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Investigations into the Etiology of Ulcerative Lesions in Atlantic Menhaden, Brevoortia tyrannus

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INVESTIGATIONS INTO THE ETIOLOGY OF ULCERATIVE LESIONS IN ATLANTIC MENHADEN, *BREVOORTIA TYRANNUS*

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ABSTRACT

Atlantic menhaden along the eastern seaboard of the United States develop characteristic ulcerative lesions, a condition termed ulcerative mycosis. These lesions are identical to those seen across Asia in fish affected by epizootic ulcerative syndrome, a condition caused by the oomycete *Aphanomyces invadans*. Recently, there has been much debate as to the cause of the lesions and massive fish kills in which the majority of menhaden exhibit these lesions. Young-of-the-year menhaden inhabiting estuarine environments are the primary species affected and little is known about the factors involved in the initiation of the lesions, or why menhaden are predominantly infected. A biological stain, fast green, was used to determine whether hypoxia, a stressor commonly encountered in estuarine environments, could cause epidermal damage, perhaps providing a means for penetration by *A. invadans*. Results indicated that fish exposed to hypoxia showed a greater percentage of stained surface area, possibly indicative of areas of cell death. Aqueous bath exposures were then carried out to determine whether exposure to hypoxia would cause a greater prevalence of lesion development in menhaden. Fish were exposed with 100 zoospores/mL for 6 hours but only a few developed lesions. While only fish exposed to the oomycete developed lesions and at least one fish in each treatment with the oomycete developed a lesion, positive controls did not develop significant percentages of lesions. No difference occurred between fish exposed to hypoxia prior to zoospore exposure and fish that did not experience hypoxia. Problems were encountered with batch production of zoospores, so a series of experiments were undertaken to optimize the methods for sporulation. The media used to grow the hyphae, the volume of the media used, the sporulation water used and the light cycle seemed to play significant roles in the outcome of the sporulation. Atlantic menhaden, hogchoker, striped killifish, mummichog and mullet were inoculated with 80 zoospores/fish to explore species differences in lesion development. All species developed lesions with the exception of mullet. Killifish developed lesions similar to menhaden while hogchoker and mummichogs developed only subdermal hemorrhaging and mummichogs showed evidence of myocyte regeneration. These experiments show that species barriers as well as ecological barriers help to explain some of the factors involved in the initiation of lesions in menhaden.

INVESTIGATIONS INTO THE ETIOLOGY OF ULCERATIVE LESIONS IN ATLANTIC MENHADEN, *BREVOORTIA TYRANNUS*

OVERVIEW

In recent years, fish kills along the eastern seaboard of the United States involving Atlantic menhaden (*Brevoortia tyrannus*) have attracted intense interest (for review see Dykstra and Kane 2000). Menhaden and other fishes develop characteristic ulcerous skin lesions that have previously been attributed to the activity of the toxic dinoflagellate, *Pfiesteria piscicida* (Burkholder et al. 1992, 1995, 2001). During 1997, *P. piscicida* was implicated in several small fish kills, outbreaks of lesions in menhaden, and adverse human health effects in Maryland portions of Chesapeake Bay (Grattan et al. 1998). Since then, lesions in menhaden have been used in conjunction with presumptive counts of *Pfiesteria*-like cells in water samples, PCR based assays of water samples, and toxic fish bioassays to monitor for *Pfiesteria* activity in Maryland and North Carolina waterways (Burkholder et al. 2001). The prevalence of lesions has been used to make decisions regarding temporary river closures. The observed co-occurrence of menhaden lesions and *Pfiesteria piscicida* in certain acute fish kills in estuaries of North Carolina and a laboratory exposure study have provided some support for these decisions. Striped bass (*Morone saxatilis*) and tilapia (*Oreochromis* spp.) exposed to sublethal levels of *P. piscicida* exhibited a complete loss of epidermis after 48 hours, followed by the development of ulcers in tilapia allowed to recover from the exposure to *Pfiesteria.* The ulcers were mainly colonized by bacteria, but oomycete hyphae were seen in one ulcer (Noga et al. 1996).

