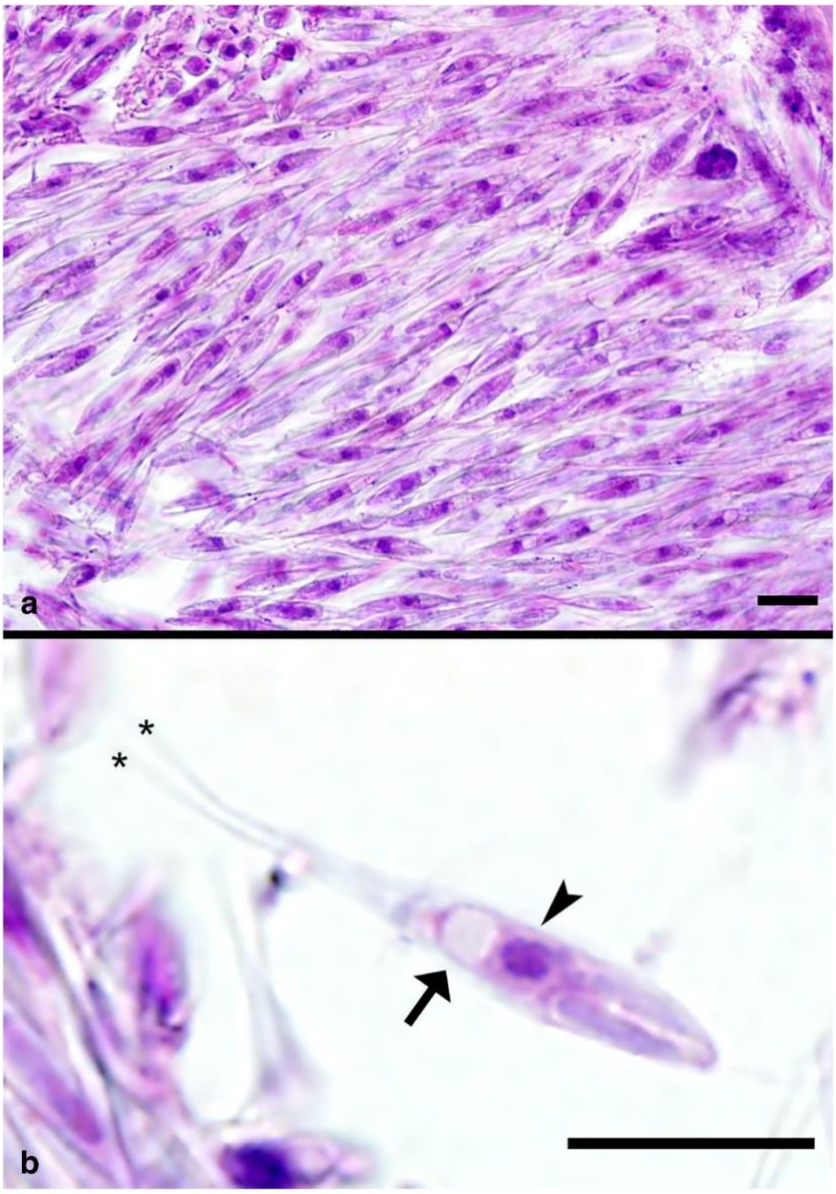

Figure 4.3 High-magnification images of *Henneguya adiposa* myxospores and their morphologic features. **a** Densely packed mature myxospores fill a plasmodium (H&E, bar = 10 μm). **b** Myxospores possess typical features of a *Henneguya* sp., including paired elongate polar capsules, a basophilic binucleated sporoplasm (arrowhead), clear posterior vacuole (arrow), and two shell valves with two long, tapering caudal processes (asterisks) (H&E, bar = 10 μm)

CHAPTER 5

PATHOLOGIC CHANGES ASSOCIATED WITH RESPIRATORY COMPROMISE AND MORBIDITY DUE TO MASSIVE INTERLAMELLAR HENNEGUYA EXILIS INFECTION IN CHANNEL × BLUE CATFISH [1]

[1] Stilwell, J.M., A.C. Camus, J.H. Leary, L.H. Khoo, & M.J. Griffin. 2019. *Journal of Parasitology*. 105(5):686-692.

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Abstract

There are multiple *Henneguya* spp. (Myxozoa: Myxobolidae) endemic to North American catfish aquaculture that affect the gills of channel catfish and their hybrids. These parasites are morphologically similar, and confusion exists regarding the predilection sites and pathologic changes associated with different species. In the spring of 2018, channel (*Ictalurus punctatus*) female × blue (*Ictalurus furcatus*) male hybrid catfish from 2 separate commercial operations in northwest Mississippi were submitted for diagnostic assessment in response to observed morbidity and reduced feeding activity. Fish presented with unusually heavy infections of *Henneguya* spp. plasmodia in the gills. The majority of gill filaments contained widespread, pinpoint, raised, white nodules corresponding microscopically to myxospore-filled plasmodia that obliterated interlamellar spaces. The bipolar myxospores were consistent with *Henneguya* spp. described from North American ictalurids, possessing slender fusiform spore bodies and elongate bifurcate caudal processes. Associated microscopic lesions included lamellar fusion, epithelial hyperplasia, infrequent, localized, granulomatous branchitis, and rare cartilage lysis, suggesting impaired gill function. Mature plasmodia were excised by laser capture microdissection from ethanol-fixed, hematoxylin and eosin–stained histologic sections for molecular analysis. Fragments (700 bp) of a highly variable region of the 18S rRNA gene, diagnostic for the Myxobolidae, were 100% similar at the nucleotide level to *Henneguya exilis*. Although mortality was negligible, fish in the affected ponds exhibited signs of respiratory distress similar to proliferative gill disease (PGD) caused by *Henneguya ictaluri* in channel and hybrid catfish. However, gross and microscopic lesions differed markedly from PGD, known colloquially as ‘‘hamburger gill disease.’’ While *H. exilis* has been reported from channel catfish, it is not typically associated with morbidity and mortality and has not previously been

reported from channel × blue catfish hybrids. This work characterizes lesions and confirms the etiology of gill disease induced by the myxozoan *H. exilis*. In addition to PGD and other non-parasitic conditions, massive interlamellar *H. exilis* infection should be a differential consideration in pond-raised channel and hybrid catfish experiencing signs of respiratory distress.

Introduction

Channel catfish (*Ictalurus punctatus*) × blue catfish (*Ictalurus furcatus*) hybrids have increased in popularity in commercial catfish aquaculture in the southeastern United States, in part due to increased resistance to common channel catfish diseases and subsequently increased growth performance (Arias et al. 2012, Torrans and Ott 2018). Differential susceptibilities exist between the parent species and their hybrid offspring against certain pathogens, including the myxozoan *Henneguya ictaluri*, the causative agent of the economically devastating proliferative gill disease (PGD) (Pote et al. 2012, Rosser et al. 2019). *Henneguya ictaluri* and *Henneguya exilis* are 2 of 10 closely related species of *Henneguya* reported from channel catfish and other North American ictalurids (Minchew 1977, Lin et al. 1999, Pote et al. 2000, 2012, Griffin et al. 2008a, Iwanowicz et al. 2008, Rosser et al. 2014b, 2015). Moreover, the actinospores of several other putative *Henneguya* spp. have been reported from catfish aquaculture ponds in the southeastern United States that have yet to be linked to a fish host (Rosser et al. 2014a). These actinospores may infect catfish or other fish species that incidentally occur in the ponds, such as the western mosquitofish, *Gambusia affinis* (Parker et al. 1971). Myxospore development of most of these *Henneguya* spp., including *H. ictaluri*, is limited to the gills, while *H. exilis* has been reported from the gills and epidermis (Kudo 1929, Rosser et al. 2016c).

Species identification of myxozoans based on myxospore morphology or histopathology alone should be approached with caution, particularly when multiple related species can occur in the same host or tissue (Eszterbauer 2002, Rosser et al., 2016c). For example, lesions of PGD were once attributed to *H. exilis* before the description of *H. ictaluri* (see McCraren et al. 1975, Duhamel et al. 1986). As a result, molecular confirmation is increasingly used for precise species identification and is important for accurate diagnosis as disease agents emerge in farmed hybrid species, such as channel × blue catfish crosses (Rosser et al. 2016c).

To date, the channel catfish has been identified as an intermediate host in the life cycles of 3 molecularly distinct, yet morphologically similar gill-infecting *Henneguya* species. Of these, *H. ictaluri*, *H. mississippiensis*, and *H. exilis* all utilize the benthic oligochaete *Dero digitata* as a definitive host, from which actinospores are released (Lin et al. 1999, Pote et al. 2000, Rosser et al. 2015). Consistent with observations by Molnár (2002), plasmodia and myxospores of *Henneguya* spp. in channel catfish appear to exhibit developmental site specificity associated with the gills (Lin et al. 1999, Rosser et al. 2014b, 2015). However, with the exception of *H. ictaluri*, little is known regarding tissue and microhabitat specificity and localization sites for vegetative stages of other ictalurid-associated *Henneguya* spp.

