8-9-2008

Development and application of a real-time polymerase chain reaction assay for the myxozoan parasite Henneguya ictaluri

Matthew J. Griffin

DEVELOPMENT AND APPLICATION OF A REAL-TIME POLYMERASE CHAIN REACTION ASSAY FOR THE MYXOZOAN PARASITE HENNEGUYA ICTALURI

By

Matthew John Griffin

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Medical Science
in the Department of Basic Sciences

Mississippi State, Mississippi

August 2008

DEVELOPMENT AND APPLICATION OF A REAL-TIME POLYMERASE CHAIN REACTION ASSAY FOR THE MYXOZOAN PARASITE HENNEGUYA ICTALURI

By

Matthew John Griffin

Approved:

David J. Wise
Extension/Research Professor
Delta Research and Extension Center
(Codirector of Dissertation)

Linda M. Pote
Professor
Department of Basic Sciences
(Codirector of Dissertation)

Alvin C. Camus
Associate Professor
University of Georgia
(Committee Member)

Michael J. Mauel
Associate Professor
Department of Pathobiology and Population Medicine
(Committee Member)

Terrence E. Greenway
Extension/Assistant Research Professor
Delta Research and Extension Center
(Committee Member)

Larry A. Hanson
Professor and Graduate Coordinator
Department of Basic Sciences

Kent H. Hoblet
Dean of the College of Veterinary Medicine

Name: Matthew John Griffin

Date of Degree: August 9, 2008

Institution: Mississippi State University

Major Field: Veterinary Medical Science

Major Professor(s): Dr. David J. Wise and Dr. Linda M. Pote

Title of Study: DEVELOPMENT AND APPLICATION OF A REAL- TIME POLYMERASE CHAIN REACTION ASSAY FOR THE MYXOZOAN PARASITE HENNEGUYA ICTALURI

Pages in Study: 130

Candidate for Degree of Doctor of Philosophy

Proliferative gill disease (PGD) caused by the myxozoan parasite *Henneguya ictaluri* is one of the most devastating parasitic infections in channel catfish aquaculture. Currently, there is no effective treatment for *H. ictaluri* and the unpredictable outbreaks can result in 100% mortality. Management strategies have been developed to prevent losses in newly stocked fingerlings by evaluating the PGD status of a pond prior to stocking, which is difficult since resident fish may not show clinical signs even when actinospore levels are lethal to naive fish. Current diagnostic methods are limited to the identification of an active infection and methods of predicting potential outbreaks have several limitations. The PGD status of a pond to be stocked can be determined using sentinel fish exposures which are labor intensive and require a source of parasite free fish. These limitations necessitated the development of more rapid and efficient means of determining actinospore concentrations to determine the risk of losing fish prior to stocking. The development of a quantitative real-time polymerase chain reaction (QPCR)

assay provided a more rapid, sensitive and quantitative method of diagnosing active infections and also provides a means to predict potential PGD outbreaks and determine the PGD status of a pond prior to stocking.

Another approach in the control of this parasite is the identification of a less susceptible culturable species or to identify traits that could be targeted in a selective breeding program. Challenge studies have shown that the closely related blue catfish (*Ictalurus furcatus*) does not exhibit as severe an inflammatory response to *H. ictaluri* and mortalities are significantly lower than in channel catfish. Comparisons of PGD severity and *H. ictaluri* infection in channel catfish, blue catfish and channel x blue catfish backcross hybrids by gross examination, histopathology and the newly developed *H. ictaluri* real-time PCR (QPCR) assay supported previous research suggesting the life cycle of the parasite can not be completed as efficiently through the blue catfish host.

This dissertation describes the development and validation of a QPCR assay to detect *H. ictaluri* in both fish tissues and environmental samples and the application of this assay in both research and production settings.

ACKNOWLEDGEMENTS

First, I would like to thank Dr. Al Camus, Dr. Linda Pote and Dr. David Wise, who all served as my co-major professor in some capacity during the course of this research. Without their help and seemingly undying patience, much of this work would never have been completed.

Secondly, I would like to extend my gratitude to Dr. Terry Greenway and Dr. Michael Mauel, for their guidance and painstaking review of this dissertation. I would also like to take this opportunity to thank the faculty and staff of the College of Veterinary Medicine, the Thad Cochran National Warmwater Aquaculture Center and the USDA CGRU for being supportive and accommodating throughout the course of my graduate studies.

Admittedly, much of the work described in this dissertation was extremely labor intensive and could not have been completed alone. As such, I would also like to acknowledge Monroe Walker, Dax Hinton, Stephen Ewing, Angela Ellington, Josh Walker, Cyndi Ware, Todd Byars and the histology labs in Starkville and Jackson for their contributions to this research.

I would also like to take the opportunity to acknowledge my fellow graduate students, Marlena Yost, Cynthia Doffitt and Angela Brandon, who were also very supportive, accommodating and tolerant over the course of this program. Their

contributions both inside and outside of the lab and classroom were invaluable. I would like to recognize my parents for all they do, my good friends Esteban Soto and Tim Streit for helping me survive Statistics and Dr. Lester Khoo for being Lester.

Lastly, I would like to express my deep appreciation and gratitude to my fiancé Shelley Meador, who not only assisted in various stages of this research, but has also demonstrated seemingly unwavering patience and understanding over the past 4 years. This could not have been done without her help and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The commercial farm-raised channel catfish industry

The channel catfish (*Ictalurus punctatus*) is the leading farm-raised fish in the United States. Since commercial channel catfish farming began in the 1960s, production and demand has increased significantly reaching annual sales of 445 million dollars in 2007. The rapid increase over the last thirty years has led channel catfish to become one of the most significant agricultural activities in the southeastern United States, with an economic impact of billions of dollars. In a number of counties in Alabama, Arkansas and Mississippi, channel catfish aquaculture is the primary source of employment and economic activity (Hargreaves and Tucker 2004). In the year 2000, the Mississippi catfish industry generated approximately 7,000 jobs, totaling nearly $102 million in wages.

Currently, there are channel catfish operations in 16 states, however, 94% of the catfish production in the United States is concentrated in Arkansas, Alabama, Louisiana and Mississippi, which has more acreage of production than the other three states combined (Hargreaves and Tucker 2004). In 2007, 360 operations utilized 92,500 water surface acres for the production of broodfish, foodfish, and fingerlings in Mississippi.

When compared with other agricultural commodities, the total crop value of $223 million was fifth to poultry ($2.3 billion), forestry ($1.11 billion), soybeans ($511 million), corn ($438 million) and cotton ($434 million) (Breazeale 2008). Although the catfish industry directly employs a significant percentage of the workforce in the Mississippi Delta region, most of the industry's influence on the economy is ancillary. The economic impact is amplified considering many of these regions are characterized by low economic development and high rates of unemployment (Dean 2003; Hanson et al. 2003).

Diseases associated with channel catfish

Intensive management schemes intended to maximize profitability have led to an increased potential for disease outbreaks due to stress from overcrowding, poor water quality and increased efficiency of disease transmission (Bellerud 1993). Increased stocking and feeding rates, the tendency to build large ponds (7-15 acres), and the introduction of multibatch systems, where younger fingerlings are stocked with older, larger fish, have led to an emergence of infectious disease problems over the last twenty years. Disease losses account for approximately 65% of fry and fingerling losses and 45% of foodfish losses, with bird depredation, dissolved oxygen depletions, and unexplained losses accounting for the remainder (Hargreaves and Tucker 2004).

Many infectious disease problems could be better managed by the use of smaller ponds, lower stocking rates, lower feed rates, and single batch aquaculture. Unfortunately, these solutions are not cost effective and are rarely economically favorable. Problems with water quality maintenance and treatment of infectious disease

would be simplified in smaller ponds but the cost of construction increases significantly with decreased pond size, and as pond sizes decrease, significantly more land acreage is used for levies and roads rather than production. The multi-batch system widely used by catfish farmers promotes disease transmission, but is economically favorable. Although the older, resident populations of fish can serve as reservoir hosts for disease and infect younger fish that are understocked into the system, the multi-batch culture provides a constant supply of on-flavor market size fish during the growout season and extends the productivity of the ponds. Lastly, lower stocking rates will improve production efficiency but may not generate sufficient income to cover the fixed and variable costs of production. Stocking rates normally fall between 100,000 - 120,000 fry per acre for fingerling production and 6,500 – 8,500 fingerlings per acre for food fish production. In short, reducing stocking rates to alleviate infectious disease concerns is not an economically favorable option for most operations (Hargreaves and Tucker 2004)

Records from the Aquatic Diagnostic Laboratory (ADL) of the Thad Cochran National Warmwater Aquaculture Center, Stoneville, MS (NWAC) show that 60% of diagnostic cases submitted can be attributed to single or mixed bacterial infections, 30% from parasitic infestation, 9% from fungal infections, and 1% from viral infections. From 1997 to 2007, the three most commonly diagnosed diseases were enteric septicemia caused by *Edwardsiella ictaluri*, columnaris disease from *Flavobacterium columnare*, and proliferative gill disease (PGD) resulting from infection by the myxozoan parasite *Henneguya ictaluri* (Khoo et al. 2008).

Proliferative gill disease in channel catfish

Proliferative gill disease (PGD) is the most prevalent parasitic disease affecting commercial catfish aquaculture in the southeast United States and has caused major economic losses since 1981 (Bowser et al. 1985; MacMillan et al. 1989; Burtle et al. 1991; Styer et al. 1991; Wise et al. 2004). Often referred to as "hamburger gill", PGD is characterized grossly by swelling, hemorrhage, inflammation and distortion of the gill filaments creating a mottled appearance, resulting in gills that resemble ground meat. Breaks in the filamental cartilage and the induced inflammatory response from localization of the developing organisms causes gill filaments to become fragile and break (Fig. 1.1). Hyperplasia of the gill epithelial cells (Fig. 1.2) and the influx of inflammatory cells associated with the developing organism (Fig. 1.3) significantly reduce the gill surface area (Bowser et al. 1985; MacMillan et al. 1989; Mitchell et al. 1998; Wise et al. 2004), inhibiting the catfish's ability to carry out vital physiological processes, namely gas exchange and osmoregulation. Severely afflicted fish swim listlessly near the water surface exhibiting behaviors consistent with oxygen stress. Because of the common occurrence and signature clinical signs of PGD, the disease is often diagnosed pondside by producers instead of diagnostic labs, resulting in the underestimation of the true prevalence of the disease throughout the industry (Wise et al. 1999; 2004).

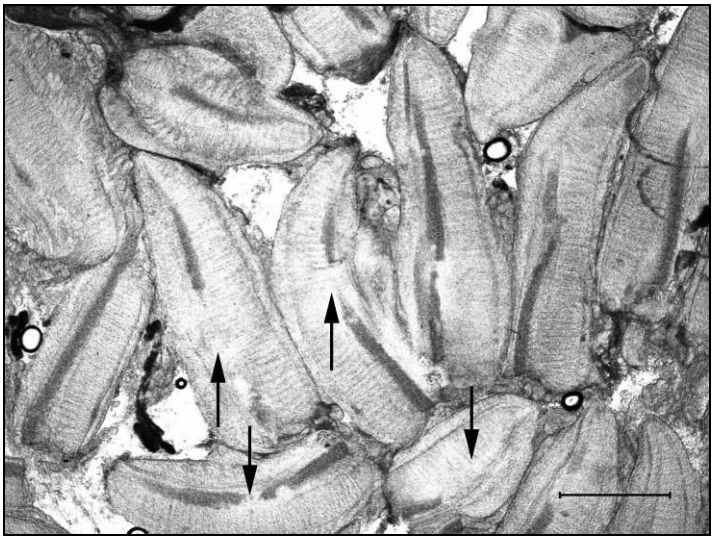


Figure 1.1. Wet mount preparation of gill clips from a channel catfish fingerling with proliferative gill disease. The chondrolytic lesions of the filamental cartilage (arrows) are indicative of proliferative gill disease. Bar = 1 mm.

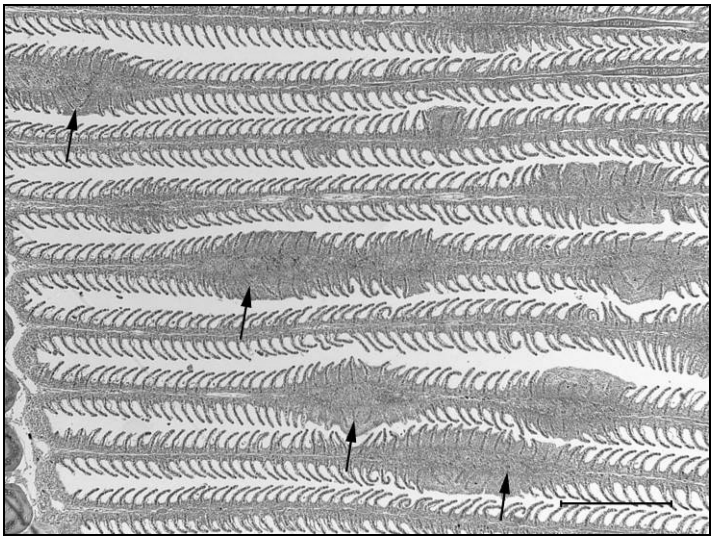


Figure 1.2. Histologic section of channel catfish fingerling with proliferative gill disease. Arrows point to diffuse fusion of gill lamellae due to epithelial hypertrophy and hyperplasia. Bar = 0.5 mm

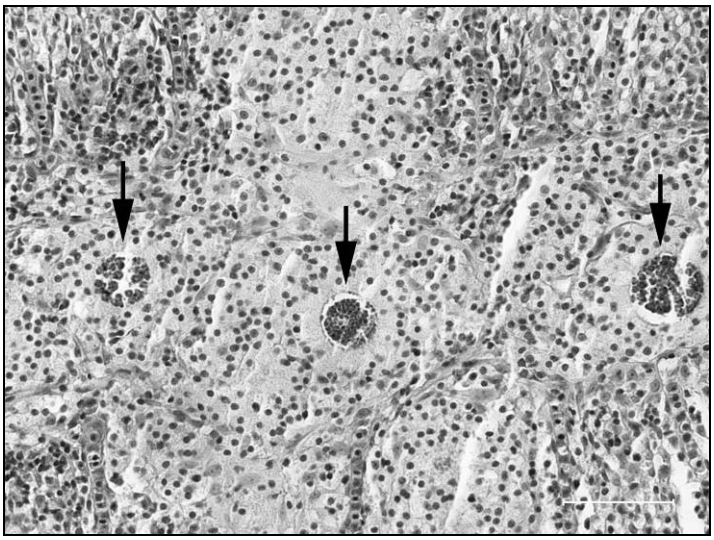


Figure 1.3. Histologic section of gill tissue from a channel catfish fingerling with proliferative gill disease. Deeply basophilic granular aggregates (arrows) are composed of multiple small trophozoites. Bar = 50 μm

Outbreaks of PGD are currently highly unpredictable and mortality rates, although highly variable, can approach 100% in severe outbreaks. The disease causes significant losses in all sizes of fish, however smaller fish tend to be more susceptible to the disease (Wise et al. 2004).

Myxozoan parasites

The myxozoans are an important group of organisms represented by more than 2000 species in over 60 genera (Lom and Dykova 2006). Primarily parasites of fish, myxozoans have been extensively studied since first identified in the early 19th century (Lom 1987). Some economically important myxozoans include *Myxobolus cerebralis* and *Ceratomyxa shasta,* which cause salmonid whirling disease and ceratomyxosis, respectively (Wolf et al. 1984; Andree et al. 1997; Bartholomew et al. 1997). Myxozoans

have been reported in a wide variety of organs in fish and are relatively host and site specific (Lom 1987). The variations in morphological characteristics and complex life cycles have added further confusion to the classification of this large group of organisms and the phylogeny of the group remains in constant flux (Kent et al. 2001).

The initial work pertaining to the myxozoan life cycle was done by Wolf and Markiw (1984). Their research with *M. cerebralis* was the first to indicate the triactinomyxon actinospore stage shed by the benthic oligochaete, *Tubifex tubifex,* was an alternate life stage of the myxospore stage found in the fish (Wolf and Markiw 1984; Wolf et al. 1986). Since then numerous lifecycles have been defined using transmission studies and molecular techniques which connect the myxospore stage in the fish to the alternate actinospore stage in the oligochaete (Wolf and Markiw 1984; Wolf et al. 1986; Andree et al. 1997; Bartholomew et al. 1997; Longshaw et al. 1999; Eszterbauer et al. 2000; Pote et al. 2000; Kent et al. 2001; Redondo et al. 2002; Liyanage et al. 2003; Koie et al. 2004; Kallert et al. 2005a; 2005b; Bartholomew et al. 2006). Although myxozoans are an intensively studied group, there are still numerous species of myxozoans in both oligochaete and fish hosts whose life cycles remain unknown. Adding further to the classification confusion is the initial placement of actinospore and myxospore stages in separate taxonomic classes (Andree et al. 1997).

Henneguya ictaluri

Researchers first suspected an infectious agent of benthic origin to be associated with PGD when investigators were able to induce PGD in specific-pathogen-free catfish

by exposure to mud extracted from a pond with an active PGD outbreak (MacMillan et al. 1989). This work narrowed the search leading to the discovery of the causative organism associated with PGD (Burtle et al. 1991; Styer et al. 1991), the myxozoan parasite *Henneguya ictaluri* (Pote et al. 2000).

An actinospore, *Aurantiactinomyxon* sp., shed by the cosmopolitan benthic oligochaete *Dero digitata* was identified as the causative agent of PGD (Styer et al. 1991) (Fig. 1.4). After release by the oligochaete, the actinospore floats throughout the water column where it comes into contact with the catfish, releases the infective sporoplasm into the host and migrates through tissues, with the final development to the myxospore stage in the gills (Pote and Waterstrat 1993; Belem and Pote 2001). Recent DNA evidence has linked the actinospore to the myxosporean parasite, *H. ictaluri*. Molecular evidence shows that the *Aurantiactinomyxon* sp. discovered by Styer et al. (1991) and *H. ictaluri* are alternate life stages of the same organism (Pote et al. 2000). To date, 30 myxozoan life cycles have been proposed, all involving alternating actinosporean stages in oligochaete worms and a myxospore life stage in fish (Kent et al. 2001; Koie et al. 2004; Kallert et al. 2005a; 2005b; Bartholomew et al. 2007).

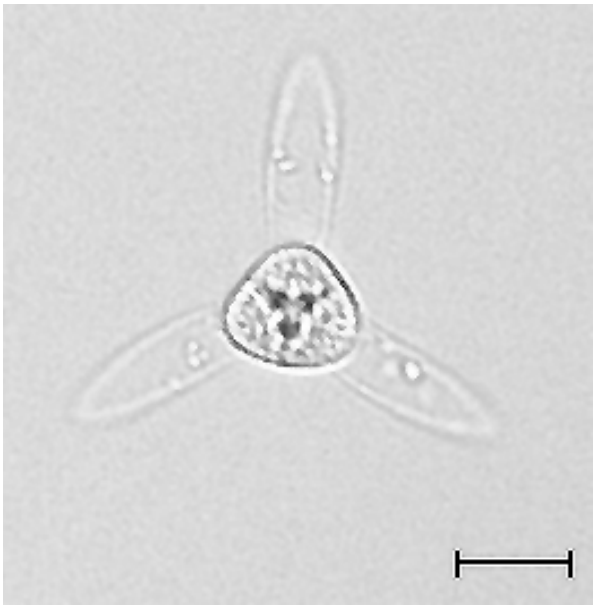


Figure 1.4. *Henneguya ictaluri* aurantiactinomyxon type actinospore. Bar = 20 µm

The environmental or chemical cues which influence the release of *H. ictaluri* actinospores from the oligochaete host have yet to be determined. The release of the triactinomyxon stage of *M. cerebralis* from the oligochaete host, *T. tubifex*, has been shown to be temperature dependent (Markiw 1992; El-Matbouli et al. 1999), and both research and case reports from the ADL, have shown that PGD outbreaks most often occur, but are not limited to, spring and fall when water temperatures are between 16-25 °C (MacMillan et al. 1989; Wise et al. 2004; Khoo et al. 2008). Sporadic outbreaks have been reported throughout the year at temperatures outside of this range but incidences are rare. The most severe outbreaks occur during the spring, from late March to early June, with a lesser incidence in the fall (Styer et al. 1994; Wise et al. 1999; 2004; Khoo et al. 2008).

PGD more commonly occurs in newly constructed or recently reworked older ponds (Macmillan et al. 1989; Johnson 1989; Styer et al. 1991). *Dero digitata* is considered a pioneering organism and it is speculated that newly constructed or reworked ponds provide an environment where *D. digitata* can establish high populations in a noncompetitive environment. As the number of potential oligochaete hosts increase, the conditions of the pond become favorable for rapid expansion of the myxozoan populations within the system. As ponds age, *D. digitata* populations may be displaced by competing benthic organisms resulting in significantly lower populations and reducing the number of hosts capable of releasing the actinospore stage infective to the fish (Killian 1994). It is still unclear how or when the myxospore stage is released by the fish or how the oligochaete host is infected, but evidence points to a bi-modal cycle. Pote et al. (2000) observed mature myxospores in channel catfish fingerlings 90 days post-exposure to *H. ictaluri* actinospores, which correlates to observed case reports of highest incidence in the spring and fall. Fish infected in spring would release mature myxospores approximately 90 days post exposure, or mid summer. Released myxospores would be ingested by the benthic oligochaetes and develop in the intestine over the course of another 90 days, undergo maturation and be released as actinospores in the fall. Ninety days post infection, approximately mid-winter, myxospores would again be released to infect the oligochaete, which undergo development and are released from the oligochaetes as actinospores in the spring. Although there is evidence through case reports and infection studies to support this hypothesis, the life cycle has yet to be completed in a laboratory setting and many questions regarding the required

environmental conditions for the life cycle remain unanswered (Styer et al. 1991; Wise et al. 1999; Pote et al. 2000; Wise et al. 2004).

A significant problem with PGD is often seen in early spring, when fingerlings understocked in production ponds for growout experience significant losses attributed to PGD, even though the resident fish population shows no clinical signs of the disease. It remains unclear whether this is due to sudden exposure of naïve fish to lethal concentrations of actinospores or if larger resident fish are more resistant to the disease.

The increased disease prevalence and severity observed in the spring often results in devastating losses to operations under-stocking ponds at the beginning of the production cycle. However, PGD is not a major problem for most fingerling operations. Typical fingerling operations stock fry in May and June and harvest fingerlings prior to the start of the next spawning season (late winter or early spring). Harvested ponds are then drained and either dried or poisoned to kill any remaining fish. Ponds are refilled and left fallow for approximately one month before hatchery fry are reintroduced into the system. This practice may inadvertently break the life cycle of the parasite as there are no fish hosts present for the actinospore stage to infect during periods of peak actinospore release. In addition since hatchery fry are raised in a PGD free environment, the myxospore stages infective to *D. digitata* are not being introduced into the system. Although speculative, these two factors may work in conjunction to minimize the incidence of PGD in fingerling operations. In support of this theory, PGD is rarely diagnosed within the first three months of production and on fingerling operations, is

most commonly found in fingerlings that have been held in ponds for more than one production cycle.

Even though recent advances in the understanding of other myxozoan life cycles have provided insight into the biology of this organism, knowledge of the epidemiology and pathogenesis of PGD remains incomplete. Therefore, to avoid significant losses, current strategies focus on manipulating stocking and culture practices aimed at reducing exposure of fish to conditions that result in severe manifestations of the disease (Wise et al. 2004). Currently there are no therapeutic or prophylactic treatments for PGD and attempted biosecurity methods have been unsuccessful (Mischke et al. 2001).

Treatment and control strategies

Although, there are no approved chemotherapeutic treatments for PGD, there has been some evidence that subcutaneous injections of the cycloxygenase inhibitor, indomethacin (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1-H-indole-3-acetic acid), can reduce the severity of gill lesions in catfish although mortalities still occur. These findings suggest that prostaglandins, products of the cyclooxygenase pathway, participate in the pathophysiologic host response to PGD and give some insight into the mechanism of the disease (Davis 1994).

Recently, there have been several reports utilizing pharmaceutical substances, particularly anticoccidials, for the treatment of myxosporean infections in fish. The anticoccidial, salinomycin, and the antimalarial, quinine, were found to be efficacious against *Henneguya* sp. infection in the tapir fish *Gnathonemus petersii* (Dohle et al.

2002). There is also evidence of successful control of *Myxobolus* sp. infections in sharpsnout sea bream using the anticoccidials toltrazuril, salinomycin and amprolium (Athanassopoulou et al. 2004). Fumagillin, an antibiotic commonly used to control the microsporean parasite *Nosema epis* in honeybees, has been shown to be effective against renal sphaerosporosis in carp (Molnar 1987), proliferative kidney disease in salmonids (Hedrick et al. 1988) and *Myxobolus* sp. infections in sharpsnout sea bream (Athanassopoulou et al. 2004). El-Matbouli and Hoffmann (1991) found fumagillin effective in preventing clinical outbreaks of whirling disease in rainbow trout experimentally infected with *M. cerebralis*, however, a later study adhering to protocols meeting U.S. Food and Drug Administration requirements for new animal drug approval, found fumagillin to be ineffective in the prevention or control of whirling disease (Gilbert and Granath 2003). There are currently no published reports regarding the efficacy of fumagillin, toltrazuril, amprolium, salinomycin or quinine to target the myxozan stage of *H. ictaluri* in channel catfish.

Chemical therapeautics which target the oligochaete host without damaging the rest of the pond ecosystem have also been investigated. In 2001, researchers investigated the ability of numerous chemical agents to interrupt the life cycle of *H. ictaluri* through the elimination of the oligochaete host, *D. digitata*. Formalin, Chloramine – T, sodium chloride (NaCl), potassium permanganate ($KMNO_4$), copper sulfate ($CuSO_4$), hydrogen peroxide (H_2O_2), Rotenone® ($C_{23}H_{22}O_5$, 5% solution, Prentiss, Inc., Sandersville, Georgia, USA) and Bayluscide® (niclosamide, 70% wettable powder, Bayer Chemical Co., Kansas City, Missouri, USA), were all tested for their ability to eliminate the

oligochaete worm without damaging the fish population. Unfortunately, doses required for these agents to successfully eradicate the *D. digitata* population are not cost effective and require multiple treatments, making them an impractical treatment option (Mischke et al. 2001). Futhermore, Bayluscide® is highly toxic to catfish.

Chemical agents are a useful tool after an outbreak has occurred and the pond has been drained, but they are not successful in eliminating the oligochaetes while fish are present. The benthic substrate and organic matter in ponds inhibit the efficacy of chemical treatments, raising chemical doses to achieve a LC50 for *D. digitata* to doses also lethal to fish (Mischke et al. 2001). It seems evident that novel management practices may be the only feasible solution to the PGD problem.

Common carp (*Cyprinus carpio*) used as a biological control agent of the oligochaete host appear to have limited success initially, but as the carp increase in size the smaller oligochaetes are no longer a preferred food organism and repeated stocking of appropriately sized carp is impractical in a commercial catfish ponds (Burtle and Styer 1996). Similarly, the fathead minnow (*Pimephales promelas*) has been proposed as a potential biological control agent in catfish ponds. Fatheads are small fish which feed primarily on oligochaetes and algae, are economically favorable, and also serve as a dietary supplement for the catfish. Unfortunately, predation by catfish will decimate a fathead population unless adequate spawning areas are provided. In order for fathead minnows to be an efficient biological control method their numbers need to be maintained above 2,000 per acre, which has proven to be a difficult task (Burtle 1998). Smallmouth buffalo (*Ictiobus bubalus)* have also been used as a possible biological

control agent with stocking densities of 2-3 fish per acre, but reported success is anecdotal, and current research is unsubstantiated.