The characteristic skin lesions in menhaden (Figure 3) are often located near the anus, and appear as deeply penetrating circular lesions with extensive necrosis and tissue loss. Histologically they are characterized by dermatitis and myositis with intense granulomatous inflammation associated with myonecrosis. A deeply penetrating, highly invasive species of oomycetes, *Aphanomyces invadans,* accompanied by an intense granulomatous inflammatory response has been consistently observed in the lesions (Dykstra et al. 1986, Noga and Dykstra 1986, Levine et al. 1990a, Noga et al. 1993, Blazer et al. 2002). This finding has brought into question the role of *P. piscicida* in the development of lesions in menhaden, and the utility of these lesions as an indicator of recent local *Pfiesteria* activity. Oomycete hyphae often penetrate the visceral organs of infected fish and a suite of bacteria and other saprophytic water molds usually co-occur as secondary invaders (Noga and Dykstra 1986, Noga et al. 1988, Levine et al. 1990). Recent investigations of menhaden from Chesapeake Bay indicate that the water mold has a very high prevalence in the lesions (> 95%), an association typical of an etiological or causative agent (Blazer et al. 1999). Further, the host exhibits a classical granulomatous cellular response to the water mold indicating that the lesions are at least a week or two old.

Vogelbein et al. (2001) contrasted lesions in wild menhaden with laboratory-induced pfiesteriosis in tilapia (*Oreochromis niloticus*). Tilapia experienced a complete erosion of the epidermis and focal hemorrhaging below the pectoral fin. The inflammation and cellular response was minimal, while the lesions in menhaden exhibited as deep penetrating ulcers with an intense cellular response. The variations between the two pathologies suggested completely different etiologies. More recently, Kiryu et al. (2002)

was able to induce lesions in menhaden similar to those seen in the wild through inoculation and bath challenges of fish with zoospores of *Aphanomyces invadans.* Menhaden exposed in bath challenges experienced a higher prevalence of lesions when abraded with a net, indicating that a portal of entry enhanced the infectivity of the water mold.

The occurrence of this disease in menhaden has been termed ulcerative mycosis (UM). Lesions such as those described have been seen in menhaden since 1984 (Levine et al. 1990a) and are identical to other ulcerative diseases seen across the world, which are now collectively termed epizootic ulcerative syndrome (EUS). EUS was first recognized in the 1970s in farmed ayu (*Plecoglossus altivelis)* and has since spread across Asia and Europe affecting numerous estuarine species such as snakehead (*Channa striatus*), grey mullet (*Mugil cephalus*), and ayu (Lilley et al. 1998). The disease is caused by *Aphanomyces invadans,* which invades the dermis presenting initially as petechia. Once established, the water mold continues to invade, causing small circular lesions that continue to develop into large necrotic ulcers (Lilley et al. 1997).

In a review article, Noga (2000) discussed risk factors that have been shown to damage the epithelium and possibly play a role in the development of skin ulcers (defined as the loss of epidermis). These included environmental factors such as hypoxia, ultraviolet radiation, salinity fluctuations, and changes in water temperature, which are common in estuarine environments. Little research has been done on possible relationships between ulcerative mycosis and environmental stressors, but there has been some investigation into relationships between EUS and environmental factors and disease events do appear to be "triggered" or promoted by certain environmental conditions.

Chinabut et al. (1995) investigated the possible effects of temperature on EUS and found that snakehead subjected to temperatures of 19°C experienced mortality when injected with zoospores of *Aphanomyces invadans*, while those individuals kept at 26°C or 31°C experienced no mortality and were able to heal lesions that formed. Catap and Munday (1998) found similar results in sand whiting (*Sillago ciliata*) kept at 17°C. This may help explain why in some areas, EUS appears to be a cyclical disease, occurring during times of lower water temperatures (Virgona 1992, Chinabut et al. 1995, Catap and Munday 1998, Lilley et al. 1998).