In channel catfish, pre-sporogonic stages of *H. ictaluri* incite intense granulomatous inflammation, epithelial hyperplasia, and cartilage lysis within gill filaments (Wise et al. 2008). The resultant respiratory compromise leads to reduced growth performance and survival due to inappetence and overt mortalities, which in severe cases can approach 100% (Bowser and Conroy 1985). Affected catfish gulp at the water's surface or congregate at water inflows and behind aerators where oxygen concentrations are highest (Wise et al. 2004). Presumptive and definitive diagnoses are made by observation of chondrolytic lesions in gill wet mount

preparations and visualization of pre-sporogonic stages within histologic lesions, respectively (Wise et al. 2008). Confirmation can be made by species-specific end-point or real-time PCR (Whitaker et al. 2001, Griffin et al. 2008b). Blue catfish are refractory to *H. ictaluri* infection, while channel × blue catfish hybrids develop acute lesions of PGD, with vegetative pre-sporogonic stages, inflammation, and gill damage consistent with lesions reported from channel catfish (Wise et al. 2008, Griffin et al. 2010, Rosser et al. 2019). However, when exposed to pond water containing *H. ictaluri* actinospores, transmission and proliferation of *H. ictaluri* myxospores in tissues of hybrid catfish are significantly reduced compared to channel catfish, and development of mature plasmodia is rare (Griffin et al. 2010, Rosser et al. 2019).

Generally, aside from *H. ictaluri*, *Henneguya* spp. reported from farm-raised catfish in the southeastern United States are not thought to induce significant pathologic changes, morbidity, or mortality. With the exception of *H. ictaluri*, pathologic descriptions of *Henneguya* spp. in hybrid catfish are lacking. Early reports of branchial *H. exilis* and *Henneguya* spp. infections in channel catfish described intralamellar and interlamellar forms of disease, though definitions and descriptions of these forms are often confusing and, at times, contradictory between reports (McCraren et al. 1975, Bowser and Conroy 1985, Duhamel et al. 1986, Molnár 2002). McCraren et al. (1975) and Bowser and Conroy (1985) identified plasmodia developing within epithelium of the lamellar troughs as the ‘‘intralamellar’’ form, which was not associated with significant morbidity or mortality. The ‘‘interlamellar’’ form depicted gill lesions within filaments, and morbidity and mortality rates suggestive of PGD. Later reports described intralamellar forms with plasmodia developing within vascular components of the lamellae, interlamellar forms with plasmodia developing in the lamellar epithelial troughs, and filamental forms with plasmodia in the gill filament collagen matrix consistent with PGD (Duhamel et al.

1986, Molnár 2002). Regardless, these reports predate the more accurate molecular approaches now applied to species identification and illustrate the limited utility of identifications based solely on myxospore morphology and tissue tropism. In this report, a distinctive form of gill pathology caused by massive, interlamellar *H. exilis* infection is described in channel × blue catfish hybrids and confirmed by laser capture microdissection and sequencing of a diagnostic variable region (DVR3; Iwanowicz et al. 2008) of the 18S rRNA gene.

Materials & Methods

In the spring of 2018, 2 commercial catfish producers located in the catfish-farming region of the Mississippi Delta (Mississippi) reported reduced feeding activity and congregation of large numbers of fish along pond banks and behind aerators, suggesting severe respiratory distress and morbidity. Five market-size, hybrid catfish from 2 separate diagnostic submissions (4 fish from Farm 1; 1 fish from Farm 2) were received by the Aquatic Research and Diagnostic Laboratory located at the Thad Cochran National Warmwater Aquaculture Center in Stoneville, Mississippi. Diagnostic workups revealed abnormally heavy infections of *Henneguya* spp. in the gills. As a result, the left and right gill arches of each fish were removed and preserved in 70% ethanol and 10% neutral buffered formalin, respectively, for histologic (both fixatives) and molecular (ethanol only) analysis.

Tissues were processed routinely (formalin-fixed) or without formalin contact (ethanol-fixed), sectioned at 4 µm, and stained with hematoxylin and eosin (H&E) for light microscopic examination. Select sections were also stained using modified Brown and Hopps Gram, Ziehl–Neelsen acid-fast, and Giemsa methods. Immunohistochemical staining (IHC) for cytokeratin analysis was performed using a cytokeratin cocktail of AE1 and AE3 mouse monoclonal antibodies (Cell Marque 313M, St. Louis, Missouri) following standard methods (Taylor et al.

2006). Plasmodia were excised from the interlamellar spaces (30/fish), filaments (10/fish), and epithelium of the gill arch (30/fish) in ethanol-fixed sections of 1 fish from each submission using laser capture microdissection (LCM). Ethanol-fixed, histologic sections were prepared on MMI nuclease-free membrane slides, and the plasmodia were excised using an MMI CellCut Plus® microdissection system (Molecular Machines & Industries, Eching, Munich, Germany). The myxozoan DNA was extracted from excised plasmodia using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Catfish were confirmed to be hybrid channel × blue crosses by PCR of ethanol-fixed tissue scrolls according to previous methods (Waldbieser and Bosworth 2008).

The excised *Henneguya* spp. were identified by conventional PCR and sequencing of a discriminatory highly variable region of the 18S small subunit rRNA gene using the H9 (TTACCTGGTCCGGACATCAA) and H2 (CGACTTTTACTTCCTCGAAATTGC) primers reported by Hanson et al. (2001). The 25-ll PCR reaction mixtures contained 0.2 lM of each primer using OneTaq® DNA Polymerase according to the manufacturer's protocol (New England BioLabs, Ipswich, Massachusetts). Amplification was carried out using a Bio-Rad T100e thermal cycler (Bio-Rad, Hercules, California) with a denaturation step of 95 C for 3 min, followed by 40 cycles of 95 C for 30 sec, 52 C for 30 sec, 68 C for 45 sec, and a final extension step of 68 C for 5 min. Amplicons were run through a 2.0% agarose gel containing 0.1 lg/ml ethidium bromide and visualized under ultraviolet light to ensure the presence of a single, appropriately sized band, estimated by direct comparison to a concurrently run molecular weight marker (exACTGene 100 bp; Fisher Scientific Inc., Hampton, New Hampshire). Bands were excised and purified (QIAquick Gel Purification Kit, Qiagen) and sequenced commercially (Genewiz, South Plainfield, New Jersey) using the same primers. Sequencing reads were

assembled in Geneious® v10.1.3 and used in a Blastn search for somewhat similar sequences in the National Center for Biotechnology Information non-redundant nucleotide (nr/nt) database for myxozoan identification.

Results

Grossly, filaments on all gill arches from each fish were severely affected by widespread, often confluent, pinpoint, raised, white nodules that distended extensive areas of individual filaments (Figs. 8.1-2). Examination of wet mount preparations revealed numerous, round to elongate nodules diffusely occluding interlamellar spaces along the gill filaments (Fig. 8.3). Microscopically, the nodules represented 50–250-µm-diameter, roughly round to oval, myxozoan plasmodia. One to 2 plasmodia frequently filled interlamellar spaces, with or without displacing adjacent lamellae, in association with lamellar fusion and epithelial hyperplasia along the entire length of nearly all filaments (Fig. 8.4). Numerous plasmodia were also present within the epithelium of the gill arch and rakers (Fig. 8.5). The plasmodia arose within stratified epithelium at the base of the interlamellar troughs of the gills and remained covered by multiple layers of epithelium and goblet cells (Fig. 8.6). Cells surrounding plasmodia were confirmed as interlamellar epithelium by strong cytoplasmic staining with anti- cytokeratin immunohistochemistry (Fig. 8.7). Infrequently, ruptured and poorly delineated plasmodia distended the subepithelial connective tissue of the filament, inciting moderate infiltration by macrophages and scattered lymphocytes (Fig. 8.6). Most plasmodia had no discernable limiting wall of parasitic origin and contained large numbers of primarily mature myxospores (Fig. 8.8). Asynchronous development was observed in less mature plasmodia that contained peripherally located pansporoblastic stages and fragments of a 4–5-µm-thick, striated, pale eosinophilic cortical zone (Fig. 8.8). In addition, infrequent lysis of filament cartilage was associated with

hemorrhage, intense inflammatory infiltrates, and, rarely, pre-sporogonic vegetative stages of myxozoans. In 1 fish, degenerate plasmodia were infiltrated by long, slender bacterial rods compatible with *Flavobacterium columnare* (Fig. 8.9). Myxospores possessed a lanceolate-shaped body with 2 shell valves, each with a long tapering caudal process, 2 elongate polar capsules, a posterior vacuole, and a binucleated sporoplasm consistent with *Henneguya* spp. reported from North American ictalurid fish (Minchew 1977, Pote et al. 2000, Rosser et al. 2014b, 2015). Polar capsules were difficult to visualize in H&E sections, but they were enhanced by Gram and Giemsa stains (Figs. 8.10-11). Shell valves stained magenta with a Ziehl–Neelsen acid fast stain (Fig. 8.12). An identical 700-bp fragment of the 18S rRNA gene was amplified from all mature plasmodia excised from interlamellar spaces, filaments, and gill arches. This fragment corresponded to a highly variable region within the 18S gene (DVR3; Iwanowicz et al. 2008) and shared 100% nucleotide similarity to the reference sequence for *H. exilis* (AF021881.1) (Hanson et al. 2001).

Discussion

The observed gross and histopathologic changes share some features of interlamellar *H. exilis* infection described in early reports from channel catfish, although the pre-sporogonic stages, as well as inflammatory and proliferative changes in some descriptions, are more consistent with PGD (McCraren et al. 1975, Duhamel et al. 1986). These reports predate the discovery of *H. ictaluri*, now known to cause PGD, and highlight the potential for species misidentification based on myxospore morphology and tissue site tropism (Pote et al. 2000, 2012). In addition to the interlamellar epithelium, plasmodial development also occurred in the gill filaments and arches of the hybrid catfish. Consistent with ultrastructural observations, fully developed plasmodia lacked the distinct cortical zones and pinocytic channels evident in

immature plasmodia with pansporoblasts, suggesting these structures may degrade prior to myxospore release (Current and Janovy 1978).