Supportive therapies for PGD involve restricted feeding to reduce the oxygen demand of the fish, and increased aeration and pond salinity to reduce oxygen stress. Although these actions may reduce mortality, reported success is anecdotal (Mitchell et al. 1998; Wise et al. 2004). Water quality should be monitored closely to prevent the onset of nitrite induced methemoglobinemia, which decreases the oxygen carrying capacity of the blood (Huey et al. 1980, Bowser et al. 1985), potentially exacerbating losses to PGD. Although mechanical aerators increase dissolved oxygen in the pond, they may contribute to the problem by increasing the dispersal of the actinospore stage throughout the water column, although this is speculative and without subsequent aeration significant losses may occur regardless. In salmonid aquaculture, the life cycle of myxozoans is broken by culturing fish in concrete raceways or other culture units that do not provide the earthen substrate required by the oligochaete host, which is not economically feasible in large catfish ponds.

Recovery has been observed in fish moved to a clean, well oxygenated environment and has proven to be effective in avoiding mortality if the outbreak is caught early enough (Wise et al. 2004). Theoretically, catfish infected with *H. ictaluri* can perpetuate the life cycle if placed in a pond without an ongoing outbreak, and there is potential to spread the parasite. Research has shown, however, that *H. ictaluri* is endemic on most catfish farms, and the parasite is likely to already be present in a majority of the commercial catfish ponds (Bellerud 1993; Bellerud et al. 1995; Wise et al. 2004; 2008).

Monitoring and surveillance of PGD using sentinel fish allows farmers to determine when it is safe to stock a pond following a PGD outbreak. Research has shown there is a strong correlation between the percentage of broken gill filaments in affected fish and pond mortalities (Wise et al. 2004; 2008). The Fish Health Management Program and the Thad Cochran National Warmwater Aquaculture Center has developed a protocol to determine severity of PGD in ponds. Using parasite free sentinel catfish, the potential for severe outbreaks in the spring or the condition of a pond following a PGD outbreak can be determined. Ten specific pathogen free (SPF) fish are held in net-pens or cages for 7 days. On day 7, fish are necropsied and gill tissues are examined microscopically for chondrolytic lysis of the gill filaments. Quantitative evaluation of the infection is determined by calculating the percent of primary gill lamellae containing lytic lesions in the cartilage of approximately 40-80 gill filaments from the same side of each fish. A mild infection, described as 1-5% of gill filaments exhibiting chondrolytic lesions, has little to no effect on the health of the fish. Moderate infections, in which no direct mortalities are observed, usually correlate with 6-15% of filaments exhibiting condrocytic lysis. Severe infections, where mortalities are observed in 1-2 weeks, will have breaks in greater than 15% of examined filaments. In the presence of a moderate to severe infection the sampling protocol is repeated for another 7 days, at which point, gills are examined again as described previously. When the severity of infection decreases from one sampling period to the next and losses do not occur in sentinel fish, the system can be stocked with little risk of losing fish. Unfortunately, severe gill damage and death often occurs in less than 7 days post exposure, resulting in a

need to repeat the protocol, resulting in a delay in determining the state of infection in a given pond (Wise et al. 1999; 2004; 2008). Other disadvantages are that the protocol requires a source of SPF fish, hauling tanks, transport equipment and the protocol takes a minimum of two weeks to determine the dynamics of the ongoing infection. This protocol is also limited in that sentinel fish are not of practical use to accurately predict when a pond will have a PGD outbreak during the PGD season and it cannot identify an increase in mild, sub-clinical infections between sampling periods.

Another potential control measure is to culture a catfish species less susceptible to PGD. Blue catfish (*Ictalurus furcatus*) possess several attributes which make them desirable for aquaculture. They have a comparable dressing percentage to channel catfish, are relatively easy to seine, have high individual weight gains in temperate regions, and are more resistant to several diseases that affect channel catfish, such as enteric septicemia and channel catfish virus (Giudice 1966; Tidwell and Mims 1990; Graham 1999). Research has also shown that channel catfish and blue catfish are affected differently by *H. ictaluri* infection. When placed in ponds with an outbreak of PGD, blue catfish displayed significantly less gill damage than channel catfish simultaneously held in the same pond (Bosworth et al. 2003). However, aquaculturists find blue catfish are slower to mature, have relatively poor food conversion rates, low captive spawning rates and are more susceptible to infection by the gram-negative bacteria *Flexibacter columnare* and the protozoan parasite *Ichthyophthirius multifiliis* than channel catfish (Dunham et al. 1993; Graham 1999).

H. ictaluri specific polymerase chain reaction

Several recent reviews have discussed advances in our understanding of the life histories and phylogeny of the myxozoa (Kent et a. 2001; Fiala 2006). Much of this knowledge is attributable to the advent of molecular methods, namely PCR and end-label sequencing of 18S small subunit (SSU) rDNA, which in addition to morphological characteristics has allowed more reliable identification and distinction between similar species. The 18S SSU rDNA sequences of many myxozoans are now readily available via Genbank, and novel sequences are added regularly. This provides the resources required to rapidly obtain a phylogenetic classification of a novel organism once the appropriate genetic sequence is obtained. Another advantage of identification by 18S SSU rDNA sequences is the ability to identify alternate actinospore and myxospore life stages in oligochaete and fish hosts, molecularly confirming many previously unknown life cycles (Andree et al. 1997; Bartholomew et al. 1997; Palenzuela et al. 1999; Lin et al. 1999; Anderson et al. 2000; Pote et al. 2000; Whitaker et al. 2001; Grossel et al. 2005; Kallert 2005a; 2005b.).

Using primers specific for the 18S SSU rDNA of *H. ictaluri*, researchers have developed a diagnostic polymerase chain reaction (PCR) assay for *H. ictaluri* by comparing the genetic sequences of four actinosporean species commonly found in catfish ponds (Lin et al. 1999; Pote et al. 2000; Hanson et al. 2001; Whitaker et al. 2001; Whitaker et al. 2005.) The ability of the PCR test to detect *H. ictaluri* infection in channel catfish was assessed by comparing the DNA based assay to conventional diagnostic methods of histology and microscopic examination of gill clip wet mounts.

Researchers found the DNA based assay to be more sensitive than conventional diagnostic methods and more accurate in detection of sub-clinical infections (Whitaker et al. 2001), although the relationship to sub-clinical infections and mortality has not been shown.

Furthur research involved utilizing the *H. ictaluri* specific PCR assay in the detection of the actinospore stage in benthic oligochaetes and water samples. The PCR assay detected early stages of *H. ictaluri* in oligochaetes and water samples, prior to infection of the channel catfish population, confirming the organism can be detected prior to infection. Correlation of the PCR assay to clinical signs of PGD is difficult however, because PCR is subjectively quantifiable (Whitaker et al. 2005). There is also some debate regarding the length of time actinospores remain infective following release by the oligochaete host. Research has identified differences based on water temperature and species, but generally the actinospores stage could remain viable from 5-12 d (Ratliff 1983; Markiw 1992; Yokoyama et al. 1993; Xio and Desser 2000; Whitaker et al. 2005). Current research regarding the viability of the *H. ictaluri* actinospore identified the infective window to be approximately 24 h post release (Wise et al. 2004; 2008), therefore PCR of water samples is representative of total DNA copies collected, and not necessarily infective actinospores.

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction assays (QPCR) have made PCR a quantitative molecular technique and are rapidly becoming standard procedures in a

wide variety of diagnostic settings (Mackay et al. 2002). Real-time PCR combines nucleic acid amplification and detection into a single step utilizing different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity. This gives researchers the ability to simultaneously detect and quantify specific nucleic acid sequences (Higuchi et al. 1993; Wong and Medrano 2005; Houghton and Cockerill 2006). QPCR reactions are characterized by the cycle in which fluorescence intensity reaches a predetermined threshold significantly greater than background fluorescence, referred to as the cycle threshold (C_T). As a consequence of PCR chemistries, the greater the initial concentration of target nucleic acid, the earlier amplification will be detected. As such, lower C_T values represent greater starting quantities of target nucleic acid (Higuchi et al. 1993; Heid et al. 1996; Wong and Medrano 2005).

There are several quantification strategies, but in cases where samples are analyzed on different days on different plates, absolute quantification by a standard curve is preferred (Wong et al. 2005). Using serially diluted standards of a known nucleic acid concentration, a standard curve can be generated that produces a linear relationship between C_T and initial concentrations of the target nucleic acid. Standards are generally the target nucleic acid sequence cloned into a plasmid. Assuming that all standards and samples have approximately equal amplification efficiencies, the concentrations of target nucleic acid sequences from unknown samples can be calculated according to their C_T values and plasmid standard equivalents (PSE) can be determined (Heid et al. 1996; Souaze et al. 1996; Wong et al. 2005). QPCR provides researchers with a nucleic acid based assay that not only confirms the presence/absence of the target organism, but can

indirectly quantify the amount of pathogen present in host tissue or environmental samples.

Detection and quantification of waterborne parasites in environmental samples

QPCR has been utilized in the detection of pathogens in a number of systems, including fish and environmental water samples (del Cerro et al. 2001; Bilodeau et al. 2003; Corbeil et al. 2003; Fontaine et al. 2003; Guy et al. 2003; Cavander et al. 2004; Chase et al. 2006; Balacázar et al. 2007; Panangala et al. 2007). Recently, a QPCR assay has been utilized to detect the presence of the actinospore stage of *C. shasta* in river water samples (Hallett and Bartholomew 2007).

Currently there are a vast array of methods utilized in the concentration, purification and detection of infective stages of waterborne parasites (Marshall et al. 1997). Filtration methods have been used to collect the actinospore stages of *M. cerebralis* and *C. shasta* from streams (Thompson and Nehring 2000; Arndt and Wagner 2003; Hallett and Bartholomew 2006), but there are two major hurdles to overcome in order to utilize a filtration method of actinospore quantification in catfish ponds. Primarily, the extremely small size of the target organism and the abundance of phytoplankton, zooplankton and fine particulates make filtering catfish pond water difficult and cumbersome as the filter is easily clogged. Secondly, humic substances, often present in environmental samples, are a major source of PCR inhibition. However, there have been several methods proposed to separate DNA from these organic inhibitors (Wilson 1997). Presently, there are commercial kits available for the separation and

purification of DNA from humic substances which provides rapid and efficient processing of DNA samples from problematic substrates (Mota et al. 2005). Using a modification of the methodologies described by Hallett and Bartholomew (2006), a commercial soil DNA extraction kit designed to specifically remove humic compounds and other potential PCR inhibitors could be used in conjunction with several centrifugation steps to isolate and quantify *H. ictaluri* DNA from pond water samples.

The water sampling protocol was used in conjunction with gross examination of gill clip wet mounts in an attempt to correlate actinospore concentrations in catfish ponds to disease severity observed in sentinel fish. Quantification of actinospore concentrations in pond water can then be used in lieu of sentinel fish for determination of the PGD status of a pond prior to stocking.

Detection and quantification of *H. ictaluri* in fish tissue

As described above, QPCR assays have been used in the detection of a number of pathogens in fish tissue (del Cerro et al. 2001; Bilodeau et al. 2003; Corbeil et al. 2003; Covander et al. 2004; Balacázar et al. 2007; Panangala et al. 2007). These assays often provide greater sensitivity than conventional diagnostic techniques and they offer the ability to quantify pathogens at low levels of infection. Based on these studies, a protocol will be developed to detect and quantify *H. ictaluri* in gill tissue in sentinel fish exposed to a PGD positive pond for 24 hours. Using a similar strategy as described above, the condition of a pond, with respect to PGD, can be determined in 1 week by sampling sentinel fish at 2 separate 24 hour periods, 7 days apart. Since *H. ictaluri* actinospores

are infective for approximately 24 hours post-release (Wise et al. 2004), molecular detection of *H. ictaluri* in fish observed 24 hours post-exposure to a pond indirectly represents the rate at which actinospores are being released into the pond.

It is hypothesized that the quantifiable nature of the assay will provide a means to identify increasing or decreasing concentrations of actinospores in the pond. In conjunction with gill clip wet mounts, a pond identified as having a moderate to mild infection with a decreasing rate of actinospore release could be restocked with a lower risk of losing fish. The QPCR methodology will provide information on the PGD status of a pond in seven days rather than the minimum of two weeks required for the current protocols being used by the industry.

H. ictaluri infection in channel catfish, blue catfish and blue x channel hybrids

Differences in susceptibility to myxozoan infection between closely related fish species is not uncommon, as demonstrated by the varying levels of susceptibility of closely related salmonids to *M. cerebralis* and *C. shasta* infection (Ibarra et al. 1992; 1994; Hedrick et al. 1999a; 1999b). The etiology of the observed differences in response of blue catfish, channel catfish and blue x channel hybrids to *H. ictaluri* exposure is unclear (Bosworth et al. 2003). It is unknown whether *H. ictaluri* is able to penetrate and establish infection in blue catfish but does not illicit the same inflammatory response as in channel catfish and hybrids or if *H. ictaluri* penetrates and enters the blue catfish but is cleared by the hosts defenses before infection can be established, or if the blue catfish possess a defense mechanism that prevents the organism from penetrating and entering

the blue catfish altogether. In hopes of providing some insight to the questions raised above, a comparative study of the response of channel catfish, blue catfish, and blue x channel hybrids to water from a pond with an active outbreak of PGD was conducted. Using all of the currently available diagnostic techniques, blood and gill tissues were analyzed for differences in the parasite loads of blue catfish, channel catfish and their hybrid cross when simultaneously exposed to a PGD positive pond.

Research goals

The objectives of this research were to develop a QPCR assay for *H. ictaluri* to be used as a diagnostic and research tool, to evaluate its ability to detect and quantify the life stages of *H. ictaluri* in fish tissue and environmental samples, and to utilize this tool in a comparative study of PGD infection in blue catfish, channel catfish and their hybrid cross. Validation of the QPCR assay will be a major building block in the development of risk assessment and epidemiological models for PGD. Once methodologies for QPCR protocols are validated, the assay can be used for PGD epidemiology studies to monitor disease activity in ponds, identify potential point sources of seasonal outbreaks, assess treatments, demonstrate the relationship between actinospore release and disease severity and provide catfish farmers with a method of determining when ponds are safer to restock.

Additionally, since DNA is conserved throughout the life cycle regardless of host, QPCR will be able to monitor the development of the organism in fish tissue. This will allow researchers to compare the course of infection of *H. ictaluri* in different species of

fish, thus expanding our understanding of host susceptibility. Identification of mechanisms used to prevent development of proliferative gill disease in blue catfish could prove invaluable in a selective breeding program.

Literature Cited

Anderson, C. L., E. U. Canning, S. M. Schafer, H. Yokoyama, B. Okamura. 2000. Molecular confirmation of the life cycle of *Thelohanellus hovorkai* Achmerov, 1960 (Myxozoa: Myxosporea). Bulletin of the European Association of Fish Pathologists. 20: 111-115.

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

Arndt R. E., E. J. Wagner. 2003. Filtering Myxobolus cerebralis triactinomyxons from contaminated water using rapid sand filtration. Aquacultural Engineering 29: 77-91

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

Balacázar J., L. D. Vendrell,I. de Blas I., I. Ruiz-Zarzuela, O. Gironés, J. L. Múzquiz. 2007. Quantitative detection of *Aeromonas salmonicida* in fish tissue by real-time PCR using a self-quenched flourogenic probe. Journal of Medical Microbiology. 56: 323-328

Bartholomew J. L., Whipple M. J., Stevens D. G., J. L. Fryer . 1997. The life cycle of *Ceratomyxa shasta*, a myxosporean parasite of salmonids, requires a freshwater polychaete as an alternate host. J Parasitol. 83: 859-68

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

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

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

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

Bilodeau, L. A., G. C. Waldbieser, J. S. Terhune, D. J. Wise, W. R. Wolters. 2003. A real-time polymerase chain reaction assay of the bacterium *Edwardsiella ictaluri* in channel catfish. Journal of Aquatic Animal Health. 15: 80-86

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

Bowser PR, JD Conroy: 1985, Histopathology of gill lesions in channel catfish associated with *Henneguya*. Journal of Wildlife Diseases 21:177-179

Breazeale, L. 2008. Mississippi's 2007 crops combine for record year. Mississippi State University Office of Agricultural Communications. Accessed online June 4, 2008. http://msucares.com/news/print/agnews/an07/071213overall.html

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

Burtle, G. J., E. L. Styer. 1996. Biological control of proliferative gill disease of channel catfish: fish as predators. Annual Report Department of Animal and Dairy Science. The University of Georgia, College of Agricultural and Environmental Sciences. 1-5.

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

Chase D. M., D. G. Elliott, R. J. Pascho. 2006. Detection and quantification of *Renibacterium salmoninarum* DNA in salmonid tissues by real-time quantitative polymerase chain reaction analysis. Journal of Veterinary Diagnostic Investigation. 18: 375-380

Corbeil, S., K. A. McColl, M. J. Craine. 2003. Development of TaqMan quantitative PCR assay for the identification of *Piscirickettsia salmonis*. Bulletin of the European Association of Fish Pathology 23: 95-101.

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

Davis, S. 1994. Effect of indomethacin on the development of proliferative gill disease. *Journal of Aquatic Animal Health*. 6: 122-125.

Dean, S., T. Hanson, S. Murray. 2003. Economic impact of the Mississippi farm-raised catfish industry at the year 2003. Mississippi State Extension Service publication no. 2317. 2003.

del Cerro, A., M. C. Mendoza, J. A. Guijarro. 2002. Usefulnes of a TaqMan-based polymerase chain reaction assay for the detection of the fish pathogen *Flavobacterium psycrophilium.* Journal of Applied Microbiology 93: 149-156.

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

Dunham, R. A., C. Hyde, M. Masser, J.A. Plumb, R.O. Smitherman, R. Perez and A.C. Ramboux. 1993. Comparison of culture traits of channel catfish, *Ictalurus punctatus*, and blue catfish, *I. furcatus*. Journal of Applied Aquaculture 3: 257-267.

El-Matbouli, A., R. W. Hoffmann. 1991. Prevention of experimentally induced whirling disease in rainbow trout *Oncorhynchus Mykiss* by Fumagillin. Diseases of Aquatic Organisms 10: 109-113.

El-Matbouli, M., T. S. McDowell, D. B. Antonio, K. B. Andree, R. P. Hedrick. 1999. Effect of water temperature on the development, release and survival of the triactinomyxon stage of *Myxobolus cerebralis* in its oligochaete host. International Journal for Parasitology. 29: 627-641.

Eszterbauer, E., C. S. Szekely, K. Molnar, F. Baska. 2000. Development of *Myxobolus bramae* (Myxosporea: myxobolidae) in an oligochaete alternate host, *Tubifex tubifex*. Journal of Fish Diseases. 23: 19-25.

Fontaine, M., E. Guillot. 2003. An immunomagnetic separation – real-time PCR method for quantification of *Cryptosporidium parvum* in water samples. Journal of Microbiological Methods. 54: 29-36.

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

Gilbert, M., W. Granath. 2003. Whirling disease of salmonid fish: life cycle, biology and disease. Journal of Parasitology. 89: 658-667.

Giudice, J.J. 1966. Growth of a blue x channel catfish hybrid compared to its parent species. Progressive Fish-Culturist 28: 142-145.

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

Grossel, G., J. Handlinger, S. Battaglene, B. Munday. 2005. Diagnostic polymerase chain reaction assay to detect *Kudoa neurophila* (Myxozoa: Multivalvulida) in a marine finfish hatchery. Diseases of Aquatic Organisms. 64: 141-149.

Guy, R. A., P. Payment, U. J. Krull, P. A. Horgen. 2003. Real-Time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Applied and Environmental Microbiology. Sept. 2003, p. 5178-5185.

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

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

Hanson, T., S. Dean, S. Spurlock. 2003. Economic impact of the farm-raised catfish industry on the Mississippi state economy. Journal of Applied Aquaculture. 15: 11-28.

Hanson, T. 2008. 2007 U. S. Catfish Database. Accessed online June 4, 2008. http://www.agecon.msstate.edu/research/catfish2007db.php

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

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

Hedrick, R. P., J. M. Groff, P. Foley, T. S. Mcdowell. 1988. Oral administration of fumagillin DHC protects chinook salmon (*Oncorhynchus tshawytscha*) from experimentally induced proliferative kidney disease. Diseases of Aquatic Organisms 4: 165–168.

Hedrick, R. P., T. S. McDowell, M. Gay, G. D. Marty, M. P. Georgiadis, E. MacConnell. 1999a. Comparative susceptibility of rainbow trout *Onchorynchus mykiss* and brown trout *Salmo trutta* to *Myxobolus cerebralis*, the cause of salmonid whirling disease. Diseases of Aquatic Organisms: 173-183

Hedrick, R.P., T. S. McDowell, K. Mukkatira, M. P. Georgiadis, E. MacConnell. 1999b. Susceptibility of selected inland salmonids to experimentally induced infections with Myxobolus cerebralis, the causative agent of whirling disease. Journal of Aquatic Animal Health. 11: 330-339.

Heid, C. A., J. Stevens, K. J. Livak, P. M. Williams. 1996. Real time quantitative PCR. Genome Research. 6: 986-994.

Higuchi, R., C. Fockler, G. Dollinger, R. Watson. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology. 11: 1026-1030.

Houghton, S. G., F. R. Cockerill. 2006. Real-time PCR: Overview and applications. Surgery. 139: 1-5.

Huey, D. W., B. A. Simco, D. W. Criswell. 1980. Nitrite-induced methemoglobin formation in channel catfish. Transactions of the American Fisheries Society. 109: 558-562

Ibarra, A. M., R. P. Hedrick, G. A. E. Gall. 1992. Inheritance of susceptibility to *Ceratomyxa shasta* (Myxozoa) in rainbow trout and the effect of legth of exposure on the liability to develop ceratomyxosis. Aquaculture. 104: 217-229

Ibarra A. M., R. P. Hedrick, G. A. E. Gall. 1994. Genetic analysis of rainbow trout susceptibility to the myxosporean, *Ceratomyxa shasta*. Aquaculture. 120: 239-262

Johnson, M. R. 1989. Culture methods and diseases of channel catfish *Ictalurus punctatus.* Pages 86-100 in Proceedings of the 93rd Annual Meeting of the United States Animal Health Association, Oct. 28-Nov. 3. Carter Publishing Company, Richmond, Virginia.

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

Kallert, D. M., E. Eszterbauer, C. Erseus, M. El-Matbouli, W. Haas. 2005b. Life cycle studies of *Myxobolus parviformis* sp. n. (Myxozoa: Myxobolidae) from bream. Diseases of Aquatic Organisms. 66: 233-243.

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

Khoo, L. H., P. Gaunt, M. Mauel. 2008. 2007 annual case summary report. Mississippi State College of Veterinary Medicine, Aquatic Diagnostic Laboratory, National Warmwater Aquaculture Center, Stoneville, MS. Accessed online July 10, 2008. http://www.msstate.edu/dept/tcnwac/cvm2007.pdf.

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

Koie, M., C. M. Whipps, M. L. Kent. 2004. *Ellipsomyxa gobii* (Myxozoa: Ceratomyxidae) in the common goby *Pomatoschistus microps* (Teleostei: Gobiidae) uses *Nereis spp.* (Annelida: Polychaeta) as invertebrate hosts. Folia Parasitologica. 51: 14-18.

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

Liyanage, Y. S, H. Yokoyama, H. Wakabayashi. 2003. Dynamics of experimental production of *Thelohanellus hovorkai* (myxozoa: Myxosporea) in fish and oligochaete alternate hosts. Journal of Fish Diseases. 26: 575-582.

Lom, J. 1987. Myxosporea: A new look at long-known parasites of fish. Parasitology Today. 3: 327-332

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

Longshaw, M., S. W. Feist, E. U. Canning, B. Okamura. 1999. Recent identification of PKX in Bryozoans from the United Kingdom – molecular evidence. Bulletin of the European Association of Fish Pathology. 19: 146-148.

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

Mackay, I. M., K. E. Arden, A. Nitsche. 2002. Real-time PCR in virology. Nucleic Acids Research. 30: 1292-1305.

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

Marshall, M. M., D. Naumovitz, Y. Ortega, C. R. Sterling. Waterborne protozoan pathogens. Clinical Microbiology Reviews. 10: 67-85.

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

Mitchell, A. J., R. M. Durborow, M. D. Crosby. 1998. Proliferative gill disease. Southeastern Regional Aquaculture Center. SRAC publication 475. United States Department of Agriculture Cooperative State Research, Education, and Extension Service. March 1998.

Molnar, K., F. Baska, C. Sze'kely. 1987. Fumagillin, an efficacious drug against renal sphaerosporosis of the common carp (*Cyprinus carpio*). Diseases of Aquatic Organisms 2: 187–190.

Mota, C., M. A. Head, J. A. Ridenoure, J. J. Cheng, F. L. de los Reyes III. 2005. Effects of aeration cycles on nitrifying bacterial populations and nitrogen removal in intermittently aerated reactors. Applied and Environmental Microbiology. 71: 8565-8572.

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

Panangala, V. S., C. A. Shoemaker, P. H. Klesius. 2007. TaqMan real-time polymerase chain reaction assay for rapid detection of *Flavobacterium columnare*. Aquaculture Research. 38: 508-517.

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

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

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

Ratliff, D.E. 1983. *Ceratomyxa shasta*: longevity, distribution, timing, and abundance of the infective stage in central Oregon. Canadian Journal of Fisheries and Auatic Sciences. 40: 1622-1632.

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

Souaze, F., A. Ntodou-Thome, C. Y. Tran, W. Rostene, P. Forgez. 1996. Quantitative RT-PCR: limits and accuracy. Biotechniques. 21: 280-285.

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

Thompson, K., R. B. Nehring. 2000. A simple technique used to filter and quantify the actinospore for *Myxobolus cerebralis* and determine its seasonal abundance in the Colorado river. Journal of Aquatic Animal Health 12: 316-323.

Tidwell, J. H., S. D. Mims. 1990. A comparison of second-year growth of blue catfish and channel catfish in Kentucky. Progressive Fish Culturist 52: 203-204.

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

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

Wilson, I. 1997. Inhibition and facilitation of nucleic acid amplification. Applied Environmental Microbioloy. 63: 3741-3751.

Wise, D., J. Terhune, L. Khoo. 1999. Proliferative gill disease more prevalent than previously thought. NWAC Newsletter. November (4-5).

Wise D. J., A. Camus, T. Schwedler, J. Terhune. 2004. Health Management. *In* Biology and culture of the channel catfish, ed. Tucker C. S., Hargreaves J.A., 1st ed., pp. 482-488. Elsevier B.V., Amsterdam, The Netherlands.

Wise, D. J., M. J. Griffin, J. S. Terhune, L. M. Pote, L. Khoo. 2008. Induction and evaluation of proliferative gill disease in channel catfish fingerlings. Journal of Aquatic Animal Health. In Press.