Virgona (1992) tracked outbreaks of red spot disease (RSD), (now considered EUS), in sea mullet (*Mugil cephalus*) in New South Wales from 1972-1988 and discovered a strong correlation between disease events and rainfall. Not only did outbreaks occur in the cooler, autumn months but they also followed periods of above average rainfall. In fact, the first ever recorded outbreak of RSD disease occurred after a period of the highest rainfall in 85 years in October of 1972. Callinan (1994) also reported a link between rainfall and disease outbreaks but believed this was related to acid sulfate soil runoff caused by the rainfall event. He hypothesized that acid sulfate runoff caused skin damage to fish allowing the fungus to attach and invade the underlying dermis. Paerl et al. (1998) did an extensive study on low oxygen in the Neuse River Estuary and found that fish kills occurred on the margins of areas where oxygen levels sank below 2mg/L. From July through September of 1994-1996, this encompassed a 25km stretch of the river and included numerous fish kills. Clearly, more research is needed to elucidate which environmental events or triggers may play a role in outbreaks of disease, but temperature, salinity and hypoxia all appear to be contributing factors.

One of the most common stressors found in the estuarine environment is hypoxia, or low levels of dissolved oxygen. Only a few studies have been done on the effects of hypoxia on fishes and these have focused on LD_{50} values, avoidance behaviors or physiological responses. Sublethal effects of hypoxia include immunosuppression, increased stress hormones, ulcerative skin lesions and loss of cellular oxidative processes (Law 2001), but these have not been explained in detail.

Plumb et al. (1976) investigated effects on channel catfish (*Ictalurus punctatus)* following a natural hypoxic event in an 8.9 ha pond. A die off of a dense bloom of *Anabaena variabilis* caused the pond to become anoxic for two days. Moribund catfish were collected and were found to exhibit hemorrhaging of the dermis, hypodermis and musculature along with necrosis of striated muscle bundles. The catfish also exhibited lesions that were later colonized by *Aeromonas hydrophila.*

Drewett and Abel (1983) subjected brown trout (*Salmo trutta*) to oxygen concentrations of 0.5, 1.0 and 1.5 mg/L and upon microscopic examination found numerous small breaks in the gill epithelium and congestion of the liver. Scott and Rogers (1980) did a similar study examining the effects of 1.5 ppm oxygen concentrations on channel catfish over 24, 48 and 72 hours. In tissues examined histologically, they found edema, hyperplasia and necrosis of the spleen, hemorrhaging and hyperaemia of the liver and kidney, edema and necrosis of the kidney and hyperplasia and hypertrophy of the gills.

The objectives of my research were to (1) investigate the effects of hypoxia on skin using Fast Green FCF as a biological stain, (2) explore the role of hypoxia in the etiology of lesions in Atlantic Menhaden, by conducting hypoxia and *A. invadans* zoospore

challenges, (3) develop a method for bath sporulation of *A. invadans* and (4) explore the response of five different estuarine species to transfection with *A. invadans*.

Chapter 1 - Literature Review

Atlantic Menhaden

The Atlantic menhaden is an abundant, commercially important clupeid fish found in coastal waters and estuaries along the eastern United States (Ahrenholz et al. 1989). Menhaden spawn during the winter months at 2 or 3 years of age (Merriner and Vaughan 1987), predominantly off the coasts of Virginia and North Carolina. The eggs hatch in waters off the continental shelf and the larvae immigrate to estuaries where they spend the first year of their life in the shallow, oligohaline zones. During the spring, the larvae metamorphose into juveniles and grow at high rates throughout the summer months (Friedland and Haas 1988).

While offshore, larvae are particulate omnivores and metamorphose into obligate planktivores in the estuaries. Menhaden remain filter feeders into adulthood, ingesting an increasingly larger particle range (greater than 16μm). While in the estuaries, schools of menhaden are distributed by phytoplankton gradients and are most abundant in areas of lower salinity (4-6 psu) near the chlorophyll maximum (Friedland et al. 1996, Friedland and Haas 1988). In autumn of their first year, as water temperatures drop below 24°C, huge schools of menhaden migrate back to the coastal ocean (Friedland and Haas 1988). Atlantic menhaden are an important component of the diet of bluefish (*Pomatomus saltrix*), striped bass (*Morone saxatilis*), black fin tuna (*Thunnus atlanticus*) and sharks. Seasonally, they are also integral members of estuarine and shelf fish

assemblages. During the migratory season in 1981 and 1982, Friedland and Haas (1988) caught up to 40,000 menhaden per pound net per day in the York River, a tributary of the Chesapeake Bay. Due to their abundance they influence the conversion and exchange of energy and organic matter throughout their extensive migratory range (Rogers and Avyle 1983). Menhaden also support a significant fishery. During the 1990s, menhaden supported the largest commercial finfish fishery in Virginia by weight and the third largest fishery on the US East Coast behind scallops and blue crabs (Kirkeley 1997). The industry annually harvests from 500-600 million pounds with a dockside value of $18-25 million. Together with Gulf menhaden (*Brevoortia patronus*), it comprises the largest US commercial fishery by weight (Rogers and Avyle 1983), and the 4th largest in Chesapeake Bay in value.