While mechanisms of myxozoan host specificity, tissue tropism, and site preference within tissues are unknown, multiple *Henneguya* spp. are reported to show affinity for epithelial tissues, connective tissues, including cartilage, or vasculature in the gills (Molnár 2002). Based on a previously described system for characterizing myxozoan gill localization sites (Molnár 2002), myxospores of *H. exilis* develop in interlamellar-epithelial plasmodia arising symmetrically from stratified epithelium at the base of interlamellar troughs to fill entire interlamellar spaces. This was confirmed here using immunohistochemistry and an anti-cytokeratin (AE1/AE3) antibody to identify gill epithelial cells. Plasmodia were confined to the interlamellar epithelium, rather than the vascular network of the lamellae, a feature ascribed to intralamellar forms in later reports (Duhamel et al. 1986, Molnár 2002).

Interpretation of early reports of *H. exilis* in channel catfish is difficult, although some images suggest large numbers of interlamellar plasmodia as seen in these hybrids (Duhamel et al. 1986). It is likely these reports described mixed infections with *H. ictaluri* and other gill-infecting *Henneguya* spp., many of which were identified at the time as *H. exilis* (see McCraren et al. 1975, Bowser and Conroy 1985, Duhamel et al. 1986). Similarly, pre-sporogonic stages and tissue changes consistent with PGD were rarely observed in the hybrid fish in this report. It is important to note that all excised mature plasmodia from all fish produced identical 18S gene sequences consistent with *H. exilis* (Lin et al. 1999, Hanson et al. 2001). At present, the pre-sporogonic stages of other gill-infecting myxozoans have not been identified, and it is plausible the pre-sporogonic stages reported in the literature represent early stages of multiple myxozoans endemic to catfish aquaculture that use the gills as a portal of entry. While it is probable that

mixed infections were involved in these fish, the small vegetative stages were rare and lost during subsequent histologic sectioning. As such, they could not be excised by LCM for species identification. Further work identifying the pre-sporogonic stages of other myxozoan species reported from catfish aquaculture ponds is needed.

Although closely related, *H. ictaluri* and *H. exilis* manifest strikingly different forms of disease in channel and hybrid catfish. Proliferative gill disease, attributed to pre-sporogonic stages of *H. ictaluri*, elicits a severe granulomatous inflammatory reaction accompanied by significant tissue destruction, primarily within filaments, and is associated with high mortality in severe outbreaks. At later stages of *H. ictaluri* infection, the inflammatory response is negligible, and mature *Henneguya* plasmodia are not thought to be associated with significant losses (Pote et al., 2012). In contrast, tissue destruction in these hybrid catfish was mild. Still, *H. exilis* produced massive interlamellar infection by mature, myxospore-filled plasmodia, resulting in respiratory compromise. Similarly, massive infections of *H. exilis* have been reported from the epidermis of channel catfish (Rosser et al. 2016c).

Though the mechanisms are poorly understood, variation in fish host species and strain susceptibility to myxozoan infections has been demonstrated for *H. ictaluri* and other myxozoans (Hedrick et al. 2003, Griffin et al. 2010, Rosser et al. 2019). While lesions of PGD can develop in hybrid catfish, diagnostic case submissions and anecdotal reports from industry suggest that incidence and severity are reduced compared to channel catfish, and in experimental challenges, parasite development in hybrids was significantly arrested prior to sporogenesis (Rosser et al. 2019). If development of *H. ictaluri* is significantly impaired in hybrid catfish, selection pressures may favor proliferation of other gill-infecting *Henneguya* spp., such as *H. exilis*, against which hybrid catfish may not have significant resistance. Alternatively, hybrid catfish

could simply have greater susceptibility to *H. exilis*. Regardless, selection for *H. exilis* over multiple growing seasons could allow for proliferation of *H. exilis* within the system, leading to the massive infections described here. Further investigation into selection pressures on myxozoan diversity in production ponds and channel, blue, and hybrid catfish susceptibility to other gill-infecting *Henneguya* spp. is needed to substantiate these claims.

Respiratory compromise in these fish and potential disturbances in acid–base and osmoregulatory balance probably resulted from physical interference with oxygen and electrolyte movements across the lamellar epithelium. Affected fish presented with signs of hypoxia similar to PGD, in which decreases in pO2 and increases in pCO2 have been documented in sublethally challenged channel catfish (Beecham et al. 2010). Additionally, *F. columnare* was recovered from the gills of fish in both *H. exilis* outbreaks and was confirmed histologically (Declercq et al. 2013). Although speculative, infiltration of plasmodia by the bacteria suggests that rupture and myxospore release could provide a portal of entry for *F. columnare*.

While mortality rates were low, signs of hypoxia were observed, along with reduced feeding activity. These signs were transient, with fish recovering within 2–3 wk, likely associated with myxospore release at maturity and subsequent recovery of the host. In 1 developmental study performed by Rosser et al. (2019), reductions in the number of *Henneguya* spp. plasmodia present at 12 wk post-challenge were observed. It is thought that once the myxospores mature and are released, the fish host rapidly recovers, and respiratory compromise associated with the mechanical disruption of gill function abates. Palliative treatments such as increased aeration and the addition of salt are recommended to reduce respiratory and osmoregulatory stress and minimize losses (Wise et al. 2004).

In this report, the gross and histologic pathology associated with outbreaks of *H. exilis* infection in hybrid catfish from 2 discrete catfish farms was confirmed by LCM and sequencing of a diagnostic variable region (DVR3; Iwanowicz et al. 2008) of the 18S rRNA gene. The heavy burdens of *H. exilis* in these cases resulted in respiratory compromise and signs of hypoxia mimicking PGD. Given that *H. exilis* and *H. ictaluri* share the same oligochaete host, similar environmental factors, including large *D. digitata* populations and temperature ranges conducive to actinospore release, may predispose outbreaks of both parasites (Wise et al. 2004, 2008, Pote et al. 2012). Additional considerations that may account for the severity of infections seen here include differences in the susceptibilities of channel, blue, and hybrid catfish to various *Henneguya* spp., including *H. exilis* (see Griffin et al. 2010). Diversity in the myxozoan communities in commercial ponds may also be influenced by the catfish species being cultured (Rosser et al. 2019). Although speculative based on the small number of cases, increased resistance of hybrid catfish to *H. ictaluri* could select for *H. exilis* proliferation over time in ponds producing hybrid catfish, leading to outbreaks like those described here. While high mortalities may not be expected, and associated morbidity may be transient, severe interlamellar *H. exilis* infection should be considered in pond-raised hybrid catfish experiencing signs of respiratory distress, and palliative treatments should be applied.

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Figures

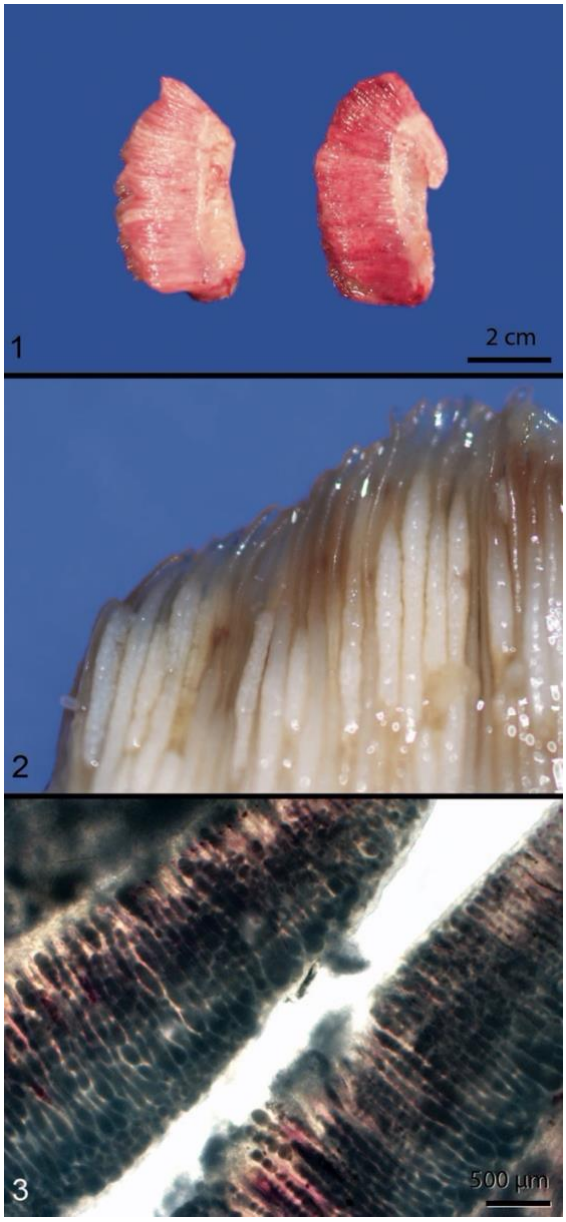

Figure 5.1-3 Gross images of fresh and formalin-fixed gill arches from channel × blue hybrid catfish infected with *Henneguya exilis*. (1) Fresh gill arches with pink to red, diffusely thickened filaments lined by indistinct, white streaks. (2) Formalin-fixed gill with locally extensive thickening of filaments by numerous, white, pinpoint nodules. (3) Wet mount cytology of the gill filaments with numerous, round to elongate plasmodia filling interlamellar spaces.