Wolf, K., M. E. Markiw. 1984. Biology contravenes taxonomy in the Myxozoa: new discoveries show alternation of invertebrate and vertebrate hosts. Science 255: 1449 – 1452.

Wolf, K., M. E. Markiw, J. K. Hiltunen. 1986. Salmonid whirling disease: *Tubifex tubifex* (Muller) identified as the essential oligochaete in the protozoan life cycle. Journal of Fish Disease. 9: 83-85.

Wong, M. L., J. F. Medrano. 2005. Real-time PCR for mRNA quantitation. Biotechniques. 39: 75-85.

Xiao, C., S.S. Desser. 2000. The longevity of actinosporeans spores from oligochaetes of Lake Sasejewun, Algnonquin Park, Ontario, and their reaction to fish mucus. Journal of Parasitology 86: 193-195.

Yokoyama, H., K. Ogawa, H. Wakabayashi. 1991. A new collection method of actinosporeans- a probable infective stage of myxosporeans to fishes- from tubificids and experimental infection of goldfish with actinosporeans, *Raabeia* sp. Fish Pathology 26: 133-138.

CHAPTER II

A REAL TIME POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF THE MYXOZOAN PARASITE *HENNEGUYA ICTALURI* IN CHANNEL CATFISH

Introduction

Proliferative gill disease (PGD), first reported in 1981, is one of the most significant parasitic infections to affect the commercial production of channel catfish, *Ictalurus punctatus* Rafinesque, 1818. Losses are greatest at temperature ranges of 16 to 25 °C and can result in mortality rates in excess of 50% (Bowser, et al. 1985; MacMillan, et al. 1989). The disease, commonly known as "hamburger gill" is characterized grossly by swelling, hemorrhage, and distortion of the gill filaments creating a mottled appearance. Chondrolysis due to localization of parasitic trophozoites causes the gill filaments to become fragile and break. Epithelial cell hyperplasia and inflammatory cell infiltration associated with the developing organism drastically reduce the gill surface area, severely inhibiting gas exchange and osmoregulation (Bowser et al. 1985; MacMillan et al. 1989). Infected fish swim listlessly near the water's surface exhibiting behaviors consistent with oxygen stress. Outbreaks of PGD are highly unpredictable and many cases go unreported (Wise et al. 2004). Losses can occur in all sizes of fish,

however, smaller fish appear to be most susceptible to the disease. Additional monetary losses may occur from sub-clinical infections which result in decreased feed intake and growth rates. Currently there are no efficacious therapeutic or prophylactic treatments for PGD and attempted biosecurity methods have been unsuccessful (Mischke et al. 2001; Wise et al. 2004).

Molecular evidence has confirmed previous work (Burtle 1991; Styer et al. 1991; Bellerud 1993; Bellerud et al. 1995) linking the cause of the disease to the myxozoan parasite *Henneguya ictaluri* (Pote et al. 2000; 2003). The complex life cycle involves a myxospore stage in channel catfish and an actinospore stage in the benthic oligochaete *Dero digitata* (Bellerud 1993; Bellerud et al. 1995; Hanson et al. 2001; Pote et al. 1994; 2000; 2003; Whitaker et al. 2001; 2005; Wise et al. 2004). Damage to gill tissue is thought to result from the development of an intense inflammatory response by the fish in response to penetration and proliferation of the actinospore stage of the parasite life cycle (Pote et al. 2000; Wise et al. 2004; 2008) (Fig. 2.1).

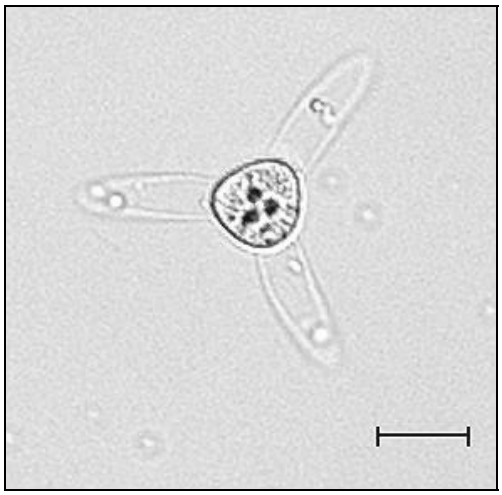


Figure 2.1. *Henneguya ictaluri* aurantiactinomyxon type actinospore. Bar = 20

A presumptive diagnosis is based on the presence of lytic areas in the filamental cartilage, seen microscopically in gill wet mounts (Pote et al. 2003; Wise et al. 2004; 2008) (Fig. 2.2), however this method is more reliable in smaller fish where size does not limit the number of filaments that can be accurately examined. Trophozoites are typically not seen unless histopathology is performed. The diagnosis is confirmed based on the presence of multinucleated trophozoites surrounded by granulomatous inflammation (Fig. 2.2, 2.3) in hematoxylin and eosin stained tissue sections or by *H. ictaluri* specific polymerase chain reaction (PCR) (Pote et al. 2000; 2003; Whitaker et al. 2001; 2005) Research has shown that although PCR is more sensitive than histology and more reliable in detecting early stages of infection (Whitaker et al. 2001), it is unable to objectively quantify the level of infection and is relatively time consuming in that it requires post-reaction processing.

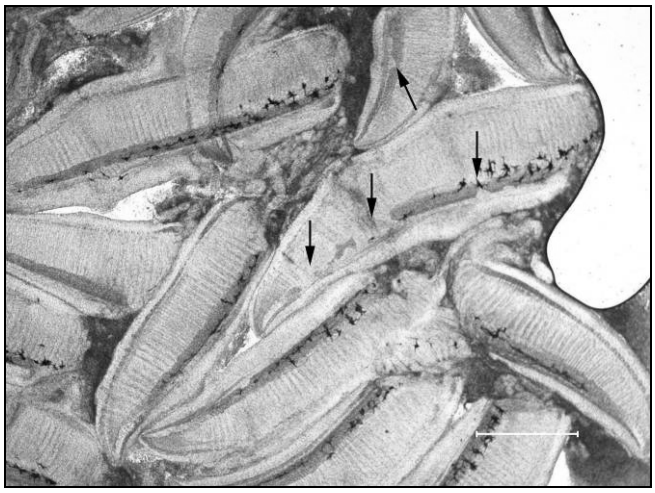


Figure 2.2. Wet mount preparation of gill clips from a channel catfish fingerling with proliferative gill disease. Note characteristic foci of chondrolysis in the filamental cartilage (arrows). Bar = 1 mm

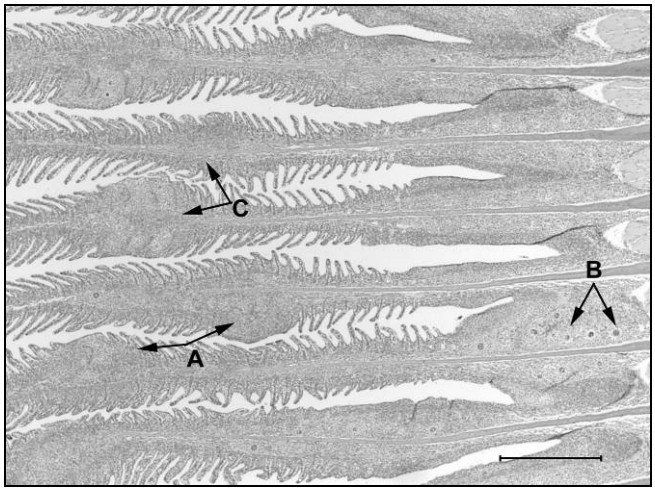


Figure 2.3 Histologic section of gill from a channel catfish fingerling with proliferative gill disease. There is widespread fusion of lamellae due to filling of lamellar troughs by epithelial hypertrophy and hyperplasia (A). Multinucleated trophozoites are surrounded by granulomatous inflammation (B) and lytic foci can be visualized within filamental cartilage (C). Hematoxylin and eosin stain; Bar = 0.5 mm

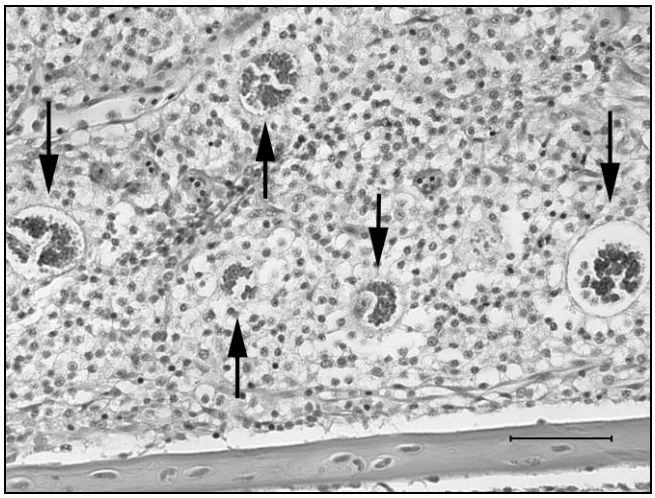


Figure 2.4 Histologic section of gill from a channel catfish fingerling with proliferative gill disease. Arrows identify deeply basophilic granular aggregates composed of multiple small trophozoites. Hematoxylin and eosin stain; Bar = 50 μm

Real-time PCR (QPCR) utilizes PCR chemistry in conjunction with fluorogenic probes to detect and monitor the amplification of reaction products after each amplification cycle (Heid et al. 1996). This allows rapid detection and quantification of PCR product as well as a means to monitor the reaction as it progresses, thereby eliminating the need for post reaction processing. Reactions are characterized by the PCR cycle in which target amplification emits sufficient fluorescence to cross a predetermined threshold significantly greater than background fluorescence, referred to as the cycle threshold (C_T) (Heid et al. 1996; Wong and Medrano 2005). Consequently, the greater the starting quantity of target DNA added to a reaction, the fewer number of cycles necessary for a significant increase in fluorescence to appear, yielding a lower C_T value. Using serially diluted standards of known concentrations to generate a standard curve, absolute quantitation of PCR product can be determined, assuming standards and

samples have approximately equal amplification efficiencies. Standards for double stranded DNA are normally created by cloning the target amplicon in a plasmid vector (Heid et al. 1996; Souze et al. 1996; Wong and Medrano 2005). The standard curve produces a linear relationship between C_T values and starting quantities of template DNA, allowing the determination of plasmid standard equivalents (PSE) for unknown samples based on their respective C_T values (Heid et al. 1996; Wong and Medrano 2005).

Quantitative real-time PCR has been utilized in the detection of pathogens in a number of aquatic systems, including fish tissues and environmental water samples (Bilodeau et al. 2003; Fontaine and Guillot 2003; Guy et al. 2003; Cavander et al. 2004; Kelley et al. 2004; Chase et al. 2006; Hallett and Bartholomew 2006; Balacázar et al. 2007). These assays are often more sensitive than conventional diagnostic techniques and offer the ability to quantify the pathogen, even at very low levels. In most cases, real-time PCR provides a rapid, more sensitive means of pathogen detection than conventional end-point PCR (Houghton and Cockerill 2006) and may be less subject to contamination than nested PCR assays.

Development of a more sensitive, quantifiable method of detecting sub-clinical PGD infections in channel catfish is important during the spring and fall when identification of a potential outbreak can avert major economic losses to the disease. A QPCR assay specific for *H. ictaluri* was developed for the detection and quantification of *H. ictaluri* infections in channel catfish and the assay's efficacy as a diagnostic tool was compared to the currently used diagnostic techniques; conventional PCR and microscopic examination of histologic sections and gill wet mounts.

Materials and methods

Actinospore collection

Benthic sediment was collected from a commercial channel catfish pond experiencing an outbreak of PGD in the resident catfish population, diagnosed by the Aquatic Diagnostic Laboratory (ADL), National Warmwater Aquaculture Center, Stoneville, MS (NWAC). *Dero digitata* were isolated from the benthic sediment (Bellerud 1993; Pote et al. 1994; Bellerud et al. 1995) and observed for 72 hours for the passing of the actinospore stages of *H. ictaluri*, *H. exilis*, and *Aurantiactinomyxon mississippiensis*. Organisms were identified morphologically according to descriptions of actinospores commonly found in commercial channel catfish ponds (Bellerud 1993). Actinospores of each species were pooled separately, enumerated and a subsample saved for molecular confirmation by sequencing (Hanson et al. 2001; Whitaker et al. 2001).

DNA extraction from actinospores

Actinospores were suspended in 20 ml of nuclease free H_2O and gently stirred with a magnetic stir bar. The concentration of actinospores/µl was determined by counting the number of actinospores in 10 separate 10 µl samples. Eight aliquots of volumes representative of 25 and 100 *H. ictaluri* actinospores were collected using a volumetric pipette and placed in individual 1.5 ml microcentrifuge tubes. Sixteen aliquots containing a single actinospore and eight aliquots containing 5 and 10 actinospores were collected using a fine glass pipette and also placed directly in individual 1.5 ml microcentrifuge tubes. The actinospores were centrifuged at 7,000 x g

for 10 min. All but 200 µl of the supernatant was removed and the pellet was resuspended in 600 µl of cell lysis solution from the Puregene® DNA isolation kit (Gentra Systems, Inc., Minneapolis). Proteinase K (20 mg/ml) was added prior to overnight incubation at 55 °C. The remainder of the isolation was carried out according to the DNA isolation kit manufacturer's suggested protocol. The purified genomic DNA was then suspended in 30 µl of Puregene® DNA hydration solution (Gentra Systems, Inc., Minneapolis) (10 mM Tris, 1 mM EDTA, pH 7.0 – 8.0) and stored at -80 °C.

Sequence amplification and cloning of SSU rRNA genes

Target regions of the small subunit (SSU) ribosomal RNA (rRNA) genes were amplified from the actinospore genomic DNA of each of the 3 myxozoan species using the universal myxozoan primers H2 and H9 (Hanson et al. 2001) (Table 2.1). The 25-µl PCR reaction mixtures contained 2.5 µl of 10X reaction buffer (10 mM of Tris, 50 mM of KCl, pH 9.0, 4.0 mM of $MgCl_2$), 2.0 µl of dNTP deoxyribonucleotide triphosphate) mixture (2.5 mM of each dNTP), 5 pM of each primer, 0.5 units of Takara® hot start *Taq* polymerase (Takara Bio USA, Madison, WI), 2 µl of DNA template, and nuclease-free H_2O to volume. The PCR was carried out using a PTC-100 thermal cycler (GMI, Inc., Ramsey, MN) programmed for 1 cycle of 95 °C for 10 min, 50 °C for 2 min, and 72 °C for 4 min, followed by 35 cycles of 92 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min, with a final extension cycle of 72 °C for 5 min. The PCR amplification products were analyzed by electrophoresis on a 1.5% agarose gel and stained with Gelstar® nucleic acid stain (Cambrex, East Rutherford, NJ) to confirm the presence of the 714-bp product.

The PCR product was then purified using a Montage® PCR centrifugal filter device (Millipore, Billareca, MD) and cloned using the PCR4-TOPO® cloning kit (Invitrogen, Carlsbad, CA). The plasmid clones with 18S SSU rRNA gene inserts from *H. ictaluri*, *A. mississippiensis*, and *H. exilis* were each grown in culture overnight and plasmid purification was performed using the Qiagen® plasmid mini-prep kit (Qiagen Inc.,Valencia, CA) The inserts were sequenced using the H2 and H9 primers (Hanson et al. 2001) (Table 2.1) on double-stranded DNA (Sanger et al. 1977) using the ABI prism dye terminator cycle sequencing ready reaction kit®, (Applied Biosystems, Foster City, CA) and Ampli*Taq* DNA polymerase (Applied Biosystems, Foster City, CA). Excess unincorporated dideoxy terminators from the completed DNA sequencing reactions were removed by spin column purification with Centrisep® spin columns (Princeton Separations, Inc., Adelphia, NJ) and the sequencing reaction products were analyzed on an ABI prism 310 genetic analyzer® (Applied Biosystems, Foster City, CA). The 714-bp amplicons were confirmed to be *H. ictaluri*, *H. exilis*, and *A. mississippiensis* by a BLASTn search (nucleotide Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) for similar sequences from the National Center for Biotechnology Information nonredundant nucleotide (nr/nt) database. The purified plasmid myxozoan DNA was quantified using a Nanodrop® spectrophotometer and accompanying software (v 3.2.1) (Nanodrop Technologies, Inc. Wilmington, DE).

TaqMan probe design

Sequence alignments of the 18S SSU rRNA genes of *H. ictaluri* (GenBank accession number AF195510), *A. mississippiensis* (GenBank accession number AF021878), and *H. exilis* (GenBank accession number AF021881) were performed using CLUSTAL software (San Diego Super Computer Biology Workbench v3.1, University of San Diego, San Diego, CA), concentrating on a 175-bp highly variable region from nt 1490 to nt 1665 in the 18S SSU rRNA genes (Hanson et al. 2001; Iwanowicz et al. 2008). A unique *H. ictaluri*–specific TaqMan probe was designed based on the 104-bp region from nt 1516 to nt 1619 amplified by the *H. ictaluri*–specific primers A1-1 and A1-2 (Whitaker et al. 2001; Pote et al. 2003; Whitaker et al. 2005) (Table 2.1). The 23-bp probe sequence (5'-TCAGCCTTGATGTTGCCACCTCA-3') beginning at nt 1573 was commercially prepared (Sigma-Genosys, Sigma-Aldrich Co., St. Louis, MO) and labeled with the fluorescent reporter dye, 6-carboxyfluorescein (FAM), on the 5' end, and the quencher dye, black hole quencher-1 (BHQ-1), on the 3' end. A BLASTn search for similar sequences was performed as described previously to ensure the sequence was unique to *H. ictaluri*.

Generation of QPCR standards

Target regions of the 18S SSU rRNA genes were amplified from genomic DNA of *H. ictaluri* actinospores and specific pathogen–free (SPF) channel catfish by use of *H. ictaluri* specific PCR primers (Whitaker et al. 2001; Pote et al. 2003) and universal 18S SSU rRNA primers (Elibol-Fleming 2006), respectively (Table 2.1). The PCR was

carried out as described previously and again the PCR amplification products were analyzed by electrophoresis to confirm the presence of a single PCR product. The target amplicons were purified and the plasmid clones with 18S SSU rRNA gene inserts from *H. ictaluri* and channel catfish were grown in culture overnight prior to plasmid purification and quantification as previously described. The plasmid standard concentrations were adjusted to 1.0 ng/µl and serially diluted to be utilized for the generation of standard curves and serve as positive controls for quantitative real-time PCR analysis.

Table 2.1. Primer and probe sequences

Name	Direction	Sequence*†‡	Reference
A1-1	Forward	5' - CAAAAGTTTCTGCTATCATTG - 3'	Whitaker et al. 2001
A1-2	Reverse	5' – AGCGCACAGATTACCTCA - 3'	Whitaker et al. 2001
H9	Forward	5' - TTACCTGGTCCGGACATCAA – 3'	Hanson et al. 2001
H2	Reverse	5' – CGACTTTTACTTCCTCGAAATTGC - 3'	Hanson et al. 2001
18SCCF	Forward	5' - CGGAGAGGGAGCCTGAGAA – 3'	Elibol-Fleming 2006
18SCCR	Reverse	5' - CGTGTCGGGAATGGGTAATTTG - 3'	Elibol Fleming 2006
HITMP	Probe	5' - [FAM] - TCAGCCTTGATGTTGCCACCTCA - [BHQ1] – 3'	This paper
18STMP	Probe	5' - [HEX] – ACCACATCCAAGGAAGGCAGCAGGC - [BHQ1] - 3'	Elibol Fleming 2006

* FAM = 6-carboxyfluorescein
† HEX = hexachloro-6-carboxy-fluorescein
‡ BHQ1 = Black Hole Quencher-1

QPCR

The 12.5-µl PCR contained 6.25 µl Biorad IQ® supermix (BioRad, Hercules, CA), 20 pM of each primer (A1-1 and A1-2), 0.25 pM of TaqMan probe (HITMP), 3 µl of

template DNA, and nuclease-free H_2O to volume. Amplifications were performed on a Biorad Icycler v3.1® (BioRad, Hercules, CA) programmed for 1 cycle of 95 °C for 3 min and 30 seconds, followed by 40 cycles of 95 °C for 30 sec, 56 °C for 1 min, and 72 °C for 30 sec. Data collection was carried out following the 72 °C elongation step at the end of each cycle.

QPCR specificity and sensitivity

The assay was tested on equal concentrations (1×10^{-2} ng) of plasmid DNA inserts of the 3 myxozoan species commonly found in commercial channel catfish ponds, along with genomic DNA from actinospores (n = 1000) of the closely related *A. mississippiensis* and SPF channel catfish. Sensitivity was tested on genomic DNA isolated as described above from 8 aliquots containing 5, 10, 25, and 100 *H. ictaluri* actinospores and 16 aliquots containing a single actinospore. Reactions for aliquots containing each of the selected numbers of actinospores were performed in triplicate. The C_T was set at 25 for all runs, and sample C_T values were compared to C_T values from a standard curve of a serially diluted plasmid of the *H. ictaluri*–specific PCR amplicon. Plasmid standard equivalents were determined for unknown samples by comparing their C_T values against that of the serially diluted plasmid standards. Data was considered valid if the slope of the standard curve was between –3.1 and –3.6, representing reaction efficiencies between 90% and 110% (Wong and Medrano 2005). Actinospore PSEs were calculated for each individual aliquot by dividing the sample PSE by the number of actinospores analyzed. Data analysis was conducted using SAS software version 9.1

(SAS Institute, Inc. Cary, NC) and actinospore PSEs per aliquot were compared using Scheffé's least significant difference test for unequal sample sizes ($P < 0.05$).

QPCR inhibition

To test for inhibition by host DNA, genomic DNA was isolated from SPF channel catfish as described above. This genomic DNA was suspended in 100 µl of Puregene® DNA hydration solution (Gentra Systems, Inc., Minneapolis, MN) (10 mM of Tris, 1 mM of EDTA, pH 7.0–8.0), spiked with 10 ng of the *H. ictaluri* plasmid DNA and serially diluted with catfish DNA. In addition, 10 ng of *H. ictaluri* plasmid DNA was suspended in 100 µl of nuclease-free water and serially diluted with nuclease-free water. Both dilution series were analyzed simultaneously according to the quantitative real-time PCR protocol described above, and the C_T was set at 25 for all runs.

Evaluation of quantitative real-time PCR as a diagnostic technique

All diagnostic methods were evaluated by testing their ability to detect *H. ictaluri* stages in fish that had been held in a PGD-positive pond for 1 or 7 d. Fifteen SPF channel catfish fingerlings were placed in each of 2 net pens, designated A and B, and held in a commercial channel catfish pond with clinically diagnosed PGD in the resident fish population. All fish (n = 15) were sampled from net pen A after 1 d in the pond. On day 6, 15 SPF channel catfish fingerlings were replaced in net pen A and held adjacent to net pen B. All fish were sampled from both cages (n = 15 fish/cage) on day 7. Upon removal from the pond, fish were transported live to the NWAC in an aerated holding

tank then euthanized immediately by an overdose (1000 mg/l) of MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate) (Argent Chemical Laboratories, Inc., Redmond, WA).

Gross examination and histology

The presence and severity of PGD lesions were determined by microscopic examination of wet mounts of gill clips (~40–80 filaments) from a left gill arch. A second gill clip (~40–80 filaments) was removed from a left gill arch and processed for molecular analysis. The presence and severity of PGD was determined by microscopic examination of histologic sections of samples from a right gill arch. Individual gill arches from the right side of the fish were removed and placed in 10% neutral buffered formalin for a minimum of 24 hr. Tissues were then processed by dehydration in a graded series of ethanol solutions of increasing strength, followed by clearing in a series of xylenes, embedding in paraffin, and sectioning at 6 µm. Prepared slides were stained with H&E (Luna 1968) and samples were designated as positive or negative based on the presence of the characteristic multinucleated trophozoites (Figs. 2.3, 2.4) (MacMillan et al. 1989; Pote et al. 2001; 2003). For wet mount examination, PGD was defined as the presence of lytic lesions in the cartilage of the gill filaments (Fig. 2.2) (Pote et al. 2003; Wise et al. 2004; 2008).

QPCR analysis

Gill clips designated for molecular analysis were placed in 600 µl of Puregene® cell lysis solution (Gentra Systems, Inc., Minneapolis, MN) and initially incubated for 10 min at 95°C prior to the addition of 3 µl of proteinase K (20 mg/ml). Samples were then incubated at 55°C until all tissue had dissolved. The remainder of the isolation was carried out according to the manufacturer's suggested protocol. After drying by vacuum centrifugation, the purified DNA was resuspended in 100 µl of Puregene DNA hydration solution (Gentra Systems, Inc., Minneapolis, MN) (10 mM of Tris, 1 mM of EDTA, pH 7.0–8.0) and quantified as previously described. At least 1 ng of total genomic DNA was added to each reaction, and all samples were analyzed in triplicate according to the quantitative real-time PCR protocol previously described. To correct for initial template variations between samples, 18S SSU rRNA PSEs were determined, along with *H. ictaluri* DNA PSEs, using the 18S SSU rRNA and probe combinations described previously (Elibol-Fleming B: 2006). Sample C_T values were compared to a standard curve based on serially diluted plasmid standards of the amplicons generated by the A1-1/A1-2 primers (Whitaker et al. 2001; Pote et al. 2003; Whitaker et al. 2005) or the 18SCCF/18SCCR (Elibol-Fleming 2006) respectively (Table 2.1). Again, data was considered valid if the slope of the standard curve was between –3.1 and 3.6, representing reaction efficiencies between 90% and 110% (Wong and Medrano 2005). Results were normalized to the equivalent of the reference gene, 18S rRNA, to prevent false negatives resulting from variations in starting template quantity.

End-point PCR sensitivity and specificity

Genomic DNA of *H. ictaluri* actinospore aliquots, along with the genomic DNA of PGD-exposed channel catfish, were analyzed by end-point PCR (Whitaker et al. 2001; Pote et al. 2003) to determine any differences in sensitivity and specificity. The PCR contained the reagents as listed previously using *H. ictaluri* specific primers (A1-1 and A1-2) and 2 µl of DNA template. The cycling conditions included an initial cycle of 95 °C for 10 min, 50 °C for 1 min, and 72 °C for 30 sec, followed by 35 cycles of 92 °C for 1 min, 50 °C for 15 sec, and 72 °C for 15 sec, with a final extension step of 72 °C for 5 min. The PCR amplification products were analyzed by electrophoresis on a 1.5% agarose gel and stained with Gelstar® nucleic acid stain (Cambrex, East Rutherford, NJ) to confirm presence of the 104-bp product.