Ulcerative mycosis

Ulcerative mycosis (UM) was first recognized as a distinct clinical entity affecting fish when an epidemic of the disease was reported in the Tar-Pamlico estuary, North Carolina and the Rappahannock River, Virginia (Levine et al. 1990a). Fish first began to display deep, crater-like lesions in Spring, 1984, and the prevalence of lesions increased with a large fish kill occurring in November of that year (Ahrenholz et al. 1987). Noga and Dykstra (1986) histologically examined 20 fish collected from pound nets set in the Pamlico River. Most fish had only one lesion, characterized by intense chronic inflammation and the formation of granulomas. A total of 56 lesions were examined microscopically and 95% were found to contain broad aseptate fungal hyphae. These were identified as primarily *Aphanomyces sp.* with a few *Saprolegnia sp.*

Noga and Dykstra (1986) highlighted the importance of these findings, as the water mold was the only organism consistently found in all lesions examined and intense granulomatous inflammation associated with infection by oomycetes had rarely been observed. While these papers reported preliminary findings, a technique for isolating the fungal hyphae had not been developed, making it difficult to confirm that the water mold was indeed the primary pathogen. Willoughby and Roberts (1994) developed an isolation method for obtaining axenic cultures of *Aphanomyces* from fish tissue, which has facilitated further research. Their method involves growing mycelia from thin slices of muscle in a broth medium containing penicillin and oxolinic acid to discourage bacterial growth followed by transfer of mycelia first after 6 and then again at 24 hours of growth to fresh broth. The colonies are then transferred to solid media and routinely maintained in glucose peptone yeast broth and sub-cultured onto solid media every 4-5 weeks.

In subsequent years, UM continued to occur in outbreaks in estuarine systems (Ahrenholz et al. 1987, Noga et al. 1988, Dykstra et al. 1989) raising concerns regarding the impact of the disease on the menhaden fishery. By 1989, UM was recognized as a regional problem throughout the mid and south Atlantic estuaries (Levine et al. 1990a). Early attempts to collect prevalence data were poor, although it became clear that young-of-the-year (YOY) Atlantic menhaden were the most affected species with far lower prevalences (0.1% - 1.2%) observed in other species such as gizzard shad (*Dorosoma cepedianum*), weakfish (*Cynoscion regalis*) and silver perch (*Bairdiella chrysura*) (Levine et al. 1990b).

Noga et al. (1988) attempted to classify the lesions into five types showing progression of the lesions. However, this was done on menhaden caught in the wild and not on experimentally infected fish so the progression of the lesions from one stage to the other was inferred. Early Type I lesions were the smallest recognized, appearing as flat, red or yellow-red areas of the skin up to 5 mm in diameter. The inflammatory response in these fish consisted primarily of macrophages with the oomycete infection appearing to originate in or near a scale pocket. Early Type II lesions were classified as raised 15-20 mm areas with a small amount of scale loss. Histologically, a small ulcerated area was seen in the center of the lesion along with an intense granulomatous response. Advanced lesions (Type III) were open ulcers up to 25 mm in diameter with numerous hyphae found penetrating into the muscle tissue. These lesions extended deep into the body, often involving surrounding organs. End-stage lesions (Type IV) occurred after the necrotic core of fungal-infected tissue was presumably sloughed leaving a crater-like cavity surrounded by dark red to white skeletal muscle tissue. Healing responses (Type V) were infrequent and were described as lesions in which the muscle tissue had been replaced by fibrous connective tissue and appeared as smooth, non-ulcerated areas of tissue loss.