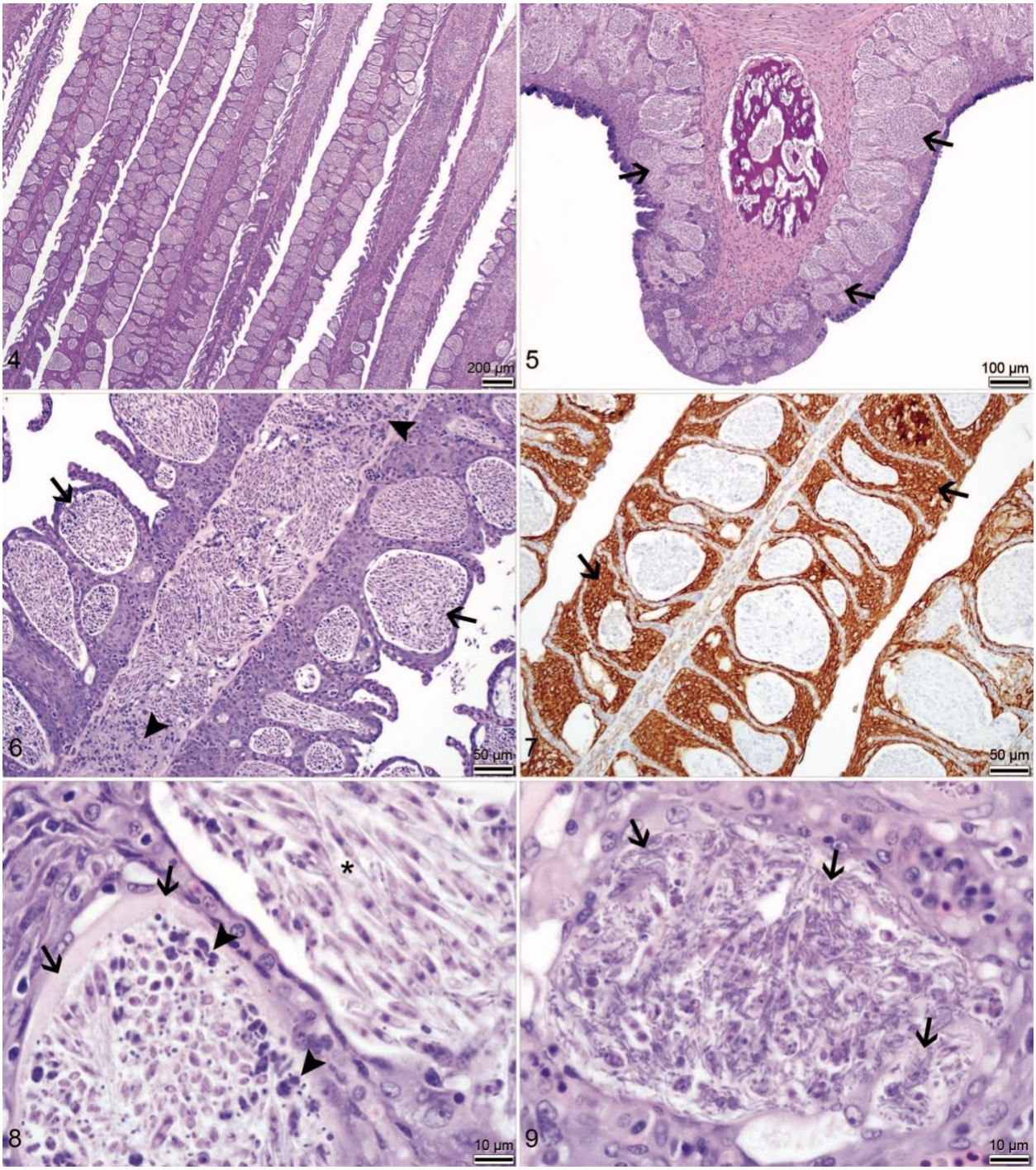

Figure 5.4-9 Histologic lesions associated with *Henneguya exilis* infection in the gills. (4) Many filaments are expanded by interlamellar plasmodia or intrafilamental inflammatory cell infiltrates (H&E). (5) Cross section through a gill raker with numerous plasmodia (arrows) within the overlying epithelium (H&E). (6) Plasmodia fill interlamellar spaces (arrows), accompanied by

epithelial hyperplasia and lamellar fusion, expand filaments, and incite infiltrates of macrophages and lymphocytes (arrowheads) (H&E). (7) Lamellar epithelial cells (arrows), surrounding interlamellar *H. exilis* plasmodia, are identified by strong immunohistochemical staining of their cytoplasm for cytokeratin (cytokeratin AE1/AE3). (8) Most plasmodia contain mature myxospores and lack a defined plasmodial wall (asterisk), while a few have an eosinophilic wall (arrows) surrounding subjacent developing pansporoblasts (arrowheads) (H&E). (9) Occasionally, plasmodia along the apical aspect of the filament are colonized by long, filamentous rods (arrows) consistent with *Flavobacterium columnare* (H&E).

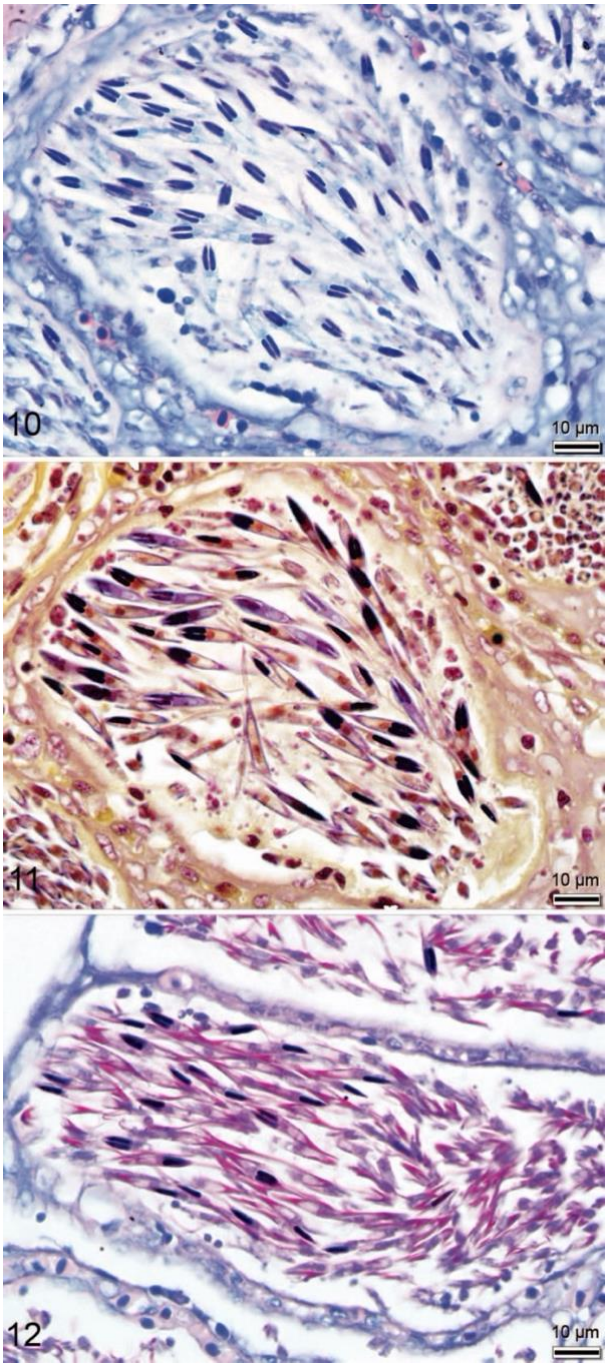

Figure 5.10-12 Histochemical stains highlighting mature myxospore components in plasmodia. (10) Polar capsules stain deep blue with Giemsa stain. (11) Polar capsules stain deep purple with Gram stain. (12) Shell valves stain bright magenta with Ziehl–Neelsen stain.

CHAPTER 6

MASSIVE BRANCHIAL HENNEGUYOSIS OF CATFISH: A DISTINCT MYXOZOAN INDUCED GILL DISEASE CAUSED BY SEVERE, INTERLAMELLAR INFECTIONS OF HENNEGUYA EXILIS IN CATFISH AQUACULTURE [1]

[1] Stilwell, J.M., M.J. Griffin, J.H. Leary, L.H. Khoo, & A.C. Camus. To be submitted to *Veterinary Pathology*.