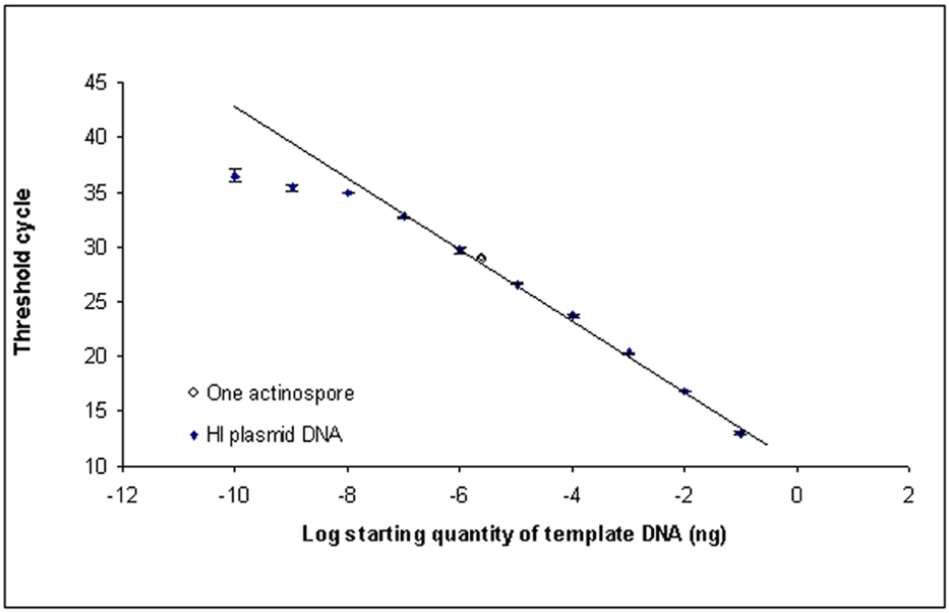
Results

The quantitative real-time PCR assay developed to detect *H. ictaluri* in channel catfish tissues was found to be highly specific and very sensitive. No amplification of the plasmid inserts of *H. exilis* and *A. mississippiensis*, extracted genomic DNA of *A. mississippiensis* actinospores (n = 1,000), or SPF channel catfish gill tissue was detected. The amplification curve of the assay was linear over 7 orders of magnitude and the sensitivity limit of the assay was 1×10^{-7} ng of purified *H. ictaluri* plasmid DNA. The assay was unable to discriminate between dilutions less than 1×10^{-8} ng of the *H. ictaluri* plasmid standard; however, the assay calculated the PSE from one *H. ictaluri* actinospore to be 9.55×10^{-6} ng of the plasmid standard. All 16 one actinospore samples were

detected, thus, the assay is sensitive enough to detect a single actinospore (Fig. 2.5). By comparison, the end-point PCR protocol was limited to definitive detection of 5 actinospores, supporting previous work (Whitaker et al. 2001). A least significant difference test performed on the calculations of spore equivalents for all aliquots revealed no significant differences ($P < 0.05$; Table 2). The mean observed DNA equivalents of the actinospore aliquots demonstrated a linear trend (Fig. 2.6). Inhibition of the PCR reaction by channel catfish DNA was not evident as SPF channel catfish samples spiked with *H. ictaluri* plasmid DNA, demonstrated the same amplification and standard curve as those suspended in nuclease-free water (Fig. 2.7).

Table 2.2. Calculated plasmid standard equivalents (PSE) for a single *Henneguya ictaluri* actinospore. There were no significant differences between calculated PSE for all aliquots; least significant difference test (Scheffe transformation) ($P<0.05$).

Aliquot	Mean C_T (± 1 S.D.)	Mean PSE (ng)	Mean Single Actinospore PSE (ng)
1 Spore (n=16)	29.7 (± 0.33)	$1.24e^{-5}$	$1.24e^{-5}$
5 Actinospores (n=8)	28.3 (± 0.27)	$3.18e^{-5}$	$6.35e^{-6}$
10 Actinospores (n=8)	27.0(± 0.31)	$6.60e^{-5}$	$6.6e^{-6}$
25 Actinospores (n=8)	25.5(± 0.48)	$2.22e^{-4}$	$8.93e^{-6}$
100 Actinospores (n=8)	23.3(± 0.26)	$1.07e^{-3}$	$1.07e^{-5}$
All Aliquots			$9.55e^{-6}$



* HI = *Henneguya ictaluri*

Figure 2.5. Real-time polymerase chain reaction amplification of a serial dilution of 104-bp *Henneguya ictaluri* plasmid inserts. The equation for the linear portion of the curve is $3.15x + 12.946$; $r^2 = .9992$; The x-axis employs a $\log_{10}$ scale and values are presented as means ± SEM;

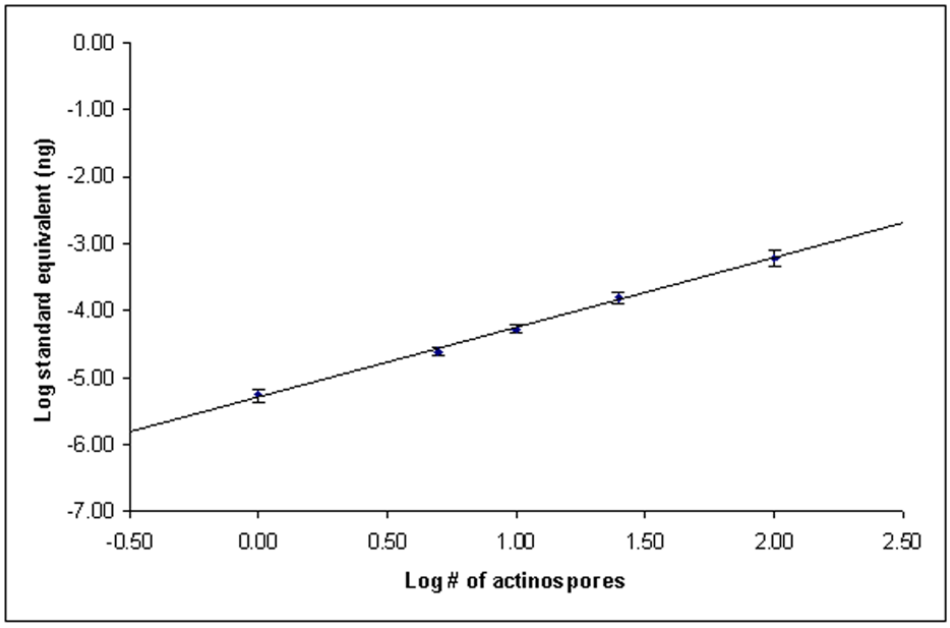


Figure 2.6. Standard curve derived from the mean plasmid standard equivalents for known numbers of actinospores. Actinospore numbers are represented by eight aliquots containing 5, 10, 25, and 100 *Henneguya ictaluri* actinospores and 16 aliquots containing a single *H. ictaluri* actinospore. The equation for the curve is $y = 1.033x - 5.2853$; $R^2 = 0.9981$. Both axes employ a $\log_{10}$ scale and values are presented as means ± SEM.

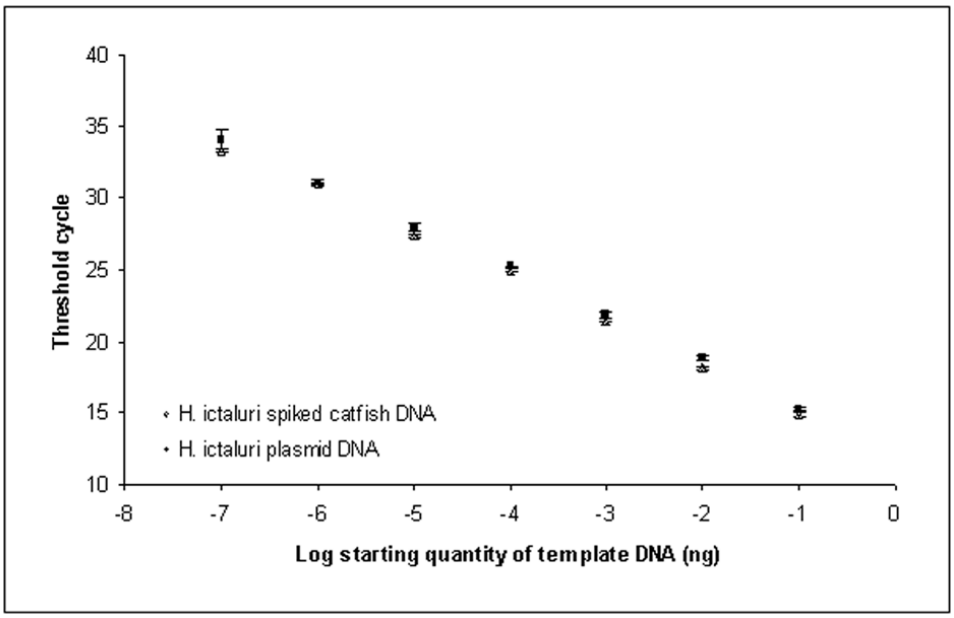


Figure 2.7. Comparison of the amplification products of pure *H. ictaluri* plasmid DNA and channel catfish genomic DNA spiked with *H. ictaluri* plasmid DNA. The x-axis employs a $\log_{10}$ scale.

The QPCR protocol detected PGD infection in all exposure groups involved in the pond study. QPCR was the only procedure to detect infection in either of the 24 hour exposure groups and was able to identify a decreased rate of infection from day 1 to day 7 in the 24 hour exposure groups (Table 2.3). End-point PCR, histology and gross examination of gill clips performed best at 168 hours post exposure, however histology and gross examination provided negative results for samples identified as positive by both PCR techniques (Table 2.3). There were no mortalities observed for any of the exposure treatments.

Table 2.3. Channel catfish determined positive for *H. ictaluri* infection by four different diagnostic techniques.

	Day 1	Day 7	
	24 hour	24 hour	168 hour
QPCR	12/15	9/15	15/15
End Point PCR	0/15	0/15	12/15
Gill Clip Wet Mount*	0/15	0/15	6/15
Histology†	0/15	0/15	12/15

* PGD diagnosed in surviving fish by presence of at least one gill filament demonstrating chondrocytic lysis
† PGD diagnosed by the presence of multinucleated trophozoites in tissue sections

Discussion

The *H. ictaluri* QPCR assay outperformed all currently practiced diagnostic techniques, including end-point PCR in the 24 hour exposure groups. The assay detected *H. ictaluri* infection 24 hours post exposure and identified a decreasing level of infectivity in the pond, which could not be determined by any of the conventional diagnostic techniques. The sensitivity of detection is a single actinospore, while the end-point PCR protocol is subjectively quantifiable and limited to detection of 5 actinospores (Whitaker et al. 2001). Although the sensitivity of the end-point PCR protocol can be increased by increasing the number of thermal cycles, this also increases the incidence of false positives (Whitaker et al. 2001). The QPCR assay provides diagnosticians and researchers with a rapid, more sensitive and quantifiable method of confirming PGD infections.

By comparison, histological preparation normally requires a 24 hour fixation period before tissue prep, and end-point PCR requires post-reaction processing. The

immediate results provided by gross examination of gill clip wet mounts are convenient in their expediency, but identify damage that has already occurred in the gills.

Significant losses can occur when catfish fingerlings are understocked for growout in the spring, even when resident fish populations demonstrate no clinical signs of disease. Previous research in the Fish Health Laboratory (FHL) of the Thad Cochran National Warm Water Aquaculture Center (TCNWAC), Stoneville, MS, showed that moving fish from a problem pond to a clean environment results in rapid recovery, creating a need for determining the PGD status of a pond prior to stocking naïve fish into the system or to identify a parasite free environment for which to relocate fish (Wise et al. 2004; 2008). This assay provides researchers with a means of determining parasite load in sentinel fish exposed to a PGD positive pond for 24 hours. Research in the Fish Health Laboratory at the Thad Cochran National Warmwater Aquaculture Center has shown the infectious window of the *H. ictaluri* actinospore stage to be approximately 24 - 48 hours (Wise 2004; 2008). As such, quantifying the parasite load in gill tissues of fish exposed to a PGD positive pond for 24 hours indirectly represents the rate at which actinospores are being released into the environment. Consequently, determination of parasite load in 24 hour exposures at two separate time points can identify increasing or decreasing rates of actinospore release, thereby providing insight into the dynamics of an ongoing outbreak. This information can prove invaluable in evaluations of potential treatments or management decisions. Quantifying the parasite load in 24 hour sentinel fish exposures prior to stocking can potentially identify problematic rates of actinospore

release in the pond and may offer producers an opportunity to take preventative measures to avoid large economic losses commonly attributed to PGD.

Although the QPCR assay is more costly and requires more specialized training than currently practiced diagnostic methods, the assay is more sensitive and provides confirmation of *H. ictaluri* infection more rapidly than histology and end-point PCR. Gross examination of gill clip wet mount preparations provides a reasonably accurate presumptive diagnosis in moderate to severe infections and while convenient, the method has limited sensitivity, especially in larger fish where size limits the number of filaments that can be accurately examined in wet mount preparation. It should be noted that this QPCR assay is not intended to replace, but rather be used in conjunction with currently practiced diagnostic techniques to provide a more rapid, sensitive and accurate diagnosis. As stated previously, the ability to quantify *H. ictaluri* DNA from fish tissue will provide an invaluable tool in epidemiological studies, treatment evaluations and management of this disease.

Literature Cited

Balcázar, J., D. Vendrell, I. de Blas, I. Ruiz-Zarzuela, O. Gironés, J. L. Múzquiz. 2007. Quantitative detection of *Aeromonas salmonicida* in fish tissue by real-time PCR using a self-quenched flourogenic probe. Journal of Medical Microbiology. 56: 323-328.

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

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

Bilodeau, L. A., G. C. Waldbieser, J. S. Terhune, D. J. Wise, W. R. Wolters. 2003. A real-time polymerase chain reaction assay of the bacterium *Edwardsiella ictaluri* in channel catfish. Journal of Aquatic Animal Health. 15: 80-86

Bowser PR, JD Conroy: 1985, Histopathology of gill lesions in channel catfish associated with *Henneguya*. J Wildl Dis 21:177-179

Burtle, G. J., L. R. Harrison, E. L. Styer. 1991. Detection of a triactinomyxid myxozoan in an oligochaete from ponds with proliferative gill disease in channel catfish. Journal of Aquatic Animal Health 3: 281-187.

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

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

Chase, D. M., D. G. Elliott, R. J. Pascho. 2006. Detection and quantification of *Renibacterium salmoninarum* DNA in salmonid tissues by real-time quantitative polymerase chain reaction analysis. Journal of Veterinary Diagnostic Investigation. 18: 375-380.

Elibol-Fleming, B. 2006. Effects of *Edwardsiella ictaluri* infection on transcriptional expression of selected immune relevant genes in channel catfish, *Ictalurus punctatus*. Doctoral dissertation. Mississippi State University, Mississippi State.

Fontaine, M, E. Guillot. 2003. An immunomagnetic separation real-time PCR method for quantification of *Cryptosporidium parvum* in water samples. Journal of Microbiological Methods 54: 29–36.

Guy, R. A., P. Payment, U. J. Krull, P. A. Horgen. 2003. Real-Time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Applied and Environmental Microbiology. Sept. 2003, p. 5178-5185.

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

Heid, C. A., J. Stevens, K. J. Livak, P. M. Williams. 1996. Real-time quantitative PCR. Genome Research 6: 986-994.

Houghton, S. G., F. R. Cockerill. 2006. Real-time PCR: Overview and applications. Surgery 139:1-5.

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

Iwanowicz, L. R., D. D. Iwanowicz, L. M. Pote, V. S. Blazer, W. B. Schill.. 2007. Morphology and 18S rDNA of *Henneguya gurlei* (Myxosporea) from *Ameiurus nebulosus* (Siluriformes) in North Carolina USA. Journal of Parasitology. 94(1): 46-57.

Kelley, G. O., F. J. Zagmutt-Vergara, C. M. Leutenegger, K. A., Myklebust, M. A. Adkison, T. S. McDowell, G. D. Marty, A. L. Kahler, A. L. Bush, I. A. Gardner, R. P. Hedrick. 2004. Evaluation of five diagnostic methods for the detection and quantification of *Myxobolus cerebralis*. Journal of Veterinary Diagnostic Investigation. 16:202-11.

Luna, L. G., editor. 1968. Manual for histologic staining methods of the Armed Forces Institute of Pathology, 3rd edition. McGraw-Hill, New York, New York.

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

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

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

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

Pote, L. M., L. A. Hanson, L. H. Khoo. 2003. Proliferative Gill Disease. *In:* Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. Blue Book 5th edition, pp. 3.7.A1, Fish Health Section, American Fisheries Society.

Sanger, F., S. Nicklen, A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences. 74: 5463–5467.

Souaze, F., A. Ntodou-Thome, C. Y. Tran, W. Rostene, P. Forgez. 1996. Quantitative RT-PCR: limits and accuracy. Biotechniques. 21: 280-285.

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

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

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

Wise D. J., A. Camus, T. Schwedler, J. Terhune. 2004. Health Management. *In* Biology and culture of the channel catfish, ed. Tucker C. S., Hargreaves J.A., 1st ed., pp. 482-488. Elsevier B.V., Amsterdam, The Netherlands.

Wise, D. J., M. J. Griffin, J. S. Terhune, L. M. Pote, L. K. Khoo. 2008. Induction and evaluation of proliferative gill disease in channel catfish fingerlings. Journal of aquatic animal health. In press.

Wong, M. L., J. F. Medrano. 2005. Real-time PCR for mRNA quantitation. Biotechniques. 39: 75-85.

CHAPTER III

APPLICATION OF A REAL-TIME POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF *HENNEGUYA ICTALURI* IN COMMERCIAL CHANNEL CATFISH PONDS

Introduction

Proliferative gill disease, caused by the myxozoan parasite *Henneguya ictaluri* (Pote et al. 2000), is one of the most prevalent parasitic infections afflicting the commercial channel catfish industry. Exposure to the actinospore stage of the organism (Fig. 3.1), released by the benthic oligochaete *Dero digitata* (Burtle et al. 1991, Styer et al. 1991, Pote et al. 2000), causes extensive gill damage, resulting in respiratory insult and osmoregulatory duress, often leading to reduced growth and eventually death (Bowser et al. 1985, Macmillan et al. 1989, Wise et al. 2004).

The syndrome is most prevalent during the spring, with a lesser incidence in the fall, when water temperatures are between 16-25 °C. Significant losses often occur when catfish fingerlings are understocked for growout in the spring, or re-stocked after a PGD outbreak, even when resident fish populations demonstrate no clinical signs of disease. Currently, there are no effective treatments for PGD, but recent research has shown that moving fish from an affected to an unaffected pond results in rapid recovery (Wise et al.

2008). These observations led to the development of methods to assess the risk of losing fish to PGD when relocating fish from an ongoing outreak or when a severe outbreak necessitates the restocking of a pond. This procedure is an indirect assessment of the level of actinospores present in the pond environment, or PGD status of the pond (Wise et al. 2004).

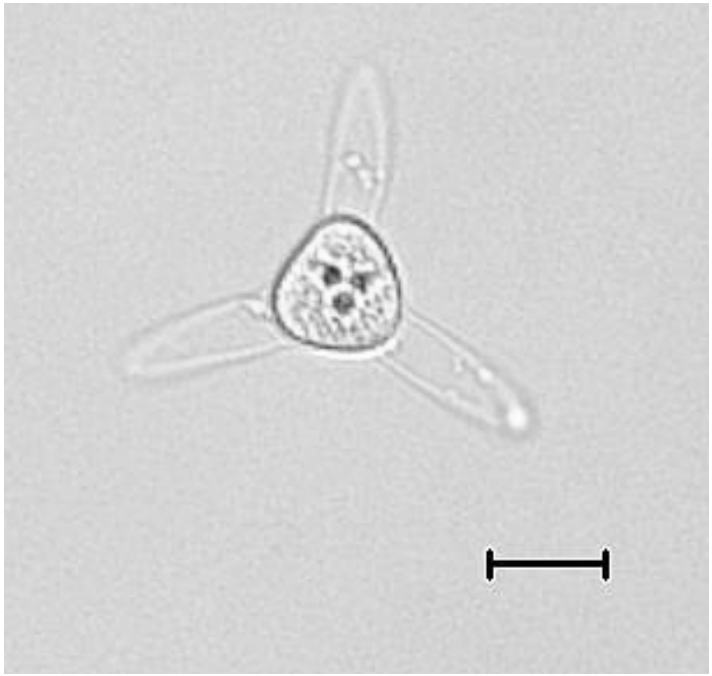


Figure 3.1. *Henneguya ictaluri* aurantiactinomyxon type actinospore. Bar = 20 µm

At present, the PGD status of a pond can be determined through sentinel fish exposures. Net-pens, constructed of 5 mm nylon mesh to allow free-exchange of water within the confined area, are suspended in the pond in question and stocked with parasite free fish. Fish are held in the pond for seven days, after which they are examined for clinical signs of disease. If PGD is present, sentinel fish are restocked for an additional 7

days to determine if the severity of the disease has increased or decreased from the first 7 day exposure. After determining the dynamics of the outbreak, decisions on restocking can be made

The percentage of gill filaments exhibiting characteristic chondrolytic lesions indicative of proliferative gill disease (Fig. 3.2) is calculated and disease severity is assessed (Table 3.3). Unfortunately, sentinel fish exposures are labor intensive, require a source of parasite-free fish and require a minimum of two weeks to yield reliable results (Wise et al. 2004). Another setback to this protocol is a bias in determining disease severity in cages where mortalities are observed. Autolysis prevents an accurate evaluation of gill damage in fish that have expired prior to sampling and usually requires the restocking of sentinel fish. In addition, without an adequate number of fish to be examined the severity of the outbreak cannot be determined. (David Wise, personal communication). The protocol is also limited in that it calculates the percent of filaments exhibiting at least one chondrolytic lesion, and does not take into consideration the number of chondrolytic lesions within each filament. In heavy infections, an individual filament may have several chondrolytic lesions, yet is considered one observation, which underestimates disease severity since each lesion is associated with an organism (David Wise, personal communication). These constraints necessitated the development of a more rapid and efficient method of determining the PGD status of a pond.

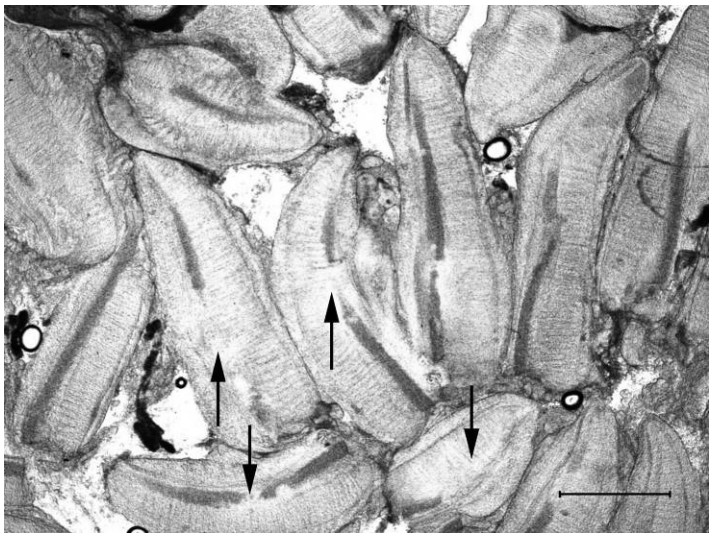


Figure 3.2. Wet mount preparation of gill clips from a channel catfish fingerling with proliferative gill disease. Foci of chondrolysis characteristic of proliferative gill disease is evident in the filamental cartilage (arrows). Bar = 1 mm

Table 3.1. Grading scale for *Henneguya ictaluri* induced lesions (Wise et al. 2004)

Percent of filaments exhibiting chondrolytic lesions	**Score**	**Effect on Fish**
1-5%	Mild	Little to no effect on fish health
6-15%	Moderate	If environmental conditions are optimal no direct mortalities involved
>15%	Severe	Expect mortalities within 2 weeks

Real-time PCR assays have been utilized in the quantification of various waterborne parasites from environmental samples, as well as the quantification of bacterial and parasitic agents in fish tissues (Bilodeau et al. 2003, Brinkman et al. 2003, Fontaine et al. 2003, Guy et al. 2003, Cavender et al. 2004, Kelley et al. 2004, Chase et

al. 2006, Hallett and Bartholomew 2006, Balacázar et al. 2007; Panangala et al. 2007). Recently, a real time PCR assay has also been developed for the quantification of *Henneguya ictaluri* in fish tissues (Griffin et al. 2008). Real-time PCR (QPCR) utilizes PCR chemistry in conjunction with fluorogenic probes to detect and monitor the amplification of reaction products after each amplification cycle (Heid et al 1996). This allows rapid detection and quantification of PCR product, as well as a means to monitor the reaction as it progresses, thereby eliminating the need for post reaction processing. Reactions are characterized by the PCR cycle in which target amplification reaches a predetermined threshold of fluorescence intensity significantly greater than the background fluorescence, referred to as the cycle threshold (C_T) (Heid et al. 1996, Wong and Medrano 2005). Consequently, the greater the starting quantity of target DNA added to a reaction, the fewer number of cycles necessary for a significant increase in fluorescence to appear, yielding a lower C_T value. Using serially diluted standards of known concentrations to generate a standard curve, absolute quantitation of PCR product can be determined, assuming standards and samples have approximately equal amplification efficiencies. Standards for double stranded DNA are normally created by cloning the target amplicon in a plasmid vector (Heid et al. 1996, Souaze et al. 1996, Wong and Medrano 2006). The standard curve produces a linear relationship between C_T values and starting quantities of template DNA, allowing the determination of plasmid standard equivalents for unknown samples based on their respective C_T values (Heid et al. 1996, Wong and Medrano 2006).

Research has shown the infectious window of the *Henneguya ictaluri* actinospore stage to be approximately 24-48 hours (Wise et al. 2004; 2008). Therefore, quantifying the parasite load in gill tissues of fish exposed to a PGD positive pond for 24 hours indirectly represents the rate at which actinospores are being released into the environment. Alternatively, correlating the relationship between actinospore concentrations in pond water to disease severity observed in sentinel fish would correlate actinospore concentrations to clinical levels of disease and be useful in assessing the potential risk of stocking fish into the pond environment. Whitaker et al. (2005) developed a PCR protocol for the detection of the actinospore stage in pond water. However, this method is subjective and results obtained from the assay are not readily quantifiable. This paper chronicles the validation of two different QPCR protocols for the identification and quantification of *H. ictaluri* actinospores in channel catfish ponds and gill tissue. Three experimental trials were conducted to: (1) determine the geospatial distribution of *H. ictaluri* actinospores in a commercial channel catfish pond (2) evaluate a pond monitoring protocol based on the determination of *H. ictaluri* actinospore concentrations in pond water (3) evaluate a pond monitoring protocol based on the determination of parasite loads following 24 hour sentinel fish exposures.

Materials and Methods

Actinospore collection

Dero digitata were isolated from benthic sediment from a commercial channel catfish pond containing fish diagnosed with clinical PGD by the Aquatic Diagnostic

Laboratory (ADL) of the TCNWAC. The oligochaetes were observed for 72 h for the shedding of *H. ictaluri* actinospores following the methods of Pote et al. (1994) and Bellerud et al. (1995) and used to validate the PCR procedures.

Collection and processing of water samples

Water samples (2 L) were collected approximately 48 cm below the surface of the pond and processed within 24 h of collection. Samples were sub-divided into 4 x 500 ml samples, transferred to 1-liter centrifuge bottles and centrifuged for 20 min at 9500 x g on a Sorvall RC-6 centrifuge (Thermo Fisher Scientific, Inc., Waltham, MA). The supernatant from each subsample was removed and the pellet resuspended in 50 ml of distilled water, then transferred to a 50 ml conical centrifuge tube and centrifuged at 7000 x g for 10 min. The supernatant was again removed and the pellet was resuspended in 1.5 ml of nuclease free H_2O and transferred to a 1.8 ml microcentrifuge tube and stored at -80 °C. Between samples, each centrifuge bottle was rinsed three times with distilled water before reuse.