Epizootic Ulcerative Syndrome (EUS)

Reports of oomycete infections in fish date back to the mid 1700s and most are characterized by a white cottony mycelium occurring on the skin of the affected animal (Neish 1980). In general, species of *Saprolegnia* are opportunistic, or secondary invaders colonizing already existing wounds and their potential as primary pathogens has been downplayed. Many teleost fishes have been experimentally infected with saprolegnian

oomycetes, yet little is known about the environmental and host conditions that allow the infection to develop (Neish 1980). While *Saprolegnia* spp. are capable of being primary pathogens, a portal of entry, such as a break in the epidermis allows the oomycete to invade the dermis and proliferate much more readily.

Ulcerative syndromes associated with the water mold *Aphanomyces* have been reported over the last 25 years, affecting many species of freshwater and estuarine fish throughout Asia and Australia. The first report of ulcerative lesions occurred in 1971 in farmed ayu. The disease was described by Miyazaki and Egusa in 1972 and called mycotic granulomatosis (MG) (Lilley et al. 1998). It was characterized by an intense granulomatous response to invasive fungal hyphae, which was isolated by Hatai et al. (1977) and named *Aphanomyces piscicida*. Epizootic ulcerative sydrome (EUS) was first reported in the mid 1970s from Papua New Guinea and has since spread westward across Asia, affecting numerous species of estuarine fishes. Around the same time, a similar ulcerative condition known as red spot disease (RSD) was spreading throughout Australia affecting estuarine fishes, particularly grey mullet. In 1992, Fraser et al. isolated an *Aphanomyces* sp. from diseased fish in Australia, which was later shown to reproduce the disease in bath challenges (Callinan 1994). Both strains of *Aphanomyces* isolated in MG and RSD have since been identified as *Aphanomyces invadans*, the cause of EUS. The disease has since been reported in 18 countries (Lilley et al. 1998).

EUS and lesions seen in menhaden are similar, both consistently show granulomatous inflammation, which arise from penetration of fungal hyphae into the skin and underlying tissue, and both develop into large necrotic ulcers (Lilley et al. 1998). Several reports have described the pathology of the disease, which is characterized by the

presence of invasive mycelia of *Aphanomyces invadans* in a single, circular, necrotic lesion (Lilley et al. 1998). The syndrome is thought to initiate as a petechia that develops on the ventrolateral surfaces of the body. As the water mold invades the tissue, small (2-4cm), circular, hemorrhagic, edematous ulcers form (Lilley et al.1997). In advanced stages the ulcers expand to form the characteristic, large, necrotic open lesions that extend into the musculature. Death is thought to result from secondary bacterial infections or from hyphal penetration into the abdominal viscera and vital organs (Lilley et al. 1998).

In Australia, outbreaks of EUS occurred following major rainfall events (Virgona 1992). This rainfall reduced salinity at outbreak sites to <2psu, a salinity which allows *A. invadans* to sporulate (Kiryu et al. 2002). These rainfall events cause acidified runoff to lower the pH (Sammut et al. 1996) or introduce organic matter, reducing dissolved oxygen concentrations. Both low pH and hypoxia can initiate epidermal damage, allowing *A. invadans* zoospores to invade the fish (Lilley et al. 1997).

Since a diverse group of abiotic and biotic factors appear to influence the occurrence of EUS, it is unlikely that a specific environmental factor is always associated with the outbreaks. It is more likely that the initiating factor varies from outbreak to outbreak in relation to the surrounding environment. Further research is needed to identify these possible relationships (Lilley et al. 1997).

Aphanomyces invadans

The genus *Aphanomyces* belongs to the Oomycota, a phylum of water molds that was previously considered a class in the fungi. It and several other classes of the lower fungi are now considered separate phyla with distinct homologies to the Protozoa (Dick

1990). Oomycetes produce motile, heterokont spores with two flagella (whiplash and tinsel type) , one of the characteristics that separates them from the true fungi. These zoospores are produced in zoosporangia that represent the primary means of asexual reproduction and dispersal. All oomycetes have a eucarpic, coenocytic thallus meaning they only use part of their cytoplasm to make spores and produce hyphae with little or no septa. The development of zoosporangium and mode of zoospore release are the two main characteristics used to delineate species of oomycetes (Neish 1980). The class Saprolegnia, to which *Aphanomyces* belongs, is diplanetic, producing two types of zoospores, with highly motile secondary zoospores emerging from cysts of the primary zoospores. In *Aphanomyces*, primary zoospores typically occur in a single row within the zoosporangium with encystment occurring at the apex of the zoosporangium. (life cycle shown in Figure 4)