Abstract

Proliferative gill disease (PGD), caused by the myxozoan parasite *Henneguya ictaluri*, is among the most devastating infectious diseases of United States catfish aquaculture. Recently, an unusual gill disease caused by massive burdens of the related myxozoan, *Henneguya exilis*, has been described in channel (*Ictalurus punctatus*) x blue (*Ictalurus furcatus*) hybrid catfish monoculture. Targeted metagenomic sequencing and in situ hybridization (ISH) were used to compare myxozoan community composition between massive *H. exilis* infections and clinical PGD cases to identify myxozoan species contributing to pathologic changes. Thirty ethanol-fixed gill holobranchs from seven cases of *H. exilis* infection in hybrid catfish were subjected to targeted amplicon sequencing of the 18S rRNA gene (Illumina MiSeq) and compared to a metagenomic dataset previously generated from PGD cases. Further, serial sections of 14 formalin-fixed gill holobranchs (2 per case) were analyzed by RNAscope® duplex chromogen ISH assays targeting eight different myxozoan species. Metagenomic and ISH data were in agreement, indicating myxozoan community composition significantly differs between PGD and branchial henneguyosis cases. Findings indicate that while PGD in farm-raised catfish can consist of mixed species infections, branchial henneguyosis results from nearly pure infections of *H. exilis*. However, *H. ictaluri* was identified by ISH in association with infrequent PGD-like lesions, suggesting coinfections and some overlap of pathologic changes in cases of massive branchial henneguyosis. Building upon previous pathologic descriptions and molecular characterization, this work provides the case definition for a potentially emerging, myxozoan-induced gill disease of farm-raised catfish known hereafter as massive branchial henneguyosis of catfish (MBHC).

Introduction

Proliferative gill disease (PGD), caused by the myxozoan parasite *Henneguya ictaluri*, has significant, negative impacts on commercial catfish production, the largest sector of foodfish aquaculture in the United States. The life cycle of *H. ictaluri* involves a myxospore stages in the gills of channel catfish and an actinospore stage released by the ubiquitous benthic oligochaete *Dero digitata* (Bellerud et al. 1995; Pote et al. 2000; 2012). Continuous exposure of catfish to the actinospore stage of *H. ictaluri* is particularly devastating, resulting in severe respiratory distress, high fish mortality, and substantial economic losses (Wise et al. 2008). Pathognomonic lesions of PGD include swollen, red and white mottled, frequently truncated gill filaments grossly, and multifocal lysis of filament cartilage seen in gill clip wet mounts. Microscopic lesions consist of epithelial hyperplasia, lamellar fusion, and intense inflammatory cell infiltrates associated with cartilage lysis and proliferation, and presporogonic stages of *H. ictaluri* (Wise et al. 2008, Chapter 3). While several other *Henneguya* spp. infect the gills of catfish, most do not cause overt disease. However, their presence in gill tissues during PGD outbreaks may induce further pathologic changes, including additional PGD-like lesions, and potentially exacerbate overall disease severity (Chapters 2 and 3).

In contrast to channel catfish, *Ictalurus punctatus*, channel catfish × blue catfish, *I. furcatus*, hybrids demonstrate decreased transmission rates and arrested development of *H. ictaluri* (Griffin et al. 2010, Rosser et al., 2019, Chapter 3). While the specific mechanisms responsible for this differential host susceptibility to the parasite are unknown, the abundance of *H. ictaluri* is suppressed in hybrid gill tissues and hybrid monoculture systems (Griffin et al. 2010, Griffin et al. 2020b, Chapter 2, Stilwell unpublished data). Additionally, monoculture of

hybrid catfish results in overall decreased myxozoan diversity and, potentially, enhanced selection of *Henneguya exilis* within the pond environment (Stilwell unpublished data).

While PGD and *H. ictaluri* are intensively studied, *H. exilis* has received relatively little attention or research interest. *H. exilis* was first morphologically described in channel catfish by Kudo (1929) followed by its molecular characterization 70 years later (Lin et al. 1999). *H. exilis* shares the same indirect life cycle as *H. ictaluri* involving the definitive oligochaete host, *Dero digitata*, and intermediate fish host, the channel catfish (Lin et al. 1999). While pathologic changes have been attributed to *H. exilis* during disease outbreaks in channel catfish, the histologic descriptions predate the molecular characterization of catfish myxozoans and overlap considerably with those of PGD, calling the diagnoses into question (Duhamel et al., 1986). More recently, gross and microscopic lesions associated with outbreaks of respiratory distress and massive, interlamellar burdens of a *Henneguya* sp. in channel catfish × blue catfish hybrids were identified as *H. exilis* by molecular sequencing of plasmodia excised from histologic sections by laser capture microdissection (Stilwell et al. 2019a). The clinical and pathologic features associated with these outbreaks differed substantially from those of PGD (Wise et al. 2008, Stilwell et al. 2019a).

Our null hypothesis assumed massive *H. exilis* infection represented another form of PGD while the alternative hypothesis assumed this disease presentation represents a distinct respiratory condition caused by *H. exilis* in channel and hybrid catfish. This work utilized metagenomic sequencing and *in situ* hybridization techniques to investigate these hypotheses.

Materials & Methods

Fish collection and histopathology

In the spring of 2018 and 2019, seven catfish case submissions to Mississippi State University Aquatic Research and Diagnostic Laboratory were diagnosed with massive *H. exilis* infection in accordance with previously reported gross and wet mount lesions (Stilwell et al. 2019a). During necropsies, right holobranchs were fixed in 10% neutral buffered formalin for histological assessment and *in situ* hybridization, while left holobranchs were fixed in 70% ethanol for laser capture microdissection and metagenomic analysis (Stilwell et al., 2019). The fish were confirmed as hybrid catfish by duplex-PCR using previously established protocols (Waldbeisser and Bosworth 2008). Tissues were processed by standard methods, embedded in paraffin, sectioned at 4 µm onto charged slides, and either left unstained for *in situ* hybridization or stained with hematoxylin and eosin for light microscopic examination.

Metagenomic sequencing and analysis

Metagenomic sequencing and analysis were performed as described previously (Chapter 2). Briefly, extracted DNA samples from ethanol-fixed gills were submitted to the Georgia Genomics and Bioinformatics Core, University of Georgia, Athens, GA, USA for library preparation and next generation sequencing (Illumina MiSeq, San Diego, CA) generating paired, 300 bp reads. General myxozoan primers were used to amplify an approximately 432 bp segment of the myxozoan 18S rRNA gene containing a diagnostic variable region used for species identification of myxobolids (Iwanowicz et al. 2008, Griffin et al. 2009a). Primer sequences were as follows: H9, forward primer, 5'-TTACCTGGTCCGGACATCAA-3' (Hanson et al. 2001); 1862R, reverse primer, 5'-ATTGTAGCGCGCGTGCAG-3' (Chapter 2). Paired end reads

were merged, trimmed, and filtered by quality (maximum expected error rate of 1.0) and sequence length (370-490 bp) using USEARCH v10.0.240 (Edgar, 2010). Unique reads were counted and binned into operational taxonomic units (OTU), which were identified through a Blastn search of the NCBI non-redundant nucleotide (nr/nt) database. OTU counts were converted to percentages to assess relative abundances of myxozoan taxa. Using PGD metagenomic data derived from 2018 and 2019 PGD cases (Chapter 2), myxozoan community composition was compared between PGD and massive *H. exilis* cases using PERMANOVA analysis of dissimilarity (ADONIS) with the vegan package (Okansen et al. 2019). Nonmetric multidimensional scaling (NMDS) plots were generated with the vegan package to visualize community differences in beta diversity.

In situ hybridization

Two blocks containing formalin-fixed gill tissue from each case were randomly chosen, serially sectioned at 4 µm, and placed onto four charged slides. The *in situ* hybridization was performed using previously developed probes and protocols (Chapter 3). *Henneguya* species-specific probes for five known and three suspect taxa were allocated into duplex assays as follows: 1) *H. ictaluri* and *H. exilis*, 2) *H. mississippiensis* and Raabeia-type TGR-2014, 3) *H. postexilis* and Myxo 12, 4) Myxo 7 and 11 (Chapter 3). The probes were diluted by half using equal parts of stock probe solution and RNAscope® probe diluent (Catalog No. 300041, Advanced Cell Diagnostics Inc., Hayward, CA). The assays were performed using the RNAscope duplex chromogen kit (RNAscope® 2.5 HD Duplex Detection Kit User Manual, Part 2, Catalog No. 322500-USM, Advanced Cell Diagnostics Inc.) according to the manufacturer's instructions with a 25-minute target retrieval step. Slides were counter stained with hematoxylin and TBS (bluing reagent) and manually coverslipped.

Results

Metagenomic analysis

Thirty gill samples from seven cases (2018, n=2; 2019, n=5) of massive *H. exilis* infection in hybrid catfish were included in the analysis. The sequencing run generated 1,931,376 paired reads with 1,483,440 reads remaining after merging and filtering for quality and sequence length (76.8%). Nearly all merged, filtered reads were related to myxozoan taxa (1,483,281 reads, 99.9%). A total of 11 myxozoan OTUs were identified in samples with all OTUs previously detected in PGD cases (Table 6.1). *H. exilis* relative abundance averaged approximately 98.5% per gill sample or nearly pure infections (Figure 6.1). *H. ictaluri* was present in all samples at an average relative abundance of 1.3% while all other taxa averaged <1% relative abundance in samples (Table 6.1). In comparison to PGD data from the same years, there was no overlap in myxozoan community composition between the two diseases ($p<0.001$, Figure 6.2, Chapter 2).

In situ hybridization

Interlamellar plasmodia and free myxospores within the fibrous stroma and vasculature of the filament demonstrated consistent positivity with only the *H. exilis* specific probe (Figure 6.3A-D). Rare presporogonic stages in association with granulomatous inflammation and filament cartilage lysis reacted with *H. ictaluri* specific probes only (Figure 6.3E-F). Other probes showed rare, scattered positivity throughout the sections, containing either few, scattered presporogonic stages (Raabeia type TGR 2014) or single plasmodia (*H. mississippiensis*, *H. postexilis*, Myxo 11) (Table 6.2).