DNA extraction

DNA was isolated from actinospore aliquots following the Powersoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) suggested protocol and re-suspended in 30 µl of Puregene® DNA hydration solution (Gentra Systems, Inc., Minneapolis, MN). Genomic DNA from pond water samples was also isolated using a Powersoil® DNA Isolation Kit but following the suggested protocol for wet samples. Genomic DNA

isolated from the pond water sub-samples were pooled, concentrated according to the manufacturer's suggested salt precipitation protocol and re-suspended into 30 µl of Puregene® DNA hydration solution.

Genomic DNA from fish tissues was isolated using a slight modification of the Puregene® DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, MN) suggested protocol for animal tissues. Gill clips (40-80 filaments) taken from a left gill arch were placed in 600 µl of cell lysis solution from Puregene® DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, MN) and initially incubated for 10 min at 95 °C prior to the addition of 3 µl of Proteinase K (20 mg/ml). Samples were then incubated at 55 °C until all tissue homogenate had dissolved. The remainder of the isolation was carried out according to the manufacturer's suggested protocol. After drying by vacuum centrifugation, the purified DNA was re-suspended in 100 µl of Puregene® DNA Hydration Solution (Gentra Systems, Inc., Minneapolis, MN) and quantified using a Nanodrop® spectrophotometer and accompanying software v 3.2.1 (Nanodrop Technologies, Inc. Wilmington, DE)

Standard design

Target regions of the 18S SSU rDNA genes were amplified from the genomic DNA of *H. ictaluri* actinospores and specific pathogen free (SPF) channel catfish by use of *H. ictaluri* specific PCR primers (Whitaker et al. 2001, Pote et al. 2003) and generic eukaryotic 18S SSU rDNA primers (Elibol-Fleming 2006), respectively (Table 3.2). The 25 µl PCR reaction mixtures contained 2.5 µl of 10X reaction buffer (10 mM Tris, 50

mM KCl, pH 9.0, 4.0 mM $MgCl_2$), 2.0 µl of dNTP mixture (2.5 mM of each dNTP), 5 pM of each primer, 0.5 units of Takara® Hot Start *Taq* Polymerase (Takara Bio USA, Madison, WI) and 2 µl of DNA template. The PCR was carried out on a PTC-100 Thermal Cycler, (Global Medical Instrumentation, Inc., Ramsey, MN) programmed for 1 cycle of 95 °C for 10 min, 50 °C for 1 min, and 72 °C for 2 min followed by 35 cycles of 92 °C for 1 min, 50 °C for 15 sec, and 72 °C for 15 sec and a final extension cycle of 72 °C for 5 min. The PCR amplification products were analyzed by electrophoresis on a 1.5% agarose gel and stained (Gelstar® nucleic acid stain, Cambrex, East Rutherford, NJ) to confirm the presence of a single PCR product.

PCR products were purified using a Montage® PCR Centrifugal Filter Device (Millipore, Billareca, MD) and cloned into a T7 vector using the PCR4-TOPO® cloning kit (Invitrogen, Carlsbad, CA). The plasmid clones with 18S SSU rDNA gene inserts from *H. ictaluri* and channel catfish were each grown in culture overnight and plasmid purification was performed using a Qiagen plasmid mini-prep kit (Qiagen Inc.,Valencia, CA). The plasmid standards were quantified as described above and their concentrations were adjusted to 1.0 ng/µl. The standards were then serially diluted and utilized to serve as positive controls and for generation of standard curves in QPCR analysis.

QPCR

The 12.5-µl PCR reactions contained BioRad IQ® supermix (Biorad, Hercules, CA), 20 pM of each primer, 0.25 pM of probe, 3 µl of template DNA and nuclease free H_2O to volume. Amplifications were performed on a BioRad Icycler v3.1® real-time

PCR system (Biorad, Hercules, CA) programmed for 1 cycle of 95 °C for 3 min 30 sec followed by 40 cycles of 95 °C for 30 sec, 56 °C for 1 min and 72 °C for 30 sec. Data collection was carried out following the 72 °C elongation step at the end of each cycle.

Determination of actinospore equivalents

Freshly released *H. ictaluri* actinospores were collected and suspended in 20 ml of nuclease free H_2O and gently stirred with a magnetic stir bar. The concentration of actinospores/µl was determined by counting the number of actinospores in 10 separate 10 µl samples. Eight aliquots of volumes representative of 25, 100 and 1000 *H. ictaluri* actinospores were collected using a volumetric pipette and placed in individual Powersoil® DNA Isolation Kit microbead tubes. Eight aliquots of 1, 5 and 10 actinospores were individually collected using a fine glass pipette and also placed directly in individual Powersoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) microbead tubes. Genomic DNA was isolated according to the manufacturer's suggested protocol. A standard curve was determined by analyzing ten replicates of the enumerated spore samples. Real-time PCR analysis of genomic DNA isolated from *H. ictaluri* actinospore aliquots was performed using *H. ictaluri*-specific primers (Whitaker et al. 2001, Pote et al. 2003) and a *H. ictaluri*-specific TaqMan probe (Griffin et al. 2008) (Table 3.2) labeled on the 5' end with the fluorescent reporter dye, 6-carboxyfluorescein (FAM), and the quencher dye, black hole quencher-1 (BHQ-1) on the 3' end. The C_T threshold was set the same for all runs and sample C_T values were used to generate a standard curve for actinospore equivalents. All samples were run in triplicate

with a negative control and a serially diluted plasmid standard which served as both a positive control and a measure of reaction efficiency. The amplification efficiency (*E)* was estimated by the formula $E = 10^{-1/s} - 1$, where s is the slope of the standard curve (Wong and Medrano 2006) and efficiency estimations between 90 and 110% were considered acceptable. Actinospore equivalents for water samples were determined by comparing the mean of the sample C_T values to the standard curve generated by the analysis of the enumerated spore samples (Fig. 3.3).

Validation of water sampling protocol

Pond water collected from a commercial channel catfish pond with no previous history of PGD was separated into eleven 500 ml water samples. *H. ictaluri* actinospores were collected from isolated *D. digitata* and enumerated. Eight aliquots of 100 actinospores were processed directly by the Powersoil DNA isolation kit. An additional eight aliquots of 100 actinospores were added directly to 500 mls (5 actinospores/ml) of pond water and the remaining pond water samples served as negative controls. All pond water samples were then processed according to the previously described protocol.

QPCR analysis of pond water samples

Parasite DNA was amplified using the *H. ictaluri* specific primers and probe combination described above. Amplifications were performed on a Biorad Icycler v3.1® (BioRad, Hercules, CA) programmed for 1 cycle of 95 °C for 3 min 30 seconds followed by 40 cycles of 95 °C for 30 sec, 56 °C for 1 min and 72 °C for 30 sec. Data collection

was carried out following the 72 °C elongation step at the end of each cycle. All samples were run in triplicate with a no template negative control and a serially diluted plasmid standard which served as both a positive control and a measure of reaction efficiency. Again, data was considered valid if the slope of the standard curve was between -3.1 and -3.6, representing reaction efficiencies between 90 and 110% (Wong and Medrano 2006).

QPCR analysis of fish tissue

Parasite DNA was amplified from host tissue using the previously described *H. ictaluri* specific primer and probe combination, while the internal standard 18S SSU rDNA was amplified using the 18SCCF/18SCCR primers and 18SCC TMP labeled on the 5' end with the fluorescent reporter dye, hexachloro-6-carboxy-fluorescein (HEX), and the quencher dye, black hole quencher-1 (BHQ-1) on the 3' end (Table 3.2). At least 1 ng of total genomic DNA (parasite and host) was added to each reaction and all samples were analyzed in triplicate according to the QPCR protocol described above. To correct for initial template variations between samples, 18S SSU rRNA PSE's were determined for each sample. Sample C_T values were compared to a standard curve based on serially diluted plasmid standards of the amplicons generated by the A1-1/A1-2 (Whitaker et al. 2001, Pote et al. 2003) primers or the 18SCCF/18SCCR (Elibol-Fleming 2006), respectively (Table 3.2). Data was considered valid if the slope of the standard curve was between -3.1 and -3.6 as described previously. Data was normalized to the equivalent of the reference gene, 18S SSU rRNA, and the log of the ratio of parasite

standard equivalent (PSE) to host standard equivalent (HSE) was used for the purpose of comparisons.

Table 3.2. Primer and probe sequences

Name	Direction	Sequence*†‡	Reference
A1-1	Forward	5' - CAAAAGTTTCTGCTATCATTG - 3'	Whitaker et al. 2001
A1-2	Reverse	5' – AGCGCACAGATTACCTCA - 3'	Whitaker et al. 2001
H9	Forward	5' - TTACCTGGTCCGGACATCAA - 3'	Hanson et al. 2001
H2	Reverse	5' - CGACTTTTACTTCCTCGAAATTGC - 3'	Hanson et al. 2001
18SCCF	Forward	5' - CGGAGAGGGAGCCTGAGAA - 3'	Elibol-Fleming 2006
18SCCR	Reverse	5' - CGTGTCGGGAATGGGTAATTTG - 3'	Elibol Fleming 2006
HITMP	Probe	5' - [FAM] - TCAGCCTTGATGTTGCCACCTCA - [BHQ1] – 3'	This paper
18STMP	Probe	5' - [HEX] – ACCACATCCAAGGAAGGCAGCAGGC - [BHQ1] - 3'	Elibol Fleming 2006

* FAM = 6-carboxyfluorescein
† HEX = hexachloro-6-carboxy-fluorescein
‡ BHQ1 = Black Hole Quencher-1

Inhibition of QPCR

Pond water samples which demonstrated PCR inhibition were diluted to reduce inhibition from environmental contaminants (Brinkman et al. 2003; Hallett and Bartholomew 2006). All pond water samples demonstrating inhibition, evident by sample amplification with a significantly reduced slope compared to the amplification slope of positive controls, were diluted with Puregene® DNA Hydration Solution (Gentra Systems, Inc., Minneapolis, MN) in two-fold increments until amplification slopes reflected that of positive controls.

Gross examination of fish tissues

Approximately 40-80 gill filaments were removed from a left gill arch and used for wet mount preparations. The percentage of gill filaments exhibiting at least one chondrocytic lesion (Fig. 3.2) was calculated and the mean gill damage for each cage was determined. PGD outbreaks were designated as mild, moderate or severe according to the sentinel fish protocol described by Wise et al. (2004) (Table 3.1).

Experimental trials

For the first experimental trial the geospatial distribution of the actinospore stage in a pond outbreak was determined. A commercial channel catfish pond (10 acres) diagnosed with clinical PGD in the resident fish population by the ADL of the TCNWAC was sectioned into a 4 x 4 grid (Fig. 3.3). Parasite free fish (n=15) were held in nylon cages positioned at each intersection of the grid for seven days. On days 0, 3 and 6 water was collected adjacent to each cage as described above. On day 6, all surviving fish were euthanized with an overdose (1000 mg/L) of MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate) (Argent Chemical Laboratories, Inc., Redmond, WA), gills were processed for gross microscopic examination, and the mean percent gill damage determined for each cage. The cage placed at location D2 was lost and subsequent water sampling on days 3 and 6 were not carried out.

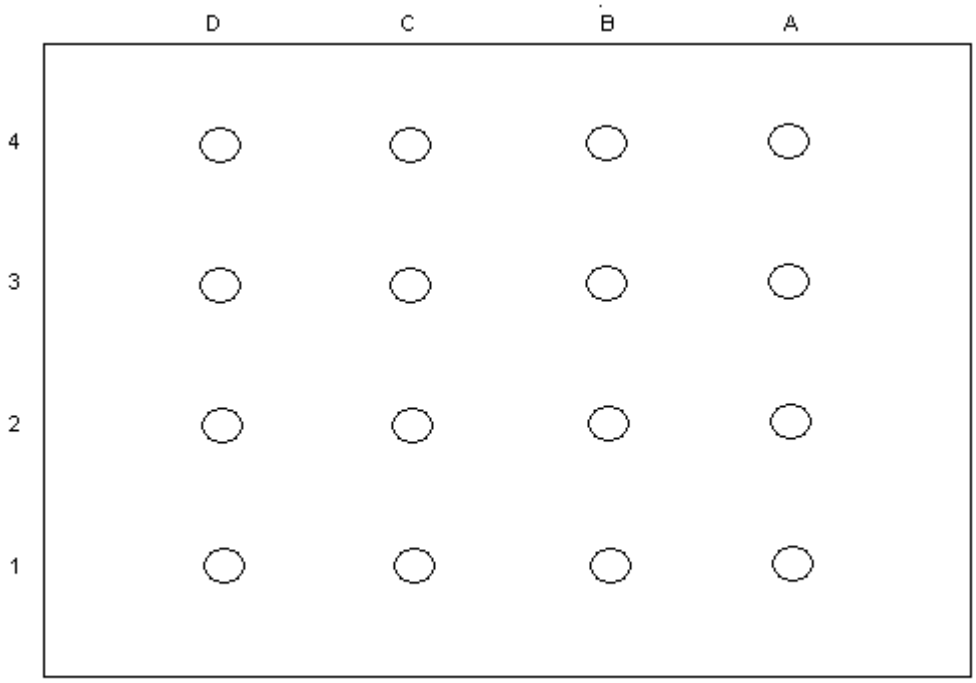


Figure 3.3. Schematic demonstrating cage and water sampling locations in a commercial channel catfish pond diagnosed with PGD in the resident fish population.

Experimental trial 2 consisted of twenty replicate week long pond trials carried out in eight different commercial channel catfish ponds (range 7-15 acres) diagnosed with clinical PGD. Pond water was sampled as described previously on days 0 and 7 in coordination with the setting out and sampling of sentinel cages. One water sample was collected adjacent to the cage and the second from a separate geographic location at least 100 m from the cage (Table 3.3). The mean actinospore equivalents l^{-1} for the two locations was determined by QPCR analysis and inhibition of the QPCR assay was corrected for by a 1:4 dilution of genomic DNA for all samples. Fifteen sentinel parasite free fish were held for seven days in nylon net pens. On day 7, surviving fish were

sampled, euthanized with an overdose (1000 mg/L) of MS-222 and processed for gross microscopic examination.

Table 3.3. Schedule of events for experimental trial 2.

Day	Cages	Water sampling
0	Placed in pond (n=15 fish/cage)	Water (2 L) collected adjacent to cage and a second separate geographic location
7	Collected from pond	Water (2 L) collected adjacent to cage and a second separate geographic location

In experimental trial 3, eight replicate week long pond trials were carried out in two different commercial channel catfish ponds diagnosed with clinical PGD. Parasite free channel catfish fingerlings were placed in two nylon net pens designated A and B (n=15 fish/pen). After 24 hours, survivors from net pen A were euthanized with an overdose (1000 mg/L) of MS-222 and processed for molecular analysis and gross examination to determine mean percent gill damage. On day 6, net pen A was re-stocked with fish from the same fish stock mentioned previously (n=15). On day 7, surviving fish from net pen A (24 hr exposure) and net pen B (7 day exposure) were sampled, euthanized and processed for molecular analysis and mean percent gill damage was determined by gross examination (Table 3.4).

Table 3.4. Schedule of events for experimental trial 3.

Day	Net pen A	Net pen B
0	Placed in pond (n = 15 fish/cage)	Placed in pond (n = 15 fish/cage)
1	All fish sampled	No action
6	Fish replaced (n = 15 fish/cage)	No action
7	All fish sampled	All fish sampled

Statistical analysis

All statistical analysis was performed using SAS Software v. 9.1 (SAS Institute, Inc., Cary, NJ). For trial 1, a one-way ANOVA and Fisher's least significant difference procedure was used to determine differences in mean percent gill damage. For trial 2, the relationship between mean actinospore concentration and mortality in 7 d sentinel fish exposures was determined by simple linear regression. For trial 3, parasite standard equivalents determined by QPCR, were normalized to the host 18S rDNA and the ratio of PSE to HSE was subjected to a log transformation prior to statistical analysis. Samples in which no parasite DNA was detected by PCR were assigned log values of -8, one log lower than the lowest observed value. Multiple comparisons of the data for trial 3 were performed using Duncan's multiple range test. The relationship between the ratio of PSE to HSE for 2 x 24 hr exposures and mortality in 7 d sentinel fish exposures was subjected to simple linear regression. For all trials, $p<0.05$ was considered significant.

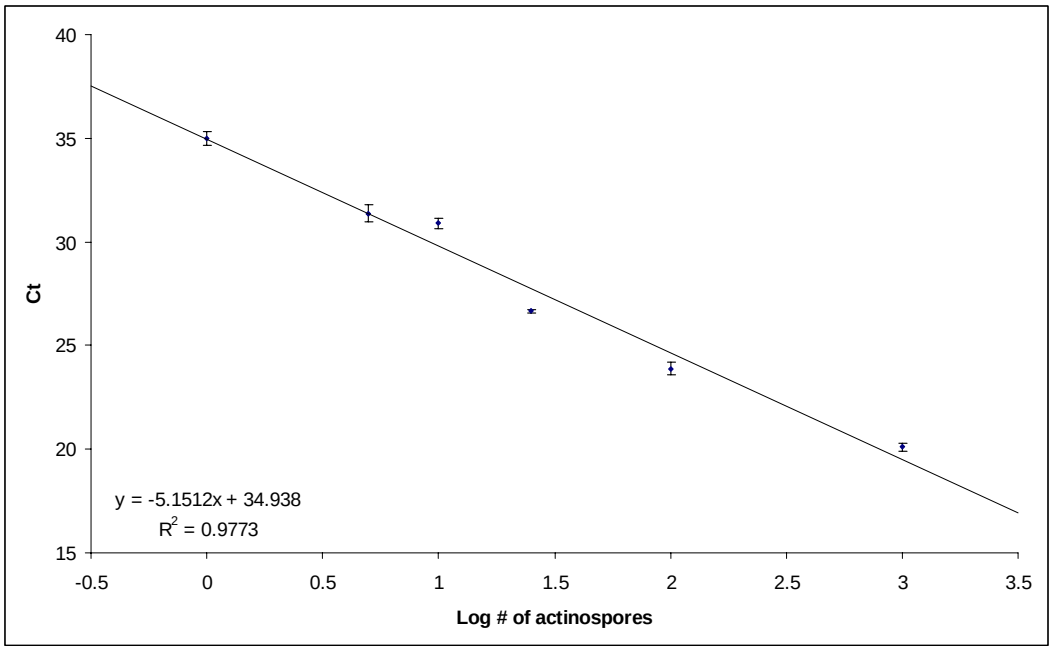


Figure 3.4. Standard curve derived from known numbers of actinospores. DNA was extracted as per pond water samples, showing log of number of spores versus mean QPCR C_T value. The x axis employs a $\log_{10}$ scale. Values are presented as means ± SEM.

Sensitivity

The C_T values for the actinospore aliquots are presented in Table 3.5. The standard curve for known starting numbers of whole spores processed and assayed as per pond water samples had an equation of -5.1512x + 34.938 with an R^2 = .9773 (Fig. 3.4). The mean C_T value for 1 actinospore was 34.54 (range 33.49 – 38.15). Pond water samples spiked with 100 *H. ictaluri* actinospores had a mean C_T value of 25.31 (range 23.91 – 26.58), which correlated to approximately ~80 actinospore equivalents, indicating an approximate 20% loss of actinospore DNA during the sampling process.

Table 3.5. Cycle threshold (C_T) values for actinospore aliquots.

Number of actinospores	C_T value
1	33.5 – 36.4 (avg. 35.0)
5	29.0 – 33.6 (avg. 31.4)
10	29.7 – 32.6 (avg. 30.9)
25	26.3 – 27.0 (avg. 26.7)
100	22.7 – 25.2 (avg. 23.9)
1000	19.4 – 20.8 (avg. 20.0)

Experimental trial 1

All surviving fish demonstrated clinical signs of PGD and only one cage location (A2) had a mean percent gill damage significantly greater than that of the other cage locations ($P<0.05$) (Table 3.5). Survival rates were variable by location and mortalities were observed in each cage. Cages from locations D1 and A1 had no surviving fish after 6 days and the cage from location D2 was lost prior to day 3 sampling. Actinospore concentrations by location were variable by sampling day and mean actinospore concentration decreased from the first to the last sampling day. All samples were positive for *H. ictaluri* DNA. The highest actinospore equivalents were observed on the first sampling day and progressively decreased across sampling days (Fig. 3.5), although the differences in actinospore concentrations for day 3 and 6 were not significant ($P<0.05$). The greatest actinospore equivalent concentration observed was ≥249 actinospore equivalents per liter, observed on day 1, and the lowest actinospore

equivalent concentration was ≥38 actinospore equivalents per liter, observed on day 6. The mean actinospore equivalent concentration for all cage locations for all days was ≥94 actinospore equivalents l^{-1}. The average mortality rate for all cages was 35.6% with mean percent gill damage for surviving fish of 11.7%.

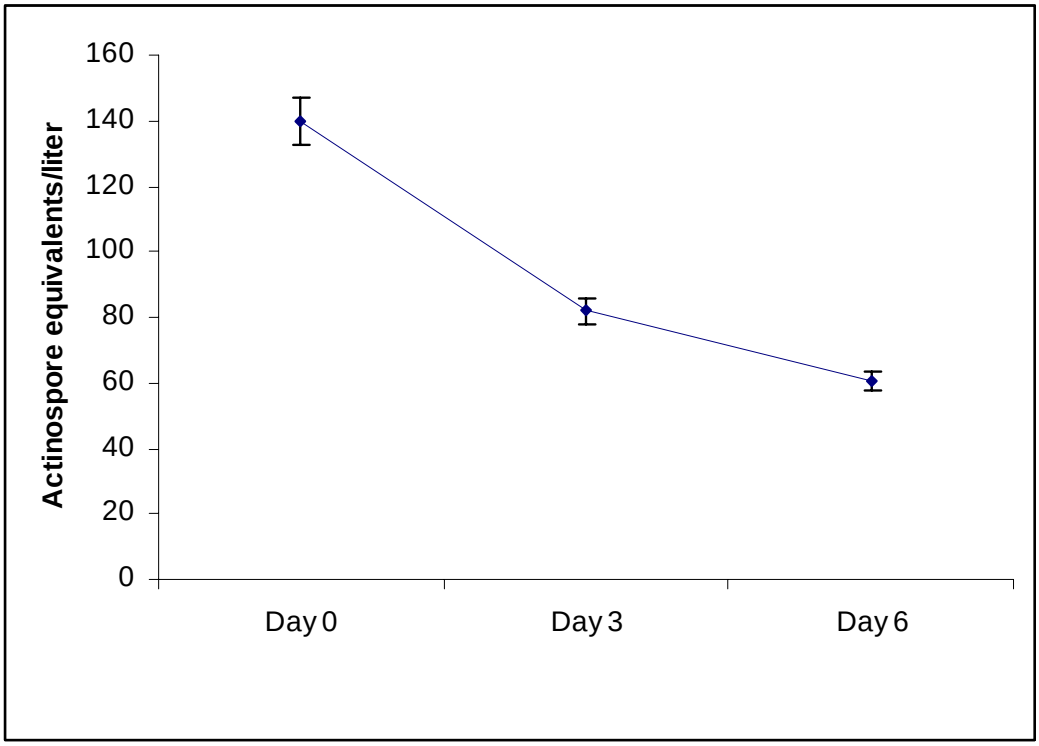


Figure 3.5. Experimental trial 1 data. Mean actinospore equivalents/liter by day for all pond locations.

Table 3.6. Experimental trial 1 data. Survival, mean percent gill damage and approximate parasite abundance determined by QPCR analysis by cage location by day.

Pond Location	% morality*	Mean % gill damage (6 d)†	Day 0‡		Day 3§		Day 6§		Mean AE l^{-1}¶
			C_T	AE l^{-1}	C_T	AE l^{-1}	C_T	AE l^{-1}	
A1	0%	N/A	28.4	74.3^{2}	26.9	73.3^{1}	27.4	57.8^{1}	$68.5^{c,1}$
A2	86.7%	30.0%[a]	27.2	127.5^{1}	25.9	116.6^{2}	27.4	58.8^{1}	$101.0^{abc,1}$
A3	13.3%	7.3%[b]	27.0	138.3^{1}	27.5	55.9^{1}	28.0	44.9^{1}	$79.8^{bc,1}$
A4	6.7%	8.5%[b]	26.4	183.3^{1}	26.9	72.5^{1}	26.5	86.7^{2}	$114.2^{ab,1}$
B1	20.0%	8.9%[b]	26.8	152.7^{1}	25.6	133.0^{2}	27.2	63.6^{1}	$116.5^{ab,1}$
B2	73.3%	17.0%[b]	28.3	77.0^{2}	26.5	88.2^{1}	26.8	78.0^{1}	$81.1^{abc,1}$
B3	20.0%	14.6%[b]	27.0	138.8^{1}	27.6	52.3^{2}	27.1	65.8^{1}	$85.7^{abc,1}$
B4	33.3%	13.5%[b]	26.8	149.0^{1}	26.8	76.9^{1}	28.0	48.8^{1}	$91.6^{abc,1}$
C1	26.7%	8.1%[b]	25.7	249.3^{3}	27.3	62.7^{1}	28.0	45.3^{1}	$119.1^{a,1}$
C2	26.7%	9.0%[b]	27.4	120.7^{1}	27.5	57.0^{1}	27.4	58.8^{1}	$78.9^{bc,1}$
C3	80.0%	9.3%[b]	27.7	101.7^{1}	25.8	122.0^{2}	28.4	37.4^{2}	$87.1^{abc,1}$
C4	60.0%	10.0%[b]	27.0	146.3^{1}	26.3	97.6^{1}	26.1	105.3^{3}	$116.5^{ab,1}$
D1	0.0%	N/A	26.3	189.8^{2}	26.6	84.9^{1}	28.1	44.7^{1}	$106.5^{abc,1}$
D3	46.7%	8.4%[b]	27.0	145.2^{1}	27.0	71.2^{1}	28.3	37.8^{2}	$85.1^{abc,1}$
D4	40.0%	8.0%[b]	27.6	108.4^{1}	27.2	64.0^{1}	27.0	69.1^{1}	$80.6^{bc,1}$
Mean (S.D.)	35.6% (28.4%)	11.7% (± 6.7%)	27.1 (± 0.8)	140.15 (± 48.0)	26.2 (± 0.7)	81.9 (± 26.1)	26.8 (± 0.7)	60.4 (± 19.4)	94.2 (± 45.9)

* Sentinel fish held in pond for 7 days. Percent mortality calculated as number of survivors out of 15 fish
† Mean percent gill damage calculated for surviving sentinel fish exposed to each location for 6 d
‡ Inhibition was corrected by an 8 fold dilution of template DNA. AE l^{-1} represents actinospore equivalents calculated by a dilution factor of 8.
§ Inhibition was corrected by a 4 fold dilution of template DNA. AE l^{-1} represents actinospore equivalents calculated by a dilution factor of 4.
¶ Average of Day 0, Day 3 and Day 6 observations for the given location
[a,b] Within columns, values with different subscript are significantly different ($p<0.05$)
[1,2,3] Within columns, values superscript numbers represent value is within 1, 2 or 3 standard deviations of the mean

Experimental trial 2

Of the twenty pond trials, six were classified as mild; five were considered moderate and nine as severe PGD outbreaks according to criteria set by Wise et al. 2004. Actinospore equivalent concentrations were variable by sampling day within trials and by trials, however, for each sampling day; actinospore equivalents between sampling locations were within one log of each other. Greater actinospore equivalent concentrations were observed in the more severe outbreaks (Table 3.7). The greatest actinospore equivalent concentration observed was ≥153 actinospore equivalents l^{-1}, while the least actinospore equivalent concentration was <1 actinospore equivalents l^{-1}, observed on three occasions, where no parasite DNA was detected. There was a positive correlation between mean actinospore equivalent concentrations and percent mortality observed in sentinel fish exposed for 7 days (Fig. 3.6). Data is summarized in Table 3.6.