Discussion

Findings from the present study indicate that massive infections of *H. exilis* represent a myxozoan induced gill disease distinct from PGD (Table 6.3), known hereafter as massive branchial henneguyosis of catfish (MBHC). Metagenomic analysis revealed that these cases resulted from nearly pure infections of *H. exilis*, a finding confirmed by only rare detection of other myxozoans, including *H. ictaluri*, by ISH. This is in contrast to cases of PGD, where metagenomic analysis and ISH studies have shown mixed infections. While *H. ictaluri* is typically the dominant organism present and usually associated with characteristic PGD lesions, it is often accompanied by mixed infections with many other myxozoans (Chapters 2 and 3). Cases of massive *H. exilis* infection also had fewer discrete OTUs present than PGD cases, further demonstrating less diversity and species evenness in the myxozoan community compared to PGD (Chapter 2). All known and potential unknown taxa detected had direct matches to sequences previously identified in association with PGD, providing additional validation for both datasets (Chapter 2).

Outbreaks of MBHC result in high morbidity, but low mortality, while PGD often results in high morbidity and mortality, differences likely related to the underlying disease pathogenesis and pathology responsible for respiratory compromise. Massive plasmodial development by *H. exilis* interferes with oxygen exchange by physical impairment of water flow through obstructed interlamellar channels but causes minimal tissue damage (Stilwell et al. 2019a). Tissue function returns rapidly following plasmodial rupture and myxospore release (Stilwell et al. 2019a). In contrast, presporogonic stages of *H. ictaluri* incite profound inflammatory, proliferative, and lytic changes, often with residual filament loss (Wise et al. 2008). Although fish may recover, the extensive tissue damage in PGD cases and persistent exposure to infective actinospores

during disease progression often becomes insurmountable, resulting in death of the fish host (Bellerud et al. 1995, Wise et al. 2004, Wise et al. 2008). Thus, respiratory distress in MBHC and PGD result from obstruction and destruction of respiratory tissue, respectively (Wise et al. 2008, Stilwell et al. 2019a). Despite differences in disease pathogenesis and pathology, similar clinical pathologic findings may result from respiratory distress in both diseases. PGD results in decreased oxygen partial pressure (pO_2) and increased carbon dioxide partial pressure (pCO_2) in the blood of affected fish (Beecham et al. 2010), although whether this occurs in MBHC affected fish remains to be determined.

The diseases both occur in mid to late spring and early summer yet involve different stages of myxozoan development, with MBHC involving numerous plasmodia with mature myxospores and PGD consisting largely of presporogonic stages associated with the destructive branchitis (Wise et al. 2008, Stilwell et al. 2019a). This implies infection with *H. exilis* occurs months before clinical presentation with MBHC while clinical PGD occurs within weeks of infection with *H. ictaluri* (Griffin et al. 2010, Rosser et al. 2019, Stilwell et al. 2019a). Given that *H. exilis* and *H. ictaluri* share the same life cycle, it would be assumed that similar factors predispose to infection and disease outbreaks, though differences in the developmental progression and seasonal timeline of these diseases suggest otherwise (Lin et al. 1999, Pote et al. 2000). These differences could be related to either differing myxozoan virulence between *H. exilis* and *H. ictaluri*, fish host susceptibility, seasonal changes in fish immune status, or a combination of factors, although these hypotheses are speculative and require further investigation.

Fish host susceptibility to infection and development influences myxozoan communities and diversity in host tissues and environments (Chapter 2, Stilwell unpub data). Differences in

susceptibility to particular myxozoans exist between fish host species as well as between different strains of the same fish species (Hedrick et al. 1999, Hedrick et al. 2003, Griffin et al. 2010, Rosser et al. 2019). Massive *H. exilis* infections in hybrid catfish potentially reflect either increased susceptibility, and subsequently positive selective pressure, to infection and development of *H. exilis*, decreased susceptibility to other *Henneguya* spp., or a combination of both. The latter is supported by work demonstrating myxozoan communities in ponds dedicated to hybrid catfish monoculture have decreased *H. ictaluri* abundance, Shannon diversity, and species evenness with a distinct peak in *H. exilis* relative abundance during each spring season (Griffin et al. 2020b, Stilwell unpub data). This suggests that *H. exilis* exhibits some competitive advantage over other myxozoans in hybrid catfish systems (Stilwell et al. 2019a, Stilwell unpub data). Progressive amplification and accumulation of *H. exilis* within continuous hybrid monoculture ponds over multiple production cycles could potentially lead to myxozoan numbers associated with debilitating parasite burdens and disease, though this is speculative and requires further study.

Few myxozoans cause disease through heavy parasite burdens alone. Similar to these *H. exilis* infections, *Myxobolus koi* in koi carp, *Cyprinus carpio*, and *Henneguya pseudoplatystoma* in pinado, a hybrid South American catfish bred from crossing *Pseudoplatystoma corruscans* and *Pseudoplatystoma fasciatum*, form large plasmodia that obscure gill filaments and reduce respiratory capacity and surface area (Naldoni et al. 2009, Camus and Griffin 2010). These outbreaks were associated with pond or aquaculture settings which presumably contained a suitable definitive host, large fish host biomass, and conditions allowing for completion and amplification of parasite replication. This suggests other myxozoans with similar tissue tropisms

may produce heavy parasite burdens and disease potential if introduced to intensive aquaculture or pond systems (Stilwell et al. 2019a, Stilwell et al. 2019c).

This work utilizing metagenomic sequencing and *in situ* hybridization techniques confirms the etiology and features of a distinct, myxozoan induced gill disease of hybrid catfish caused by *H. exilis*. While MBHC and PGD both induce respiratory distress, massive *H. exilis* infections are differentiated from PGD clinically based on levels of morbidity and mortality. Microscopic distinctions include the location and severity of tissue changes, as well as the myxozoan life stages present and their specific tissue tropisms. Molecular evidence indicates near pure infections of *H. exilis* in cases of MBHC, while cases of PGD involve a diverse community of myxozoans dominated by *H. ictaluri* (Chapters 2 and 3). While epidemiologic factors leading to outbreaks of *H. exilis* are unknown, metagenomic analysis of controlled pond water environments dedicated to hybrid catfish monoculture revealed distinct peaks in relative abundance of *H. exilis* (Stilwell unpublished data), suggesting increased host susceptibility and selection for *H. exilis* in hybrid catfish ponds (Stilwell unpublished data). The repeated diagnosis of MBHC in conjunction with increased production of hybrid catfish during 2018 and 2019 suggest that this condition may represent an emerging disease of hybrid catfish monoculture. Further investigations are needed to determine the true prevalence of the disease in the catfish aquaculture industry and epidemiologic factors associated with outbreaks.

Figures

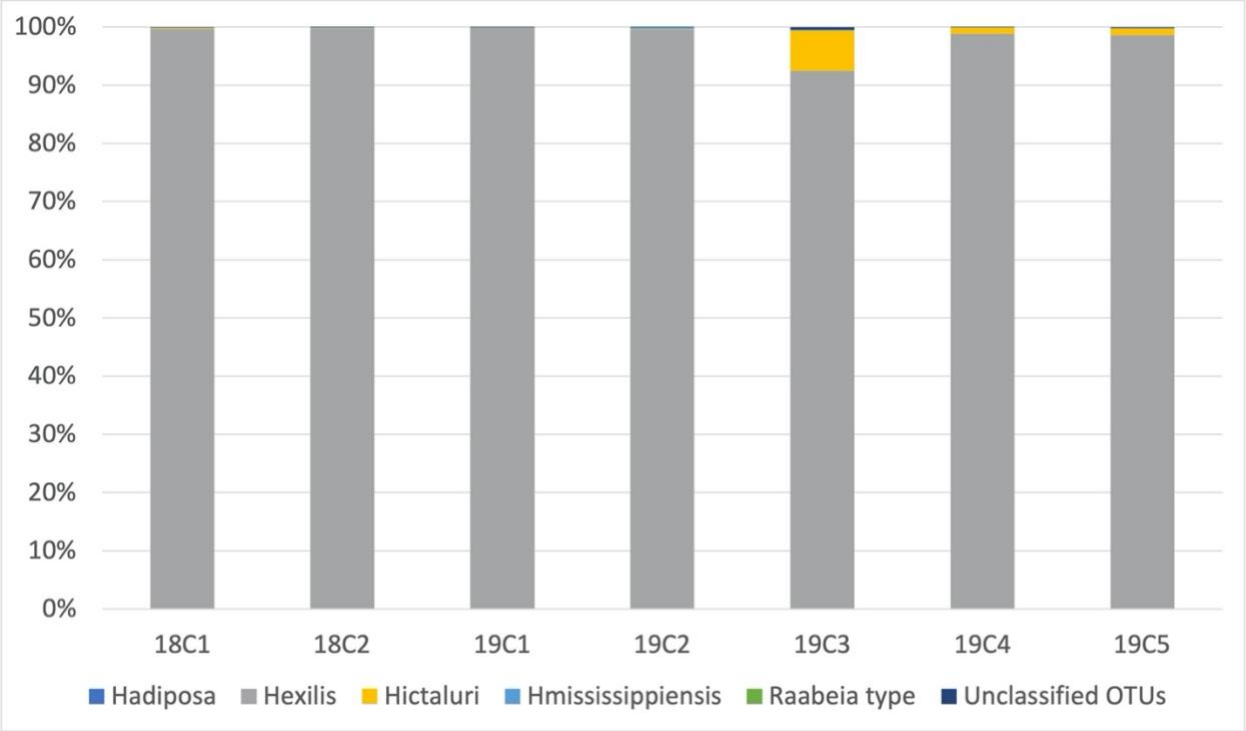

Figure 6.1 Relative abundances of myxozoan taxa in cases of massive branchial henneguyosis of catfish. The y-axis represents myxozoan relative abundance as stacked percentages while the x-axis lists case numbers. Each case represents a nearly pure infection of *H. exilis* with few other myxozoans detected.

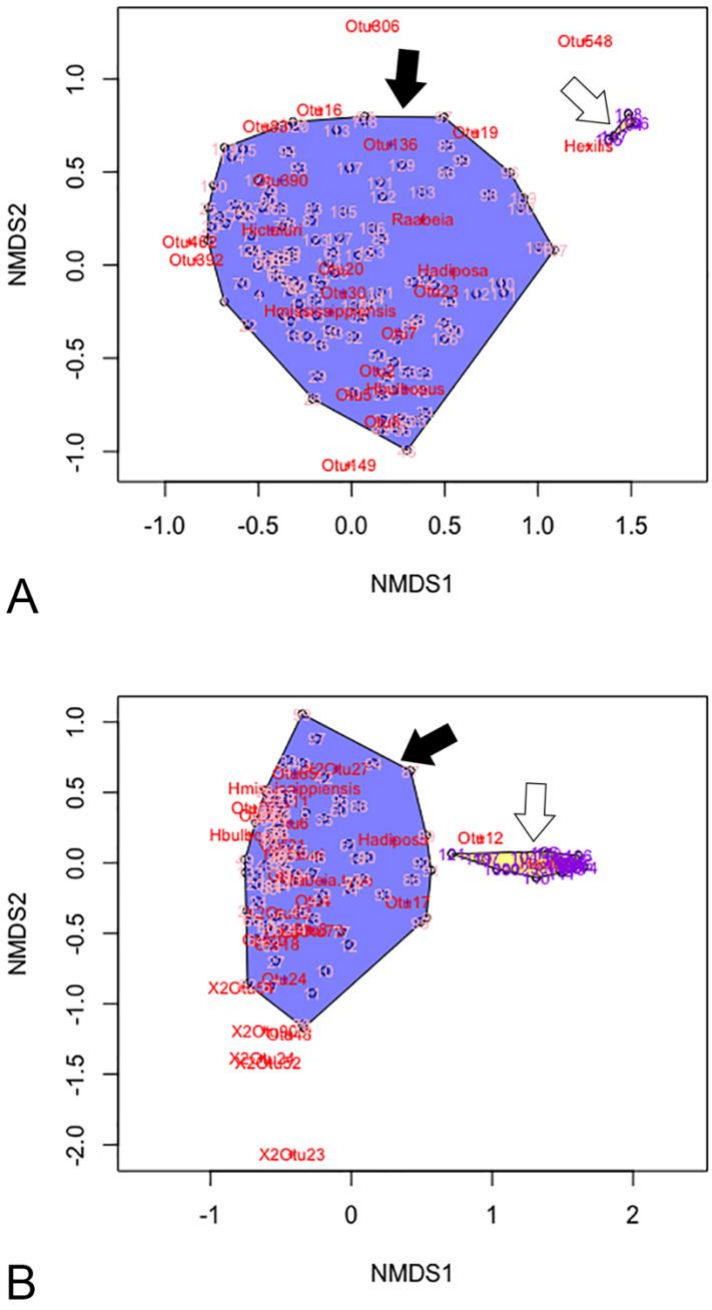

Figure 6.2 Non-metric multidimensional scaling (NMDS) plots comparing myxozoan community composition and beta diversity between PGD samples (open arrows) and samples of Massive Branchial Henneguyosis of Catfish (MBHC) infections (solid arrows) in 2018 **(A)** and 2019 **(B)**. Pink numbers within blue regions represent PGD communities while purple numbers

in yellow regions represent MBHC communities. Note there is no overlap in myxozoan community composition between the two disease conditions with PGD gills demonstrating considerable variability in community structure while MBH cases cluster in tight association with *H. exilis*. NMDS Stress values: 0.1452, 2018; 0.1299, 2019.

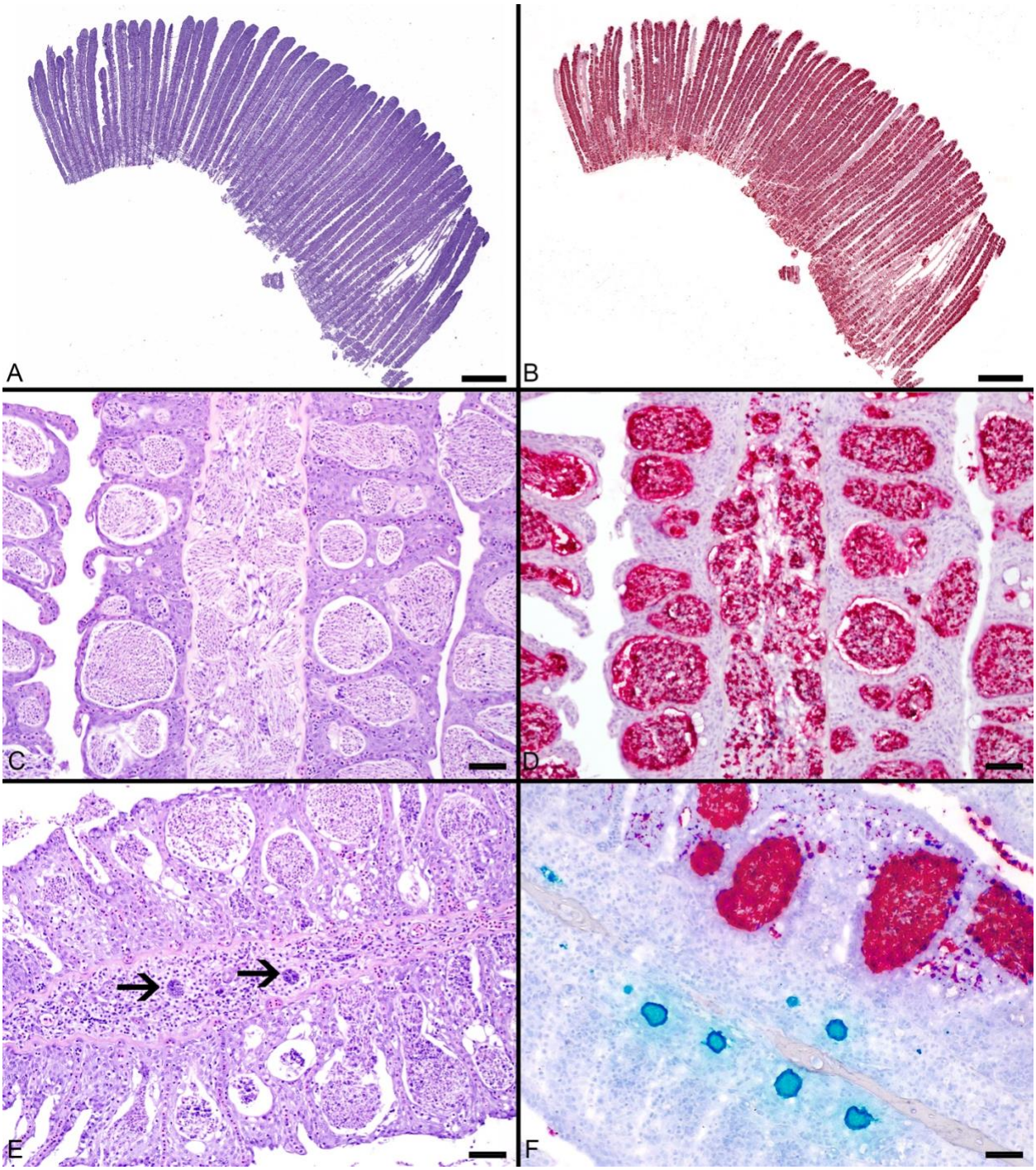

Figure 6.3 Histologic lesions associated with Massive Branchial Henneguyosis infection in the gills of hybrid catfish. (A) Subgross magnification of a gill arch with diffuse thickening of filaments due to numerous interlamellar plasmodia. H&E, Bar = 2 mm. (B) Nearly all plasmodia are identified as *H. exilis* by red chromogen staining, Duplex *H. ictaluri/H. exilis* ISH, Bar = 2

mm. (C) Typical histologic lesions associated with MBH with interlamellar spaces filled by epithelial hyperplasia, embedded myxozoan plasmodia, and inward rupture of plasmodia releasing myxospores into the filament stroma. H&E, Bar = 50 µm. (D) All plasmodia and free myxospores are identified as *H. exilis* by red chromogen staining. Duplex *H. ictaluri/H. exilis* ISH, Bar = 50 µm. (E) Presporogonic life stages of a myxozoan (arrows) and granulomatous inflammation expanding the filament with *H. exilis* plasmodia filling interlamellar spaces. H&E, Bar = 50 µm. (F) Presporogonic life stages are identified as *H. ictaluri* by green chromogen staining in association with PGD-like lesions while interlamellar plasmodia are *H. exilis*. Duplex *H. ictaluri/H. exilis* ISH, Bar = 50 µm.