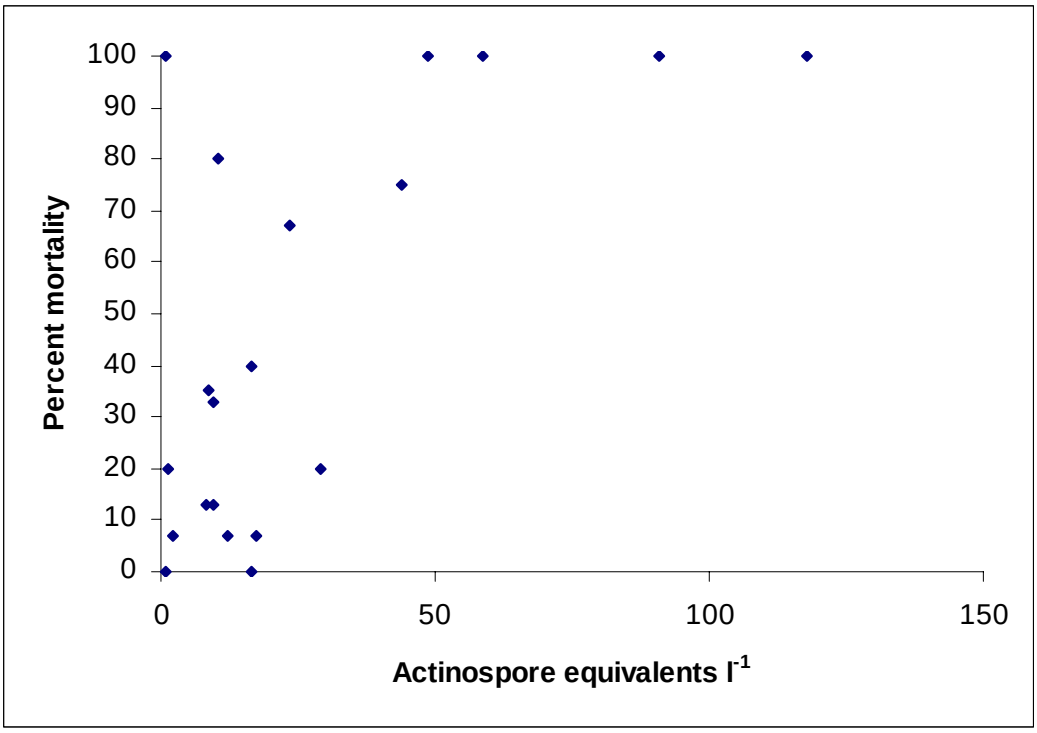


Figure 3.6. Experimental trial 2 data. Scatterplot demonstrating positive correlation between mean actinospore equivalents l^{-1} for two separate sampling days and percent mortality in sentinel fish exposed to PGD positive pond for 7 d.

Table 3.7. Experimental trial 2 data. Approximate abundance of *Henneguya ictaluri* actinospores in 20 commercial channel catfish ponds with varying proliferative gill disease severity. Data represented by mean percent gill damage of survivors from sentinel fish exposures, QPCR cycle threshold (C_T) values and approximate spore abundance from water samples; N/D = No parasites detected

					C_T values		AE l^{-1} †		
Rep	Pond	Survival (% mortality)*	% Gill damage (7 d)	Outbreak score[a]	Day 0	Day 7	Day 0	Day 7	Mean AE l^{-1} (Trial)‡
1	1	5 (66.6%)	10.0%	Moderate	35.4	30.5	≥ 1.7	≥ 15.4	≥ 8.6
2	1	4 (73.3%)	3.0%	Severe	27.0	33.3	≥ 82.6	≥ 5.3	≥ 43.9
3	1	12 (20.0%)	19.0%	Severe	28.6	29.5	≥ 34.3	≥ 23.7	≥ 28.9
4	1	3 (80.0%)	10.0%	Severe	31.7	31.1	≥ 9.1	≥ 11.6	≥ 10.3
5	1	0 (100.0%)	N/A	Severe	37.1	N/D	≥ 1.4	N/D	≥ 0.7
6	1	15 (0%)	2.0%	Mild	N/D	37.2	N/D	≥ 1.4	≥ 0.7
7	2	6(60.0%)	5.0%	Mild	33.3	29.2	≥ 4.4	≥ 28.7	≥ 16.6
8	2	14 (6.7%)	14.0%	Moderate	30.3	32.6	≥ 17.4	≥ 7.1	≥ 12.3
9	2	5 (66.6%)	9.0%	Severe	29.9	30.0	≥ 27.6	≥ 19.1	≥ 23.4
10	2	10 (33.3%)	4.0%	Mild	33.2	31.0	≥ 6.5	≥ 12.8	≥ 9.7
11	2	13 (13.3%)	1.0%	Mild	31.0	33.1	≥ 12.8	≥ 5.8	≥ 9.3
12	3	0 (100.0%)	N/A	Severe	25.3	26.7	≥ 152.9	≥ 82.6	≥ 117.8
13	3	0 (100.0%)	N/A	Severe	26.2	26.7	≥ 101.4	≥ 80.1	≥ 90.8
14	3	0 (100.0%)	N/A	Severe	26.7	29.1	≥ 82.6	≥ 34.5	≥ 58.6
15	3	0 (100.0%)	N/A	Severe	26.7	30.4	≥ 80.1	≥ 16.7	≥ 48.4
16	4	15 (0.0%)	10.0%	Moderate	29.0	33.8	≥ 29.2	≥ 3.5	≥ 16.4
17	5	14 (6.7%)	9.0%	Moderate	29.3	32.9	≥ 24.8	≥ 9.6	≥ 17.2
18	6	12 (20.0%)	1.0%	Mild	N/D	34.7	N/D	≥ 2.4	≥ 1.2
19	7	14 (6.7%)	6.0%	Moderate	36.6	34.9	≥ 2.2	≥ 2.1	≥ 2.2
20	8	13 (13.3%)	2.0%	Mild	36.0	35.3	≥ 8.5	≥ 1.7	≥ 5.1

* Sentinel fish held in pond for 7 days. Percent mortality calculated as number of survivors out of 15 fish
† Inhibition was corrected by a 4 fold dilution of template DNA. AE l^{-1} represents actinospore equivalents calculated by a dilution factor of 4
‡ Average of Day 0 and Day 7 observations

Experimental trial 3

Of the eight pond trials, five were classified as severe outbreaks while three were considered mild (Table 3.8), according to the criteria in the grading scale by Wise et al. (2004). Mean percent gill damage after 7 d exposure in ponds with survivors of severe outbreaks was 34% and 23%, but only 3%, 2% and <1% from ponds with mild PGD. Mortality ranged from 33.3% to 100% and 7 to 27% in severe and mild outbreaks respectively. There was no observed gill damage in any of the 24 hour exposure treatments for the mild ponds. Conversely, gill damage was observed within 24 hr exposures in three of the five severe ponds experiencing 100% mortality 7 d post-exposure (Table 3.8).

Parasite loads in 24 hr exposure treatments characterized by the ratio of parasite PSE to HSE for both 24 hr exposures ranged from -1.419 in the most severe outbreak to -8.000 in the mildest outbreak, where no parasite DNA was detected in any sentinel fish from the second 24 hr exposure. Log mean PSE/HSE was significantly higher in ponds considered to have a severe outbreak and there was a strong positive correlation between QPCR determined parasite loads and percent mortality in 7 d sentinel fish exposures (Fig. 3.7). All trials which qualified as severe outbreaks had log mean PSE/HSE values greater than -5.16.

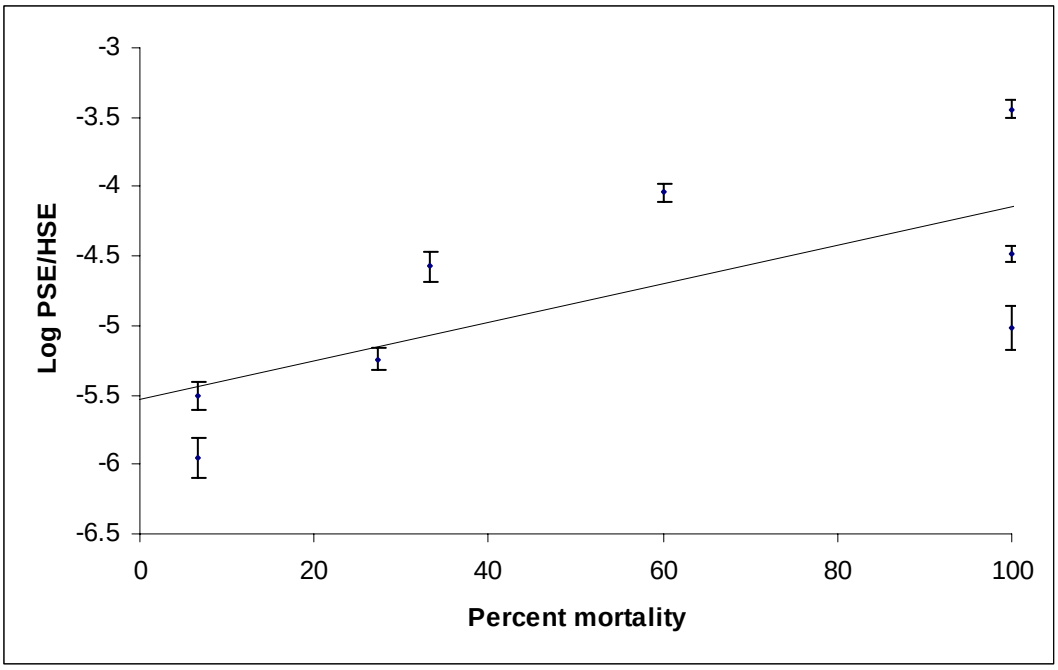


Figure 3.7. Experimental trial 3 data. Scatterplot demonstrating correlation between mean parasite level as determined by QPCR in sentinel fish exposed to PGD positive pond for 2 24 hr exposures and percent mortality observed in sentinel fish exposed for 7 d. QPCR data is presented in terms of the log of the ratio of mean parasite standard equivalents (PSE) and host standard equivalents (HSE).

Table 3.8. Experimental trial 3 data. Utilization of a *Henneguya ictaluri* specific QPCR assay to determine the parasite level in sentinel fish for 2 - 24 hour exposures and one 7 day exposure to ponds of varying proliferative gill disease severity.

Rep	Pond	Survivors* (% mortality)	Mean % gill damage (7d)	Pond score†	Mean % gill damage 24 hr (1)	Mean % gill damage 24 hr (2)	24 hr (1) Log PSE/HSE‡	24 hr (2) Log PSE/HSE‡	Mean Log PSE/HSE‡
1	1	0 (100.0%)	N/A	Severe	14.0%	13.0%	-3.212	-3.667	-3.440[a]
2	1	0 (100.0%)	N/A	Severe	9.0%	0%	-3.611	-6.16	-5.155[d]
3	1	6 (60.0%)	34.0%	Severe	0.0%	1.0%	-4.32	-3.78	-4.038[b]
4	1	10 (33.3%)	23.0%	Severe	0.0%	0.0%	-4.025	-5.086	-4.576[b]
5	1	0 (100.0%)	N/A	Severe	0.0%	0.0%	-4.690	-4.339	-4.487[b]
6	2	11 (27.3%)	3.0%	Mild	0.0%	0.0%	-5.287	-5.198	-5.242[d]
7	2	14 (6.7%)	2.0%	Mild	0.0%	0.0%	-5.559	-5.873	-5.716[e]
8	2	14 (6.7%)	0.0%	Mild	0.0%	0.0%	-5.402	-8.000	-6.701[f]

* Sentinel fish held in pond for 7 days. Percent mortality calculated as number of survivors out of 15 fish
† According to Wise et al. (2004) (Table 1).
‡ PSE = Parasite standard equivalent normalized to host 18S SSU rDNA
a,b,c Within columns, values with different superscript letters are significantly different ($p<0.05$)

Discussion

Based on findings from experimental trial 1, *H. ictaluri* actinospores are relatively evenly distributed throughout a commercial channel catfish pond during a PGD outbreak. Research conducted by Bellerud et al. (1995) demonstrated that populations of *D. digitata* are not equally distributed throughout the pond, and that populations of *D. digitata* within the pond are variable in their rates of actinospore release, making benthic sampling a poor predictor of potential outbreaks. However, data from experimental trial 1 demonstrated that although variation was observed in several sampling sites, most sample sites were within one standard deviation of the mean actinospore concentration of the pond for that day and the locations in the sampling grid with the highest and lowest concentrations of actinospores varied with the day of collection. Variations in localized high and low concentrations of actinospores were not consistently found in the same locations. The observed homogeneity and variation in localized high and low concentrations of actinospores indicates that although variations in oligochaete populations and their rate of actinospore release could result in areas with high concentrations of actinospores, diffusion from wind, aeration and other physical processes are more significant factors in actinospore distribution than the location of *D. digitata* in the benthic environment. Since channel catfish move freely in the pond and pond water diffusion occurs, these variations in actinospores concentrations may have little effect on the rate of infection in the pond as a whole. However, since water samples only capture the approximate actinospore concentrations from a given location at the time of sampling, it is recommended that a minimum of 2-3 pond water samples be taken from

separate geographic locations to reflect the actual concentration of actinospores in the pond environment. Alternatively, sentinel fish exposures represent a continuous sampling period over time, therefore multiple sentinel fish cages in multiple locations may not be necessary and one cage would be sufficient for an accurate estimation of the rate of infection for the pond.

The identification of a potentially lethal outbreak of PGD in a commercial channel catfish pond is a complicated task that faces channel catfish producers every spring. The sentinel fish protocol described by Wise et al. (2004) is reliable if the cage system does not fail within the sampling period, but is time consuming, labor intensive and requires assistance of trained lab personnel. A major drawback of sentinel fish exposures is the inability to determine the cause of death in a cage system in which 100% of the sentinel fish die prior to sampling. In the early spring, failure to properly acclimate sentinel fish to ambient pond temperatures can result in mortalities or predispose fish to other conditions such as saprolegniasis and columnaris disease that may be misinterpreted as PGD related. Alternatively, mortalities in sentinel fish also frequently occur in the summer months when heavy algal blooms create conditions conducive to the fouling of net-pens, which restricts water flow to the fish, and oxygen depletions in the pond environment. To overcome incidences of oxygen depletions, net-pens are often placed adjacent to mechanical aerators, which can result in significant currents flowing through the cage and fish may subsequently die of exhaustion. Both QPCR assay protocols eliminate this problem as PGD can be ruled out as the cause of death when PSE to HSE ratio of the 24 hr sentinel fish exposures or mean actinospore equivalents l^{-1} are

relatively low. This is evident in several of the experimental pond trials in which there was greater than 50% mortality in sentinel fish, yet PSE to HSE ratios or actinospore concentrations were low leading researchers to conclude that the cause of death may not be directly related to PGD (Table 3.7,3.8).

A distinct advantage of this water sampling protocol is it significantly reduces the time required to determine the presence of a pathogen in a commercial catfish pond compared to the sentinel fish protocol described by Wise et al. (2004) or the sentinel fish protocol described here. However, it should be noted that since DNA is conserved throughout the life cycle of the parasite, it is possible that DNA detected in the pond water samples could also represent myxospore stages that are not infective to the fish. Even so, the dynamics of the life cycle leads us to believe that during periods of peak actinospore release, there is a lower prevalence of the myxospore stage in the pond system, therefore the effect of the myxospore stage on the assay is likely to be negligible.

One major hindrance to the research conducted in this study was ponds used for experimental trials were initially identified through ADL case submissions. As such, most of the ponds sampled had outbreaks in which mortalities were already subsiding, which may account for the observed decreases in PSE to HSE ratio and actinospore concentrations from day 1 to day 7 in most trials. Further research needs to be conducted to determine the temporal variability of actinospore abundance as well as the variation in actinospore release over the course of a given day to determine the optimal sampling time for outbreak determination, as well as the relationship between PGD induced mortality

and various environmental conditions. Also, the effects of water quality parameters such as salinity and dissolved oxygen on PGD related mortalities need to be determined.

At this time, both QPCR assays are able to identify potentially lethal concentrations of *H. ictaluri* actinospores in ponds prior to stocking and have application in preventing PGD induced mortalities in fingerlings stocked in the spring of the year or for identifying a *H. ictaluri* free environment in which to relocate fish. This work has demonstrated that *H. ictaluri* actinospore abundance is fairly homogonous throughout the pond with actinospore numbers decreasing over the course of an outbreak which corresponds with previous research that states the rates of infection increase to a peak, then decrease gradually over time. Research in this laboratory has shown that once an outbreak begins to subside it does not tend to relapse within a given season, however this relationship needs further investigation (Wise et al. 2004). However, once a pond reaches a safe parasite threshold, the outbreak for that season is most likely over or on the decline and the pond can be stocked with little risk of losing fish to PGD. Although estimates based on this research are conservative, ponds with a log PSE to HSE ratio less than -5.2 in 24 hr sentinel fish exposures or actinospore equivalent concentrations less than 10 actinospores per liter, can be stocked with lower risk of losing fish, as long as log PSE to HSE ratio or actinospore equivalents is decreasing from the first to second sampling period. Although this research has identified parasite levels that correlate to high and low risk of losing fish stocked into the system, these values are not absolute and many other variables must be taken into consideration such as temperature, water quality and stocking densities when making management decisions.

Although, analysis of the data presented here indicates that the determination of parasite load in sentinel fish exposures is a more reliable method of determining PGD severity, the benefits of the water sampling protocol should not be overlooked. There were several instances in the second experimental trial where sentinel fish mortality was most likely not attributable to PGD, as evidenced by the low concentrations of actinospores detected in water samples. These ponds point out a flaw in the sentinel fish exposure method as mortalities observed in sentinel fish may be incorrectly attributed to PGD, when in fact PGD is not the cause of death and other factors need to be considered. This research provides another tool to be used in conjunction with currently practiced diagnostic and management protocols which will provide producers with the maximum amount of information for which to make management decisions.

Literature Cited

Balacázar J., L. D. Vendrell,I. de Blas I., I. Ruiz-Zarzuela, O. Gironés, J. L. Múzquiz. 2007. Quantitative detection of *Aeromonas salmonicida* in fish tissue by real-time PCR using a self-quenched flourogenic probe. Journal of Medical Microbiology. 56: 323-328

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

Bilodeau A. L., G. C. Waldbieser, J. S. Terhune, D. J. Wise, W. R. Wolters. 2003. A real-time polymerase chain reaction assay of the bacterium *Edwardsiella ictaluri* in channel catfish. Journal of Aquatic Animal Health. 15: 80-86

Bowser PR, JD Conroy: 1985, Histopathology of gill lesions in channel catfish associated with *Henneguya*. J Wildl Dis 21:177-179

Brinkman, N. E., R. A. Haughland, L. J. Wymer, M. Byappanahalli, R. L. Whitman, S/ J. Vesper. 2003. Evaluation of a rapid quantitative real-time PCR method for enumeration of pathogenic *Candida* cells in water. Applied Environmental Microbiology 69: 1775-1782

Burtle G. J., L. R. Harrison, E. L. Styer. 1991. Detection of a triactinomyxid myxozoan in an oligochaete from ponds with proliferative gill disease in channel catfish. Journal of Aquatic Animal Health. 3: 281-187

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

Chase D. M., D. G. Elliott, R. J. Pascho. 2006. Detection and quantification of *Renibacterium salmoninarum* DNA in salmonid tissues by real-time quantitative polymerase chain reaction analysis. Journal of Veterinary Diagnostic Investigation. 18: 375-380

Elibol-Fleming, B. 2006. Effects of *Edwardsiella ictaluri* infection on transcriptional expression of selected immune relevant genes in channel catfish, *Ictalurus punctatus*. PhD dissertation, Mississippi State University, Mississippi State, MS

Fontaine, M., E. Guillot E. 2003. An immunomagnetic separation real-time PCR method for quantification of *Cryptosporidium parvum* in water samples. Journal of Microbiological Methods 54: 29–36.

Griffin, M. J., D. J. Wise, A. C. Camus, M. J. Mauel, T. E. Greenway, L. M. Pote. 2008. A real-time polymerase chain reaction assay for the detection of *Henneguya ictaluri* in channel catfish. Journal of Veterinary Diagnostic Investigation. In Press.

Guy, R. A., P. Payment, U. J. Krull, P. A. Horgen. 2003. Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Applied Environmental Microbiology. 69: 5178–5185.

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

Heid, C. A., J. Stevens, K. J. Livak, P. M. Williams. 1996. Real-time quantitative PCR. Genome Research. 6: 986-994

Kelley, G.O., F. J. Zagmutt-Vergara, C. M. Leutenegger, K. A. Muklebust, M. A. Adkison, T. S. McDowell, G. D. Marty, A. L. Kahler, A. L. Bush, I. A. Gardner, R. P. Hedrick. 2004. Evaluation of five diagnostic methods for the detection and quantification of *Myxobolus cerebralis*. Journal of Veterinary Diagnostic Investigation. 16: 202-11

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

Panangala, V. S., C. A. Shoemaker, P. H. Klesius. 2007. TaqMan real-time polymerase chain reaction assay for rapid detection of *Flavobacterium columnare*. Aquaculture Research. 38: 508-517.

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

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

Pote, L. M, L. A. Hanson, L. Khoo. 2003. Proliferative Gill Disease. In: Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. Blue Book 5th edition, pp. 3.7.A1, Fish Health Section, American Fisheries Society

Souaze, F. A. Ntodou-Thome, C. Y. Tran, W. Rosténe, P. Forgez . 1996. Quantitative RT-PCR: limits and accuracy. Biotechniques 21: 280-285

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

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

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

Wise D. J., A. Camus, T. Schwedler, J. Terhune. 2004. Health Management. *In* Biology and culture of the channel catfish, ed. Tucker C. S., Hargreaves J.A., 1st ed., pp. 482-488. Elsevier B.V., Amsterdam, The Netherlands.

Wise, D. J., M. J. Griffin, J. Terhune, L. M. Pote, L. H. Khoo. 2008. Induction and evaluation of proliferative gill disease in channel catfish fingerlings. Journal of Aquatic Animal Health. In Press.

Wong, M., J. Medrano. 2005. Real-time PCR for mRNA quantitation. Biotechniques 39: 75-85.

CHAPTER IV

VARIATION IN SUSCEPTIBILITY TO *HENNEGUYA ICTALURI* INFECTION OF TWO SPECIES OF CATFISH AND THEIR HYBRID CROSS

Introduction

Since its discovery in 1981, proliferative gill disease (PGD), caused by the myxozoan parasite *Henneguya ictaluri* (Pote et al. 2000), has been one of the most damaging diseases affecting the commercial aquaculture of the channel catfish *Ictalurus punctatus* Rafinesque, 1918. The complex parasitic life cycle involves a myxospore stage in the fish and an actinospore stage shed by the ubiquitous benthic oligochaete, *Dero digitata* (Styer et al. 1991; Pote et al. 2000). At present, there are no effective prophylactic or therapeutic treatments for PGD and mortalities can exceed 50% in severe outbreaks (Bowser et al. 1985; Wise et al. 2004).

Bosworth et al. (2003) examined the genetic variation for PGD resistance between blue catfish *Ictalurus furcatus* Rafinesque, 1918 and channel catfish and determined blue catfish were more resistant to PGD. Although the culture of blue catfish is limited due to other unfavourable production characteristics (Dunham et al. 1993), there is evidence that blue catfish are not only more resistant to PGD but are also resistant to *Edwardsiella ictaluri* and channel catfish herpes virus (Graham 1999).

Although myxozoan host specificity is open to debate, there is evidence that some myxozoan life cycles demonstrate high degrees of host specificity (Molnar 1994; Hervio et al. 1997; Molnar et al. 2002; Eszterbauer 2002; Eszterbauer 2004). Alternatively, it has been shown that the actinospore stages of *Myxobolus cerebralis* and *Ceratomyxa shasta* do not demonstrate high levels of host specificity; however, susceptibility to infection by these parasites is highly variable amongst closely related salmonids (Ibarra et al. 1992; 1994; Hedrick et al. 1999a; 1999b).

Bosworth et al. (2003) confirmed that blue catfish and channel catfish x blue catfish F1 hybrids exhibit less gill damage than channel catfish when placed in ponds experiencing PGD-related fish mortalities. This current study was designed to confirm findings by Bosworth et al. (2003) and to compare the resistance of blue catfish, channel catfish and channel catfish x blue catfish hybrids to PGD to provide insight into the underlying causes for resistance in blue catfish.

Materials and Methods

Experimental design

Three replicate week long pond trials were conducted in commercial channel catfish ponds containing fish diagnosed with PGD in the resident fish population. All diagnostic evaluations on commercially produced fish were conducted by the Aquatic Diagnostic Laboratory (ADL) of the Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS. Specific pathogen free catfish fingerlings of each genetic strain were obtained from the United States Department of Agriculture Catfish Genetics

Research Unit in Stoneville, MS. For each genetic strain, three net-pens, constructed of 5 mm nylon mesh to allow free-exchange of water within the confined area, were each stocked with 20 fish. Five fish were sampled from each cage on days 1, 3, 5 and 7 post-exposure and processed for gross examination, molecular analysis and histology. Upon removal from the pond, fish were transported live to the NWAC in an aerated holding tank and held until sampling.

Blood collection, gross examination and histology

Immediately prior to sampling, fish were euthanized by an overdose (1000 mg/L) of MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate) (Argent Laboratories, Redmond, WA). Approximately 100 µl of blood was collected from the caudal vein of each fish sampled on the last day of each trial and transferred to a 1.5 µl microcentrifuge tube and stored at -80 °C until further processing. The presence and severity of PGD lesions was determined by microscopic examination of gill clip wet mounts (~40-80 filaments) from a left gill arch. PGD was defined as the presence of lytic lesions in the cartilage of the gill filaments (Fig. 2.2) (Wise et al. 2004; 2008; Griffin et al. 2008). Another ~40-80 filaments were removed from a left arch and processed for molecular analysis. Five fish from each genetic strain had individual right gill arches removed and placed in 10% neutral buffered formalin for a minimum of 24 h. Tissues were then processed by dehydration in a graded series of ethanol solutions of increasing strength, followed by clearing in a series of xylenes, embedding in paraffin, and sectioning at 6 µm. Prepared slides were stained with hematoxylin and eosin (H&E). Samples were

designated as positive or negative based on the presence of the characteristic changes including foci of osteochrondrolysis, epithelial hyperplasia, granulomatous inflammation and multinucleated trophozoites (Fig. 2.3, 2.4) (MacMillan et al., 1989; Pote et al. 2000; Pote et al. 2003; Griffin et al. 2008; Wise et al. 2008).

Actinospore collection

Benthic sediment was collected from a commercial channel catfish pond experiencing an outbreak of PGD in the resident catfish population. *D. digitata* were isolated from the benthic sediment and observed for 72 hours for the passing of the actinospore stages of *H. ictaluri* (Bellerud 1993; Pote et al. 1994; Bellerud et al. 1995). Organisms were identified morphologically according to descriptions of actinospores commonly found in commercial channel catfish ponds (Bellerud 1993).

DNA extraction from actinospores and SPF channel catfish

The genomic DNA from *H. ictaluri* actinospores and SPF channel catfish was isolated by suspending actinospores and tissues in 600 µl of cell lysis solution from the Gentra® Puregene® DNA isolation kit for fresh or frozen animal tissue (QIAGEN, Valencia, CA). Proteinase K (20 mg/ml) was added prior to overnight incubation at 55 °C. The remainder of the isolation was carried out according to the manufacturer's suggested protocol. The purified genomic DNA was then suspended in 30 µl of TE Buffer[b] (10 mM Tris, 1 mM EDTA, pH 7.0 – 8.0) and stored at -80 °C.

DNA extraction from blood

The genomic DNA of blood collected from each fish was isolated using the Gentra® Puregene® blood DNA isolation kit (QIAGEN, Valencia, CA) and using the manufacturer's suggested protocol for extracting and isolating genomic DNA from frozen whole blood. The purified genomic DNA was then suspended in 200 µl of TE Buffer[b] (10 mM Tris, 1 mM EDTA, pH 7.0 – 8.0) and stored at -80 °C.

Generation of QPCR standards

Target regions of the 18S small subunit (SSU) rDNA genes were amplified from genomic DNA of *H. ictaluri* actinospores and specific pathogen free (SPF) channel catfish by use of *H. ictaluri* specific PCR primers (Whitaker et al. 2001; Pote et al. 2003; Whitaker et al. 2005) and generic eukaryotic 18S SSU rRNA primers (Elibol 2006), respectively (Table 1). The 25 µl PCR reaction mixtures contained 2.5 µl of 10X reaction buffer (10 mM Tris, 50 mM KCl, pH 9.0, 4.0 mM $MgCl_2$), 2.0 µl of dNTP mixture (2.5 mM of each dNTP), 5 pM of each primer, 0.5 units of hot start *Taq* DNA polymerase (Takara® hot start *Taq* polymerase, Takara Bio USA, Madison, WI) and 2 µl of DNA template. The PCR was carried out on a PTC-100 thermal cycler, (GMI, Inc., Ramsey, MN) programmed for 1 cycle of 95 °C for 10 min, 50 °C for 2 min, and 72 °C for 4 min followed by 35 cycles of 92 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min with a final extension cycle of 72 °C for 5 min. The PCR amplification products were analyzed by electrophoresis on a 1.5% agarose gel and stained (Gelstar® nucleic acid stain, Cambrex, East Rutherford, NJ) to confirm the presence of a single PCR product.

The PCR products were purified using a Montage® PCR centrifugal filter device, (Millipore, Billareca, MD) and cloned using the PCR4-TOPO® cloning kit (Invitrogen, Carlsbad, CA). The plasmid clones with 18S SSU rDNA gene inserts from *H. ictaluri* and SPF channel catfish were each grown in culture overnight and plasmid purification was performed using the Qiagen plasmid mini-prep kit (Qiagen Inc.,Valencia, CA). The plasmid standards were quantified as described above and their concentrations were adjusted to 1.0 ng/µl. The standards were then serially diluted and utilized for the generation of standard curves and to serve as positive controls for QPCR analysis.

QPCR

Gill clips designated for molecular analysis were placed in 600 µl of cell lysis solution from the Puregene® DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN) and DNA isolation and quantification was carried out as described previously. At least 1 ng of total genomic DNA was added to each reaction and all samples were analyzed in triplicate according to the QPCR protocol described by Griffin et al. (2008). The *H. ictaluri* specific TaqMan probe was labeled on the 5' end with the fluorescent reporter dye, 6-carboxyfluorescein (FAM), on the 5' end and the quencher dye, black hole quencher-1 (BHQ-1), on the 3' end, while the generic 18S SSU rDNA TaqMan probe was labeled with the fluorescent reporter dye hexachloro-6-carboxy-fluorescein and also quenched by black hole quencher-1 (BHQ-1), on the 3' end (Table 1). The 12.5 µl PCR reactions contained BioRad IQ® supermix (Biorad, Hercules, CA), 20 pM of each primer, 0.25 pM of TaqMan probe, 3 µl of template DNA and nuclease free water to volume.

Amplifications were performed on a BioRad Icycler v3.1® real-time PCR system (Biorad, Hercules, CA) programmed for 1 cycle of 95 °C for 3 min 30 seconds followed by 40 cycles of 95 °C for 30 seconds, 56 °C for 1 min and 72 °C for 30 seconds. Data collection was carried out following the 72 °C elongation step at the end of each cycle. To correct for initial template variations between samples, 18S SSU rDNA plasmid standard equivalents (PSE's) were determined along with *H. ictaluri* DNA PSE's, using the 18S SSU rDNA and probe combinations described by Elibol (2006). Sample C_T values were compared to a standard curve based on serially diluted plasmid standards of the amplicons generated by the *H. ictaluri* specific primers (Whitaker et al. 2001; Pote et al. 2003; Whitaker et al. 2005) or the 18SCCF/18SCCR (Elibol 2006), respectively (Table 1). Again, data was considered valid if the slope of the standard curve was between -3.1 and -3.6 representing reaction efficiencies between 90 and 110% (Wong and Medrano 2006). Results were normalized against the initial concentration of 18S SSU rDNA to prevent false negatives resulting from variations in starting template quantity and the ratio between parasite standard equivalents and host standard equivalents was used for the purpose of comparisons.

Table 4.1 Primer and probe sequences

Name	Direction	Sequence*†‡	Reference
A1-1	Forward	5' - CAAAAGTTTCTGCTATCATTG - 3'	Whitaker et al. 2001
A1-2	Reverse	5' – AGCGCACAGATTACCTCA - 3'	Whitaker et al. 2001
H9	Forward	5' - TTACCTGGTCCGGACATCAA - 3'	Hanson et al. 2001
H2	Reverse	5' - CGACTTTTACTTCCTCGAAATTGC - 3'	Hanson et al. 2001
18SCCF	Forward	5' - CGGAGAGGGAGCCTGAGAA - 3'	Elibol-Fleming 2006
18SCCR	Reverse	5' - CGTGTCGGGAATGGGTAATTTG - 3'	Elibol Fleming 2006
HITMP	Probe	5' - [FAM] - TCAGCCTTGATGTTGCCACCTCA - [BHQ1] - 3'	This paper
18STMP	Probe	5' - [HEX] – ACCACATCCAAGGAAGGCAGCAGGC - [BHQ1] - 3'	Elibol Fleming 2006

* FAM = 6-carboxyfluorescein
† HEX = hexachloro-6-carboxy-fluorescein
‡ BHQ1 = Black Hole Quencher-1

Statistical analysis

All statistical analysis was performed using SAS Software v. 9.1 (SAS Institute, Inc., Cary, NJ). Parasite standard equivalents determined by QPCR, were normalized to the host 18S rDNA and the ratio of PSE to HSE was subjected to a log transformation prior to statistical analysis. All samples in which were negative for *H. ictaluri* DNA were assigned a log value of -9. Multiple comparisons for weight, mean percent gill damage and mean log PSE to HSE ratio were analyzed by a one-way ANOVA and Duncan's multiple range test. For all trials, $p<0.05$ was considered significant.

Results

Mortalities

Mortalities or fish loss occurred in only two of the three experimental trials, with the greatest incidence occurring in experimental trial 1. Seven fish were lost from one channel catfish cage on the third day of the trial. Subsequently, there were no fish left to sample from that cage on day 5. There was one mortality observed from a second channel catfish cage on day 5, resulting in 9 total channel catfish being sampled on the fifth day of the trial. Also in experimental trial 1, one cage of blue catfish was lost by the fifth day of the trial leaving no fish to sample from that cage. At this point the trial was terminated. For the second and third trials, one mortality was observed in blue catfish from trial 2, resulting in only 14 fish being sampled on day 7. No mortalities or fish losses occurred in trial 3.

Gross examination of gill clip wet mounts

Based on microscopic examination of gill wet mounts, the severity of PGD in the first trial was significantly greater than from the second and third trial with both channel catfish and hybrid catfish showing mean percent gill damage greater than 30% after 5 d in the pond (Table 4.2). Alternatively, in trials 2 and 3, mean percent gill damage in channels and hybrids after 7 d exposures was less than 21%, and no chondrocytic lesions were observed grossly in any of the blue catfish from the second and third trials. The only blue catfish which demonstrated any chondrolysis of the filamental cartilage were from trial 1.

QPCR

For all trials, blue catfish had significantly lower parasite loads in gill tissue than channel and hybrid catfish, averaging nearly 2 orders of magnitude less PSE per HSE in trial 1, and nearly 4 and 5 orders of magnitude less in trials 2 and 3, respectively Comparatively, hybrid catfish gill tissue consistently had less parasite DNA than channel catfish through all three trials, but PSE per HSE for hybrids and channels were different by more than one order of magnitude (Tables 4.2, 4.3; Fig. 4.1). By the third day of each trial, 100% of channel and hybrid catfish gill tissues were PCR positive for *H. ictaluri,* which remained consistent for the remaining sampling days. Conversely, the number of blue catfish with gill tissue PCR positive for *H. ictaluri* was variable by day and did not increase consistently over time, as would be expected if the pathology of the organism were consistent with that observed in channels and hybrids. Molecular analysis of blood from fish on the last day of each trial showed the presence of *H. ictaluri* DNA in only one blue catfish, from experimental trial 1. Three of the blood samples from day 7 of trial 3 were compromised during processing resulting in the analysis of only 12 channel catfish blood samples for that day. Parasite DNA was detected in channel and hybrid catfish blood on the last day of the trials, but the greatest percentage of fish with *H. ictaluri* DNA in the blood in all three trials was in channel catfish.

Table 4.2. Data summary for all three experimental trials

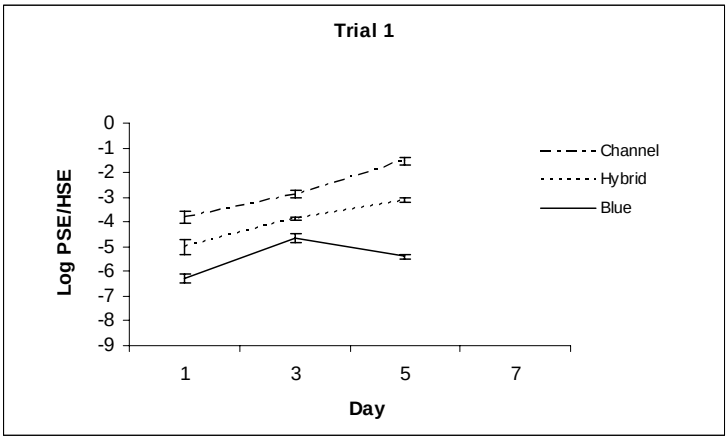
Trial	Day	Species	Weight (g)*	Mean % gill damage†	PGD positive Wet mount†	QPCR positive gills	Mean PSE/HSE‡	Log mean PSE/HSE
1		Blue	14.0^{kl}	0.3^{e}	1/15	7/15	5.0×10^{-6}	-6.25^{mn}
	1	Channel	56.0^{ab}	1.0^{e}	2/15	14/15	2.2×10^{-3}	-3.80^{ghi}
		Hybrid	44.2^{cde}	0.2^{e}	1/15	13/15	3.4×10^{-4}	-5.03^{jk}
		Blue	14.7^{jkl}	0.0^{e}	0/15	14/15	7.8×10^{-5}	-4.66^{j}
	3	Channel	48.3^{bcd}	56.7^{a}	13/13	13/13	7.4×10^{-3}	-2.87^{de}
		Hybrid	32.1^{fgh}	46.0^{ab}	15/15	15/15	1.8×10^{-4}	-3.89^{hi}
		Blue	19.1^{jkl}	6.6^{de}	5/10	7/10	3.4×10^{-4}	-5.39^{k}
	5	Channel	61.9^{a}	44.9^{ab}	9/9	9/9	9.3×10^{-2}	-1.63^{ab}
		Hybrid	34.1^{efgh}	33.1^{bc}	15/15	15/15	1.8×10^{-3}	-3.12^{ef}
2		Blue	15.4^{jkl}	0.0^{e}	0/15	4/15	$<1 \times 10^{-6}$	-8.82^{q}
	1	Channel	38.5^{def}	0.0^{e}	0/15	14/15	1×10^{-6}	-6.32^{l}
		Hybrid	31.5^{fgh}	0.0^{e}	0/15	10/15	1×10^{-6}	-7.13^{m}
		Blue	15.4^{ijkl}	0.0^{e}	0/15	4/15	$<1 \times 10^{-6}$	-8.26^{po}
	3	Channel	33.0^{efgh}	4.9^{de}	9/15	15/15	1.6×10^{-5}	-4.86^{jk}
		Hybrid	39.5^{cdef}	1.4^{de}	6/15	15/15	1.3×10^{-5}	-5.26^{k}
		Blue	15.7^{jkl}	0.0	0/15	3/15	1.5×10^{-4}	-8.18^{po}
	5	Channel	39.2^{cdef}	5.7^{de}	14/15	15/15	6.5×10^{-4}	-3.33^{efg}
		Hybrid	31.0^{fghi}	2.6^{de}	7/15	15/15	3.5×10^{-4}	-3.70^{ghi}
		Blue	11.7^{l}	0.0^{e}	0/14	9/15	2.1×10^{-3}	-7.03^{m}
	7	Channel	25.8^{ghij}	9.0^{de}	13/15	15/15	1.8×10^{-1}	-1.74^{ab}
		Hybrid	25.2^{ghijk}	9.6^{de}	15/15	15/15	5.0×10^{-2}	-2.74^{de}
3		Blue	16.7^{jkl}	0.0^{e}	0/15	2/15	1×10^{-6}	-8.50^{pq}
	1	Channel	60.8^{a}	0.3^{e}	2/15	15/15	8.1×10^{-4}	-3.62^{fgh}
		Hybrid	41.6^{cdef}	0.2^{e}	2/15	15/15	2.1×10^{-4}	-4.17^{i}
		Blue	20.5^{ijkl}	0.0^{e}	0/15	6/15	$<1 \times 10^{-6}$	-7.77^{no}
	3	Channel	49.9^{bc}	12.2^{de}	15/15	15/15	7.2×10^{-4}	-3.64^{fghi}
		Hybrid	46.2^{bcd}	15.7^{cde}	15/15	15/15	1.2×10^{-4}	-4.14^{hi}
		Blue	17.7^{jkl}	0.0^{e}	0/15	12/15	5.0×10^{-6}	-6.49^{l}
	5	Channel	63.0^{a}	14.9^{cde}	15/15	15/15	5.3×10^{-2}	-1.46^{a}
		Hybrid	34.6^{efg}	15.5^{cde}	15/15	15/15	3.0×10^{-2}	-1.83^{ab}
		Blue	23.0^{hijkl}	0.0^{e}	0/15	5/15	1×10^{-6}	-7.93^{o}
	7	Channel	63.0^{a}	8.5^{de}	12/15	15/15	1.1×10^{-2}	-2^{bc}
		Hybrid	34.6^{efg}	21.1^{cd}	14/15	15/15	2.9×10^{-2}	-2.48^{cd}

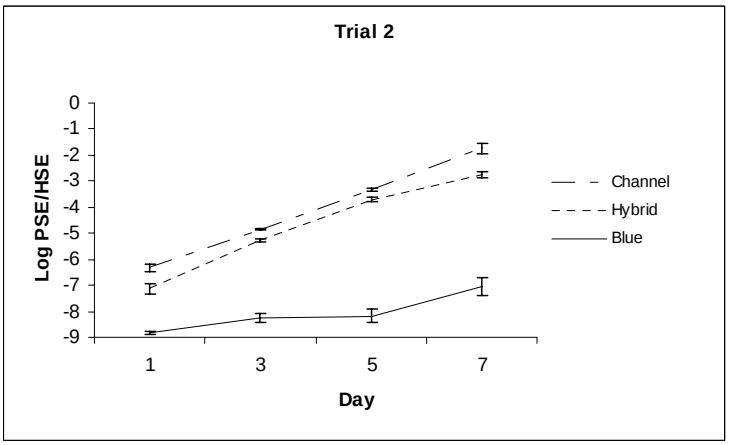
* Mean percent of gill filaments from surviving fish demonstrating at least one chondrolytic lesion
† PGD diagnosed in surviving fish by presence of at least one gill filament demonstrating chondrolytic lysis
‡ Data normalized to the initial quanitity of host 18S SSU rDNA
[a,b] Within columns, values with different letters are significantly different ($P<0.05$)

Table 4.3. Summary statistics for all three experimental trials.

Trial		Blue	Channel	Hybrid
1	Weight (g)	15.6[a]	54.7[b]	36.8[c]
	Mean % gill damage[*]	1.7[a]	31.2[b]	26.4[b]
	Mean log PSE/HSE -gill[†]	-5.3[a]	-2.9[b]	-4.0[c]
	PCR positive - gills[‡]	28/40	36/37	43/45
	PCR positive – blood[‡]	1/10	7/9	3/15
	PGD positive – wet mount[§]	6/40	24/37	31/45
2	Weight (g)	15.8[a]	34.1[b]	31.8[b]
	Mean % gill damage[*]	0[a]	4.9[b]	3.4[b]
	Mean log PSE/HSE - gill[†]	-8.1[a]	-4.3[b]	-4.8[c]
	PCR positive - gills[‡]	19/59	58/60	54/60
	PCR positive – blood[‡]	0/14	8/15	4/15
	PGD positive – wet mount[§]	0/59	36/60	28/60
3	Weight (g)	19.5[a]	59.2[b]	39.2[c]
	Mean % gill damage[*]	0.0[a]	9.0[b]	13.3[c]
	Mean log PSE/HSE - gill[†]	-7.7[a]	-2.7[b]	-3.2[c]
	PCR positive – gills[‡]	25/60	60/60	60/60
	PCR positive – blood[‡]	0/15	11/12	7/15
	PGD positive – wet mount[§]	0/60	44/60	46/60
All trials	Weight (g)	17.1[a]	48.5[b]	35.9[b]
	Mean % gill damage[*]	0.4[a]	12.7[b]	13.4[b]
	Mean log PSE/HSE - gill[†]	-7.3[a]	-3.3[b]	-4.0[c]
	PCR positive - gills[‡]	73/159	155/157	158/165
	PCR positive – blood[‡]	1/39	32/36	14/45
	PGD positive – wet mount[§]	6/159	104/157	105/165

* Mean percent of gill filaments from surviving fish demonstrating at least one chondrolytic lesion
† Data normalized to the initial quanitity of host 18S SSU rDNA
‡ PGD diagnosed by the amplification of *H. ictaluri* DNA by QPCR
§ PGD diagnosed in surviving fish by presence of at least one gill filament demonstrating chondrolytic lysis
[a,b] Within rows, values with different letters are significantly different ($P<0.05$)





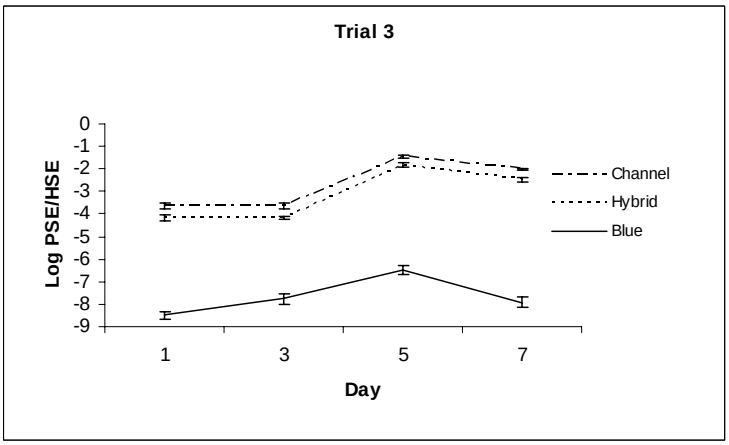


Figure 4.1. Parasite DNA equivalents in gill tissue detected by QPCR analysis. Data was normalized to initial concentration of 18S SSU rDNA. Values are presented as means ± SEM

Histology

For the first trial, histological changes observed in the channel catfish on day 1 were predominated by congestion, hemorrhage, and edema (Fig. 4.2). There was widespread lifting of lamellar epithelial cells from capillary endothelia creating clear spaces occasionally traversed by strands of fibrillar pale eosinophilic material. There were multifocal widely scattered aneurysmal dilations of lamellar capillaries, which often ruptured and hemorrhaged into the subepthelial spaces described above. These small focal hemorrhages occasionally obliterated the normal architecture of lamellar troughs. More significant extensive areas of hemorrhage were widely present and traversed long distances along filamental bone and cartilage. Large hemorrhages spanned up to 15-20 lamellar capillaries and expanded the overall width of affected filaments. Rarely within these areas of hemorrhage were found small focal collections of mononuclear inflammatory cells and foci of lysis involving the adjacent bone and cartilage (Fig. 4.2). However, organisms were not visualized. Epithelial changes were limited to minimal to mild hypertrophy and hyperplasia, typically in association with foci of lamellar hemorrhage and the bases of lamellar troughs. Moderately sized round leukocytes, characterized by moderate amounts of finely granular pale eosinophilic cytoplasm and slightly oval, eccentric nuclei with stippled chromatin were common within the filament vasculature. A band of identical cells of varying thickness dissected between the epithelial basement membrane and bone of large numbers of filaments. Changes in hybrids were comparable in all respects to those seen in channel catfish, but were encountered slightly less frequently. In contrast to the severe changes described for the

channel catfish, sections in hybrids were characterized by diffuse minimal congestion and rare, widely scattered, small foci of lamellar hemorrhage and minimal foci of epithelial hyperplasia involving small groups of lamellar troughs.

By the third day of trial 1, hemorrhage remains a significant finding in both channel and hybrid catfish gill tissues, but was reduced in severity (Fig. 4.2). Concomitant with this was an increase in the extent of epithelial hyperplasia, severity of inflammatory infiltrates, and size and number of lytic bone and cartilage lesions. Epithelial hypertrophy and hyperplasia was widespread and partially to completely filled focally extensive areas of consecutive lamellar troughs. Thrombosed lamellar capillaries were occasionally present within areas of hyperplasia. Inflammatory infiltrates shifted from predominantly granulocytic to epithelioid-like macrophages, with indistinct cell borders, finely vacuolated pale eosinophilic cytoplasm, and central nuclei with vesicular to marginated chromatin. Epithelial hyperplasia and macrophage infiltrates were most prominent adjacent to foci of osteochondrolysis. Mantles of macrophages were immediately apposed to lytic gill filaments and were separated from epithelial cells by a basement membrane. Macrophages were further distinguished from epithelial cells, which also had vesicular nuclei, but typically possessed a prominent nucleolus and have finely granular amphophilic cytoplasm. Lymphocytic infiltrates were increased and moderate in number, but limited primarily to filament and lamellar epithelial surfaces. For blue catfish, histological examination revealed widely scattered, small foci of minimal hemorrhage limited predominantly to lamellar capillaries. Larger hemorrhages associated with gill filaments, as described for channel catfish, were extremely rare.

There were also scattered, multifocal, mild foci of epithelial hyperplasia that typically filled lamellar troughs resulting in the fusion of 2-3 lamellae. Thrombosed capillaries were occasionally present within these foci. Rare focal lysis of filament bone and cartilage were present, but was not associated with severe epithelial hyperplasia or inflammatory infiltrates as seen in channel and hybrid catfish.

By the fifth day of trial 1, for both channels and hybrids, hemorrhage continued to diminish, while hyperplasia and macrophage infiltrates, as described previously, continued to increase (Fig. 4.2). Small numbers of elongated plump fibroblasts were visible in areas of osteochondrolysis, and bordered rare irregular foci of proliferating dysplastic cartilage. Located adjacent to foci of osteochondrolysis and less frequently within lamellar troughs, surrounded by mantles of macrophages, were large spherical structures (plasmodia) with numerous, centrally located, small, hyperbasophilic nuclei and an outer rim of clear to pale eosinophilic cytoplasm (Fig. 4.5). Conversely, examination of blue catfish tissues revealed changes that closely resembled those seen in channel and hybrid catfish from the first day of the trial, including the scattered presence of osteochondrolysis however no parasitic organisms were observed.

For the second trial, significant lesions were not observed in channel and hybrid catfish until the third day of the experimental trial, at which point, there were rare, small foci of epithelial hypertrophy and hyperplasia filling small numbers of consecutive lamellar troughs, often accompanied by mild lamellar and filamental hemorrhage. Rare foci of ostrochondrolysis were associated with some foci of hyperplasia and hemorrhage. Examination of blue catfish tissues at this stage revealed no significant findings.

On the fifth day of the second trial, changes in the channel and hybrid catfish typical of moderate to severe proliferative gill disease. There were multifocal, widespread, large foci of osteochondrolysis surrounded by broad zones of macrophages that expanded filaments and more superficial epithelial hypertrophy and hyperplasia that filled long expanses of lamellar troughs. Hemorrhage was mild. At this time, two of the five blue catfish examined had rare, small foci of epithelial hyperplasia that filled 2-3 consecutive troughs or mild hyperplasia that expanded the entire filament. By the seventh day of the trial changes in channel and hybrid catfish were severe but did not differ significantly from those described in the Day 5 sample, with the exception that parasitic organisms were now common. Only one of the blue catfish examined from this time point had rare, focal, minimal epithelial hyperplasia commonly associated with PGD, but no organisms were present.

Findings for the third trial did not differ from those of the second trial, except that changes were observed on the first day of the study. Again, changes observed in channels and hybrids were mild and limited to multifocal, widely scattered foci of epithelial hyperplasia that partially to completely filled typically 2-3, occasionally up to 8, consecutive lamellar troughs, resulting in focal expansion of filaments. By the third day changes were moderate to severe and typical of PGD. Lesions were widely distributed, with large areas of epithelial hyperplasia and infiltrates of macrophages surrounding areas of ostrochondrolysis. Hemorrhage was mild and organisms were not present. On the fifth day of the trial changes were typical of severe PGD. There were multifocal, widespread, large foci of osteochondrolysis surrounded by broad zones of

macrophages that expanded filaments and more superficial epithelial hypertrophy and hyperplasia that filled long expanses of lamellar troughs. Hemorrhage was mild and unlike the fifth day from the second trial, organisms were common in these sections.

On the seventh day of the third trial, the first significant findings were seen in four of the five blue catfish examined. Changes were minimal and limited to rare, isolated, small foci of epithelial hyperplasia, typically limited to 1-2 consecutive lamellar troughs. Although widely scattered and overall damage to the gills is interpreted as mild, one fish had well developed lesions typical of PGD in all respects, but again lacking visible organisms. In contrast, changes observed for channels and hybrids were typical of severe PGD. Lesions were large, widely distributed and parasitic organisms were common (Figs. 4.3, 4.4).