Tables

Table 6.1 Operational Taxonomic Unit table detailing counts of merged myxozoan sequences detected by targeted metagenomic sequencing in the gills of hybrid catfish with massive branchial henneguyosis of catfish

Sample ID	*H. adiposa* (OTU14)	*H. exilis* (OTU1)	*H. ictaluri* (OTU2)	*H. mississippiensis* (OTU3)	OTU4*	OTU6*	OTU8*	OTU9*	OTU10*	OTU11*	Raabeia type TGR 2014 (OTU5)
18C1-1	0	26989	22	42	0	3	1	0	0	0	1
18C2-1	0	11750	20	8	0	3	0	0	0	0	0
18C2-2	0	31676	21	14	0	43	1	26	0	0	1
18C2-3	0	23116	34	26	0	0	0	0	0	3	0
!8C2-4	0	6037	20	13	0	0	0	0	0	0	0
19C1-1	0	43659	8	22	0	1	0	3	0	0	2
19C1-2	0	38710	7	13	0	4	2	2	0	0	0
19C1-3	0	10305	20	9	0	5	0	2	0	2	0
19C1-4	0	47916	5	36	0	8	2	1	1	0	0
19C1-5	0	41685	13	10	0	3	11	0	1	0	2
19C1-6	18	99085	32	18	0	1	5	0	3	0	0
19C1-7	0	82033	8	11	0	0	1	0	1	2	0
19C1-8	0	113055	20	28	0	2	0	0	0	0	0
19C2-1	0	74052	39	202	0	0	1	0	1	0	0

19C2-2	0	109352	38	292	0	1	1	0	1	0	0
19C2-3	0	67832	19	295	0	0	0	0	3	0	4
19C3-1	0	26568	1846	30	50	0	3	1	0	0	10
19C3-2	0	62276	57	25	0	0	0	0	0	0	0
19C3-3	0	21469	5839	67	441	3	2	10	2	0	30
19C3-4	0	60728	106	14	11	3	1	0	1	0	0
19C4-1	0	31460	98	16	3	0	3	0	3	0	3
19C4-2	0	33671	31	9	0	0	0	0	0	0	0
19C4-3	0	72052	1867	114	5	0	7	0	0	5	0
19C4-4	0	28191	399	36	3	0	3	0	5	0	0
19C4-5	0	66469	605	55	8	0	8	0	3	0	15
19C5-1	0	56862	53	16	0	0	2	0	0	0	32
19C5-2	0	27511	371	6	69	0	0	0	1	0	16
19C5-3	0	39103	84	21	37	3	2	0	0	0	13
19C5-4	0	68526	43	4	14	0	0	0	0	0	5
19C5-5	0	44840	1823	69	156	4	6	0	0	0	43

*OTUs 4, 6, 8, 9, 10, and 11 correspond to Unclassified Taxa 8, 1, 11, 4, 12, and 7 identified from PGD cases, respectively (Chapter 2)

Table 6.2 Presence of developing myxozoan life stages in the gills of hybrid catfish with natural, massive branchial henneguyosis determined by *in situ* hybridization

Case Number	*H. exilis*	*H. ictaluri*	*H. mississippiensis*	*H. postexilis*	Raabeia type	Myxo 7	Myxo 11	Myxo 12	Number of species per case
18C1	2/2	2/2	0/2	1/2	1/2	0/2	1/2	0/2	5/8
18C2	2/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	1/8
19C1	2/2	0/2	0/2	2/2	2/2	0/2	0/2	0/2	3/8
19C2	2/2	0/2	2/2	0/2	0/2	0/2	0/2	0/2	2/8
19C3	2/2	2/2	0/2	0/2	0/2	0/2	0/2	0/2	2/8
19C4	2/2	2/2	0/2	0/2	0/2	0/2	0/2	0/2	2/8
19C5	2/2	2/2	2/2	0/2	0/2	0/2	0/2	0/2	3/8
Total	14/14	8/14	4/14	3/14	3/14	0/21	1/21	0/21	

Table 6.3 Case definitions for massive branchial henneguyosis of catfish and proliferative gill disease

	Massive Branchial Henneguyosis of Catfish	Proliferative Gill Disease
Etiologic Agent	*Henneguya exilis*	*Henneguya ictaluri*
Clinical Presentation	Respiratory distress with adequate dissolved oxygen concentrations	Respiratory distress with adequate dissolved oxygen concentrations
Gross Findings	Swollen, thickened, red gill filaments; multifocal, pinpoint to linear, white-gray, slightly raised lesions	Swollen, blunted, red and white mottled, hemorrhagic gill filaments
Wet Mount Gill Clip Findings	Interlamellar spaces filled with fully developed plasmodia containing mainly mature myxospores, hemorrhage	Multifocal cartilage lysis with or without proliferation and hemorrhage within filaments, epithelial hyperplasia
Key Microscopic Findings	Interlamellar plasmodia filled with mature myxospores embedded within the lamellar epithelium, epithelial hyperplasia, minimal tissue destruction, mild granulomatous inflammation in filament in association with internally ruptured plasmodia and free myxospores	Acute: cartilage fractures and lysis; blunted, collapsed filaments; hemorrhage; intense granulomatous inflammatory reaction to multinucleated presporogonic stages associated with gill filaments; epithelial hyperplasia, hypertrophy, and lamellar fusion; plasmodia in various stages of development unassociated with cartilage fracture and inflammation Chronic: bridging cartilage proliferation, plasmodia with myxospores in various stages of maturation
Morbidity/Mortality	High/Low	High/High
Myxozoan Developmental Stage	Mature	Presporogonic
Purity of Infection	Nearly pure infection of *H. exilis*	Mixed

CONCLUSION

Despite advancements in our understanding of proliferative gill disease (PGD) over 40 years of study, PGD persists within both channel and hybrid catfish monoculture systems in the catfish industry. Major milestones in PGD research included identifying the etiologic agent and its life cycle, developing rapid, molecular diagnostic techniques, determining predisposing factors for outbreaks, illustrating variability in fish host susceptibility to infection and parasite development, and investigations into potential management strategies and treatments to mitigate and prevent disease outbreaks. Key knowledge gaps and questions remained unanswered particularly how fish hosts influence myxozoan communities in ponds, whether other non-*H. ictaluri* myxozoans contribute to or can cause PGD lesions, and how *H. ictaluri* persists in hybrid catfish monoculture ponds despite evidence of impaired development in the fish host. This dissertation provided additional support for these former milestones while building upon them to answer these pressing questions.

Differences in *H. ictaluri* relative abundance were evident between channel and hybrid catfish monoculture in the disease process with suppression of *H. ictaluri* relative abundance in hybrid catfish. Infection challenge experiments have confirmed presporogonic *H. ictaluri* infection in hybrid catfish by *in situ* hybridization but mature plasmodia or myxospores were not observed, even 20 weeks post-challenge. This is consistent with previous work identifying decreased rates of infection and arrested parasite development in hybrid catfish. These findings

suggest crop rotation strategies, alternating between channel and hybrid stocks, could be developed and optimized to mitigate *H. ictaluri* prevalence in ponds and prevent or mitigate disease outbreaks. Combined with established management strategies such as pre-screening ponds, utilizing crop rotation in the industry could prevent millions of dollars in losses each year. Future work must also address how *H. ictaluri* is introduced to and persists within ponds, particularly hybrid catfish monoculture systems.

In addition to *H. ictaluri*, numerous known and unknown myxozoans were detected in gill lesions, illustrating that a considerable amount of previously unappreciated myxozoan diversity exists in catfish aquaculture. These myxozoans contributed their own pathology to PGD by causing localized inflammation, hemorrhage, and epithelial hyperplasia. Plasmodia embedded in the epithelium caused physical impediments to gas exchange with the blood in lamellar capillaries. While these myxozoans on their own typically only cause subclinical disease, these lesions presumably exacerbate PGD by further impairing respiratory function. The presporogonic stages associated with characteristic filament cartilage lysis and granulomatous inflammation were consistently identified as *H. ictaluri* by *in situ* hybridization, confirming it as the etiologic agent of PGD. Rarely, presporogonic stages of other myxozoans were associated with PGD-like lesions, suggesting other myxozoans may be capable of producing similar lesions. Controlled challenges using pure actinospore isolates are needed to determine the ability of other myxozoans to produce PGD lesions.

Other myxozoans, particularly *H. adiposa* and *H. exilis*, contributed subclinical or clinical pathology and disease independently of PGD outbreaks. In the last three years, diagnosticians have encountered 10 cases of an unusual, myxozoan-induced gill disease associated with massive, interlamellar burdens of *H. exilis* infection. Rare, historic cases in

channel catfish were included in early descriptions of PGD, contributing to conflicting histopathologic descriptions. Recent cases have been associated with commercial hybrid catfish monoculture. Through laser capture microdissection, metagenomic analysis and *in situ* hybridization techniques on infected gill tissue, this disease was determined as a separate entity from PGD, known hereafter as Massive Branchial Henneguyosis of Catfish. The diseases differ in their etiology, pathogenesis, purity of infection, myxozoan community composition, myxozoan developmental stages, and level of mortality, though co-infections can lead to overlapping gross and microscopic lesions. In combination with metagenomic analysis of water from hybrid catfish monoculture ponds showing significantly greater relative abundance of *H. exilis* compared to channel catfish ponds, hybrid catfish monoculture may apply selective pressures allowing for proliferation of *H. exilis* and suppression of *H. ictaluri* and other myxozoans in pond environments and gill diseases (Stilwell unpub data). More work is needed to understand the etiopathogenesis, predisposing factors and prevalence of this putative emerging disease in the catfish aquaculture industry.

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