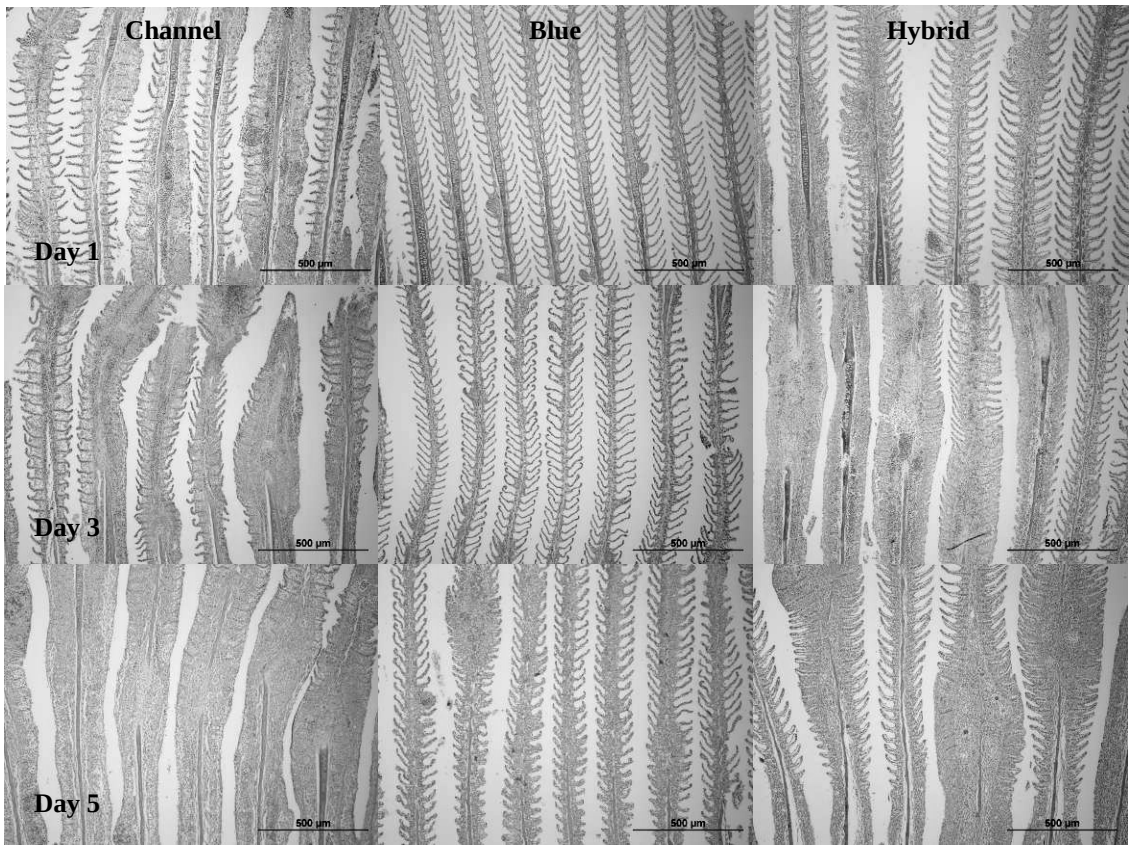


Figure 4.2. Experimental trial 1. Histological sections demonstrating pathological changes over time in fish held in a PGD positive pond

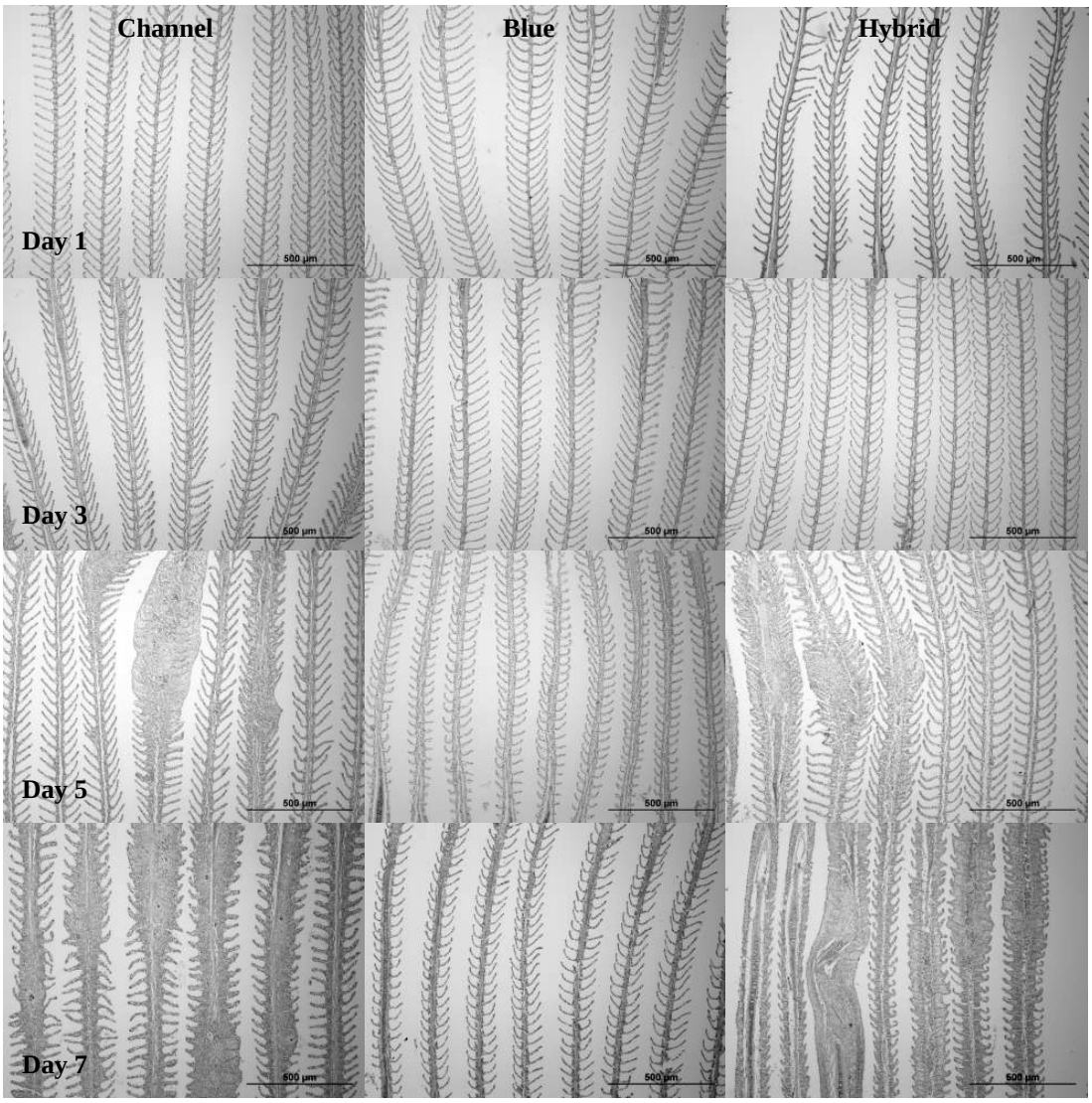


Figure 4.3. Experimental trial 2. Histological sections demonstrating pathological changes over time in fish held in a PGD positive pond

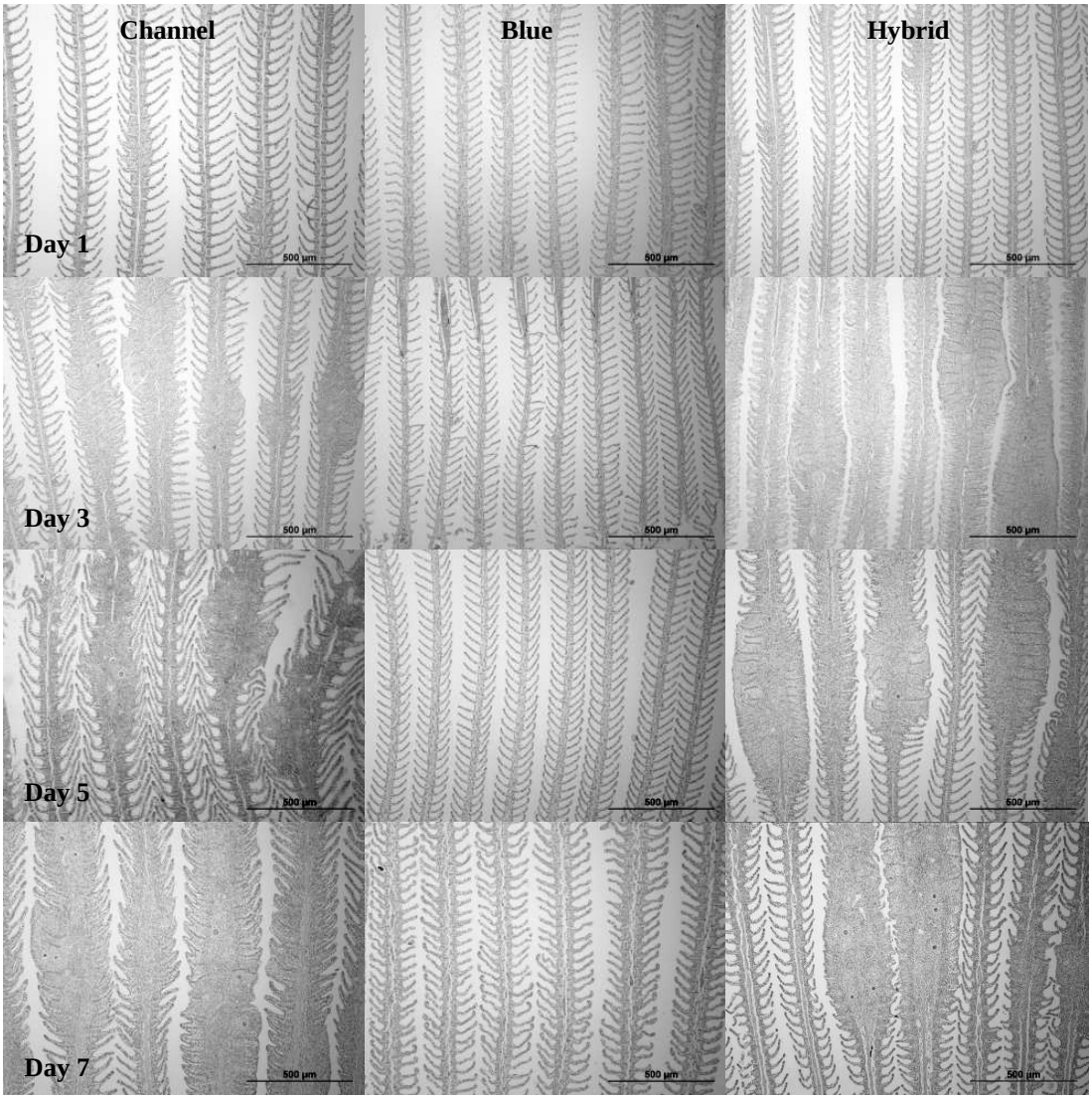


Figure 4.4. Experimental trial 3. Histological sections demonstrating pathological changes over time in fish held in a PGD positive pond

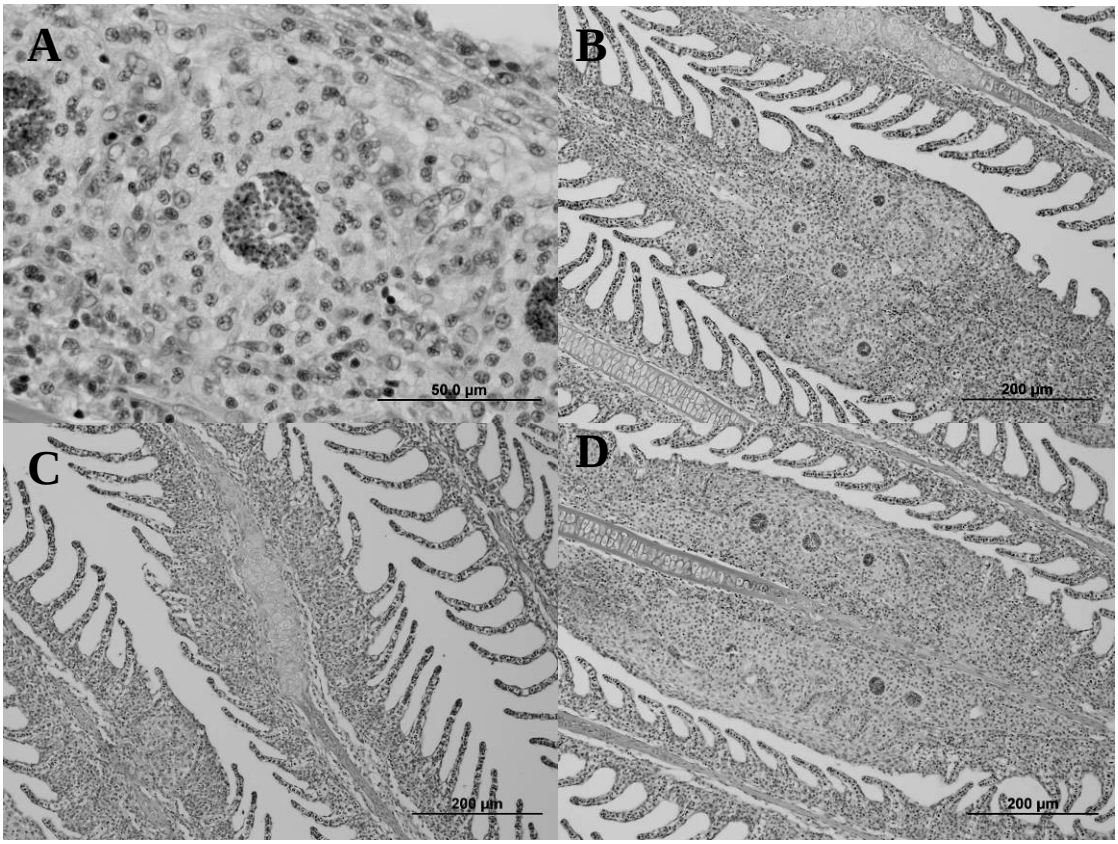


Figure 4.5. Histological sections of fill of channel catfish fingerling with proliferative gill disease (Day 7; Trial 3). Spherical structures (plasmodia) with numerous, centrally located, small, hyperbasophilic nuclei and an outer rim of clear to pale eosinophilic cytoplasm (A, B, D) are located adjacent to foci of osteochondrolysis (C,D) and less frequently within lamellar troughs (B).

Discussion

Data from these experimental trials supports the hypothesis that *H. ictaluri* may be unable to successfully complete its life cycle in blue catfish. This data also supports previous work stating that blue catfish will develop lesions characteristic of proliferative gill disease during severe outbreaks, but are significantly more resistant to PGD than both channels and hybrids (Bosworth et al. 2003). Only 6 of the 160 blue catfish examined demonstrated gross lesions indicative of PGD, and all blue catfish demonstrating chondrolytic lesions were from the first trial, in which the most severe lesions were observed in both the channel and hybrid catfish, suggesting a larger number of infective actinospores in the pond environment.

QPCR analysis did detect *H. ictaluri* DNA in the gill tissues of several blue catfish during the course of this study; however, there was no evidence of developing plasmodia in the histological sections for these fish. A limitation of PCR analysis is that the assay can only determine whether or not target DNA is present in a given sample, and not the viability of the organism itself. Assuming the actinospore stage of the organism is present in pond water at the time of sampling and considering the sensitivity of the QPCR assay, it cannot be stated with certainty that the small amounts of *H. ictaluri* DNA detected represents true infection, curtailed prior to the development of typical lesions, or surface associated contaminants trapped in gill tissues at sampling. Since amplification of target DNA does not distinguish between an active infection and DNA artifact, the presence of *H. ictaluri* DNA may not necessarily signify an active infection. Conversely, in all three trials, channel catfish and hybrid catfish gills consistently demonstrated a

significant increase in parasite DNA equivalents when compared to blue catfish. This finding, coupled with the presence of developing trophozoites in histologic sections indicates an active infection and subsequent proliferation or increased uptake of the parasite in channels and hybrids to a degree which was not observed in blue catfish.

QPCR analysis of the blood taken from fish on the last day of each experimental trial indicated that even in heavily infected channels and hybrids, a greater percentage of channel catfish have *H. ictaluri* DNA in their blood than hybrids suggesting the pathology of infection and degree of tissue migration of *H. ictaluri* is not identical in channel and hybrid catfish even though gross representation of the disease is similar. Although the biological significance of this finding remains unclear, it suggests that *H. ictaluri* may initiate infection but may not complete its life cycle in hybrid catfish as efficiently as it does in channel catfish. The presence of *H. ictaluri* DNA in the blood of one blue catfish from the heaviest infection (Trial 1), suggests that, although they demonstrate a high degree of resistance, the parasite can still overwhelm host defenses when exposed to large numbers of actinospores but in a more resistant host such as the blue catfish, the infection may be rapidly eliminated, preventing the parasite from completing its life cycle as efficiently as in channel and hybrid catfish.

Histologically, lesion development was qualitatively similar throughout the respective treatment groups in all three studies, varying only in severity over time. This variation is speculated to be related to challenge dose. In general, blue catfish were significantly less affected, although changes typical of PGD were present in some fish. Importantly, even though osteochondrolysis and inflammatory infiltrates were present in

the gills, these changes are non-specific and could be attributed to other non-PGD related factors. Most importantly, organisms were not visualized in blue catfish from any trial, despite being numerous in channel and hybrid catfish gills from the same exposure group.

The findings from this study beg the question of host specificity over host susceptibility. Although there is evidence of host specificity of the actinospore stage of select myxozoans (Molnar 1994; Hervio et al. 1997; Molnar et al. 2002; Eszterbauer 2002; Eszterbauer et al. 2002; Eszterbauer 2004) there is also evidence of a lack of host specificity but variable host susceptibility to the developmental stages of *M. cerebralis* and *C. shasta* in salmonids (Ibarra et al. 1992; 1994; Hedrick et al. 1999a;1999b). Regardless of the whether resistance is attributed to host specificity of the parasite or parasite resistance by the host, findings here thus supporting the earlier findings of Bosworth et al. (2003) demonstrating a significantly lower susceptibility to *H. ictaluri* infection in blue catfish than channel catfish and channel catfish x blue catfish hybrids.

The data presented here identifies another favorable characteristic of blue catfish, demonstrating a lower susceptibility to another significant channel catfish pathogen. The significantly increased disease severity and levels of parasite DNA in channel and hybrid catfish, coupled with the lack of developing organisms in blue catfish tissue sections indicates that *H. ictaluri* may be unable to successfully complete its life cycle in the blue catfish. The presence of a low level of *H. ictaluri* DNA in the blue catfish gills may be attributed to the presence of the organism in the gill mucus or a consequence of the organism being present in the water and may not signify an active infection. The length of time the organism remains in contact with the gill tissue after initial penetration

potentially fails or the length of time gill tissue remains PCR positive after infection has been eliminated by the host is not known. The lack of developing organisms in blue catfish tissue sections and negligible gill damage observed throughout the study indicates that further life stages of this organism did not occur and the infection was unsuccessful.

Furthur research is needed to identify the specific protective mechanisms utilized by blue catfish to prevent *H. ictaluri* infection which catfish could prove invaluable in a selective breeding program. The inability of *H. ictaluri* to successfully complete its life cycle in blue catfish also suggests that this could be utilized to potentially interrupt the life cycle through crop rotations, preventing the accumulation of *H. ictaluri* in pond systems that can result in devastating losses for commercial channel catfish operations.

Literature cited

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

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

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

Bowser PR, JD Conroy: 1985, Histopathology of gill lesions in channel catfish associated with *Henneguya*. J Wildl Dis 21:177-179

Dunham, R. A., C. Hyde, M. Masser, J.A. Plumb, R.O. Smitherman, R. Perez and A.C. Ramboux. 1993. Comparison of culture traits of channel catfish, *Ictalurus punctatus*, and blue catfish, *I. furcatus*. Journal of Applied Aquaculture 3: 257-267.

Elibol-Fleming B. 2006. Effects of *Edwardsiella ictaluri* infection on transcriptional expression of selected immune relevant genes in channel catfish, *Ictalurus punctatus*. PhD dissertation, Mississippi State University, Mississippi State, MS

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

Eszterbauer, E. 2002. Molecular biology can differentiate morphologically indistinguishable myxozporean species: *Myxobolus elegans* and *M. hungaricus*. Acta Veterinaria Hungarica. 50: 51-57.

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

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

Hedrick, R. P., T. S. McDowell, M. Gay, G. D. Marty, M. P. Georgiadis, E. MacConnell. 1999a. Comparative susceptibility of rainbow trout *Onchorynchus mykiss* and brown trout *Salmo trutta* to *Myxobolus cerebralis*, the cause of salmonid whirling disease. Diseases of Aquatic Organisms: 173-183

Hedrick, R.P., T. S. McDowell, K. Mukkatira, M. P. Georgiadis, E. MacConnell. 1999b. Susceptibility of selected inland salmonids to experimentally induced infections with Myxobolus cerebralis, the causative agent of whirling disease. Journal of Aquatic Animal Health. 11: 330-339.

Hervio, D. M., M. L. Kent, J. Khattra, J. Sakanari, H. Yokoyama, R. H. Devlin. 1997. Taxonomy of *Kudoa* species (Myxosporea), using small-subunit ribosomal DNA sequence. Canadian Journal of Zoology. 75: 2112-2119.

Ibarra, A. M., R. P. Hedrick, G. A. E. Gall. 1992. Inheritance of susceptibility to *Ceratomyxa shasta* (Myxozoa) in rainbow trout and the effect of legth of exposure on the liability to develop ceratomyxosis. Aquaculture. 104: 217-229

Ibarra A. M., R. P. Hedrick, G. A. E. Gall. 1994. Genetic analysis of rainbow trout susceptibility to the myxosporean, *Ceratomyxa shasta*. Aquaculture. 120: 239-262

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

Molnar, K. 1994. Comments on the host, organ and tissue-specificity of fish myxosporeans and on the types of their intrapiscine development. Parasitologia Hungarica. 27: 5-20.

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

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

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

Pote, L. M., L. A. Hanson, L. H. Khoo. 2003. Proliferative Gill Disease. *In*: Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. Blue Book 5th edition, pp. 3.7.A1, Fish Health Section, American Fisheries Society

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

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

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

Wise D. J., A. Camus, T. Schwedler, J. Terhune. 2004. Health Management. *In* Biology and culture of the channel catfish, ed. Tucker C. S., Hargreaves J.A., 1st ed., pp. 482-488. Elsevier B.V., Amsterdam, The Netherlands.

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

Wong, M. L., J. F. Medrano. 2005. Real-time PCR for mRNA quantitation. Biotechniques. 39: 75-85.

CHAPTER V

CONCLUSION

Proliferative gill disease (PGD) has been a major problem for the channel catfish industry for over thirty years, and only recently have researchers gained enough understanding of PGD to implement management strategies for the disease. Until now, the most practical method of controlling PGD was avoidance through management practices and not stocking fingerlings into ponds with high actinospore concentrations. The PGD status of a pond to be stocked had previously been determined through sentinel fish exposures, which although accurate in their prognosis, have limitations that necessitated the development of a more rapid and efficient means of determining actinospore concentrations.

The *Henneguya ictaluri* specific QPCR assay developed for this study was found to be highly specific and very sensitive and although the assay is more costly and requires more specialized training to perform, it outperformed all currently practiced diagnostic methods providing confirmation of *H. ictaluri* infection more rapidly than histology and end-point PCR. While gross examination of gill clip wet mount preparations provide reasonably accurate presumptive diagnosis and while convenient in their expediency, the method has limited sensitivity, especially in larger fish where size limits the number of filaments that can be accurately examined.

From a management perspective, the QPCR assay has tremendous value in identifying potentially lethal concentrations of *H. ictaluri* actinospores in ponds prior to stocking and preventing PGD induced mortalities in fingerlings stocked in the spring of the year. The assay and concurrent methodologies described in this dissertation not only provide a more rapid and efficient method of determining actinospore concentrations in a pond prior to stocking, they also eliminate the need for sentinel fish. Although the relationship between *H. ictaluri* actinospore concentration and PGD related mortalities have been demonstrated, the levels determined in this study to be low risk are conservative. The QPCR assay can provide information that is valuable in making management decisions but it can only provide one piece of the puzzle. It is important to note that the relationship between actinospore levels and PGD related mortalities is not absolute and other variables such as water quality, temperature, salinity and stocking densities also need to be taken into consideration when deciding when a pond is safe to stock.

This study has also demonstrated the potential of the QPCR assay as a research tool. Since DNA is conserved throughout the life cycle of an organism, the QPCR assay adds a molecular aspect to investigations regarding life cycle studies and the development and pathogenesis of *H. ictaluri* in the fish or oligochaete hosts. In conjunction with gross examination and histological analysis of fish tissues, QPCR helped demonstrate another favorable characteristic of blue catfish; lower susceptibility to PGD. The results generated by QPCR added one more level of analysis to this study, which supported the earlier findings of Bosworth et al. (2003) and support host specificity among select

myxozoans. Admittedly, further research is needed to identify the specific protective mechanisms utilized by blue catfish which may prevent the completion of the life cycle in this fish host, however, these findings suggest that blue catfish could be utilized to potentially interrupt the life cycle through crop rotations, thereby preventing the accumulation of *H. ictaluri* in pond systems that can result in devastating losses.

The *H. ictaluri* specific QPCR assay developed for this study was found to be successful in the detection of *H. ictaluri* in from fish tissues and environmental samples and the assay shows promise in both research and production settings. As stated previously, the ability to quantify *H. ictaluri* DNA from fish tissue and environmental samples provides an invaluable tool in diagnostics, epidemiological studies, treatment evaluations and management of this disease.

APPENDIX A

COPYRIGHT PERMISSION

THAD COCHRAN
NATIONAL WARMWATER AQUACULTURE CENTER
Delta Branch Experiment Station

Mississippi State UNIVERSITY

July 23, 2008

Matt J. Griffin
Thad Cochran National Warmwater Aquaculture Center
127 Experiment Station Road
Stoneville, MS 38776

I request permission for reprinting the following material appearing in the Journal of Veterinary Diagnostic Investigation vol. 20, issue 5 (September 2008); "A real-time polymerase chain reaction assay for the detection of the myxozoan parasite *Henneguya ictaluri* in channel catfish" by Matt J. Griffin, David J. Wise, Alvin C. Camus, Michael J. Mauel, Terrence E. Greenway and Linda M. Pote.

The requested material will appear in "Development and application of a real-time polymerase chain reaction assay for the myxozoan parasite *Henneguya ictaluri*"; Matthew J. Griffin; PhD Dissertation; Mississippi State University, Mississippi State, MS; pp. 34-61.

I understand you have sole rights to this material. Permission is desired for publication in this book and in future editions and revisions thereof in all languages and countries. I shall use the conventional form of acknowledgment, including author, title, publisher's name, and date.

Permission may be granted by filling in the bottom portion of this letter and returning it to me.

If you are not the copyright holder or if I need other permissions for world rights, kindly indicate that fact.

Sincerely,

Matt J. Griffin

Provided proper acknowledgment is given, I hereby grant permission to use the material requested above.

Approved by:  Date: 7-23-2008

The American Association of Veterinary Laboratory Diagnosticians holds the copyright to the requested material. Acknowledgment should be given to AAVLD.

127 Experiment Station Road, P.O. Box 197, Stoneville, MS 38776-0197
Phone (662) 686-3242 • Fax (662) 686-3320