Aphanomyces invadans is a slow-growing, aseptate oomycete with intercalary and terminal zoosporangia. Zoosporangia produce non-motile primary zoospores that encyst and then excyst to produce biflagellate secondary zoospores (Lilley et al. 1998). The water mold is slow growing in culture; gaining only 4 mm/day and cultures are frequently overgrown by faster growing, free-living saprophytic species and bacteria. No sexual structures have been observed. However, mature zoospores that fail to develop will germinate as a large unit, a giant cyst derived from several fused zoospores (Willoughby et al. 1995). Sporulation occurs below salinities of 2 psu, but zoospores can tolerate salinities up to 20 psu, though this may be a strain-dependent trait. Optimum growth of *A. invadans* occurs between 20 to 30°C and below 8 psu (Fraser et al. 1992, Lilley and Roberts 1997, Shaheen et al. 1999). Fowles (1976) reported an optimum pH of 7 for

other species of *Aphanomyces* and our lab has found 6.8 to be the optimal pH for growth of our strain of *A. invadans* (unpublished data).

Role of stress

Stress often plays an important role in disease outbreaks (Snieszko 1974). The term stress has been defined in various ways but here will be used as defined by Selye (in Wedemeyer 1970), who stated that stress is "the sum of all the physiological responses by which an animal tries to maintain or re-establish a normal metabolism in the face of a physical or chemical force". A stressor, therefore, is the chemical or physical force that is causing the stress.

The morphological, biochemical and physiological changes that occur as the result of stressors are collectively termed the general adaption syndrome (GAS), which has three stages: the alarm reaction, the stage of resistance, and the stage of exhaustion. The metabolic changes occurring in these stages are not species specific and are generally similar for all stressors (Wedemeyer 1970). The earliest consequences of stress are endocrine changes and these are referred to as primary effects. Primary effects include the release of corticosteroids and catecholamines. These both lead to secondary effects including decreased white blood cell counts, decreased muscle protein, increased heart rate, and increased blood glucose levels. Secondary effects are those that occur as a result of these endocrine changes (Mazeaud et al. 1977). The duration of stress has some bearing on the physiological outcome (Wedemeyer 1970), but it has been shown that even brief exposure to a stressor can bring about long lasting effects (Mazeaud et al. 1977). Wedemeyer and Goodyear (1984) included a tertiary level of effects. These include decreased inflammatory response, poor antibody response and poor healing of

wounds. These result from increased plasma corticosteroid levels leading to protein deficiency (Neish 1980). Effects on growth, survival, longevity and reproduction are likely to be seen even at the population level (Wedemeyer and Goodyear 1984).

The theory that environmental stress can trigger outbreaks of infectious diseases in fish populations (Meyer 1970, Wedemeyer 1970, Snieszko 1974) is based primarily on the coincidence of stress with outbreaks of infectious diseases (Snieszko 1974). There is still much to be learned about the relationship between the stress response of the fish and the subsequent increase in its susceptibility to disease (Pickering and Dunston 1983). At the present time, tolerances to specific stressors are not well defined for most species, even with those stressors that occur singly. This problem is made more complicated by the fact that fish populations are normally exposed to many stressors (Wedemeyer 1984).

A few recent studies have provided evidence that skin damage following exposure to environmental stressors can be mediated through the actions of stress hormones and can result in increased susceptibility to opportunistic microbial infections (Pickering and Dunston 1983, Iger et al. 1995). Noga et al. (1998) induced profound skin ulceration in striped bass and hybrid bass (*M. saxatilis* female x *M. chrysops* male) subjected to acute confinement stress or epinephrine injection. Cultures from the fish were streaked on blood agar plates, showing that the lesions supported little microbial growth, ruling out an infectious etiology. However, in a previous study, Noga et al. (1994), observed rapidly developing opportunistic infections in bass subjected to acute confinement stress. Harms et al. (1996) observed a similar response in net-stressed striped bass with red tail. Another stressor, hypoxia has also been shown to increase the susceptibility of fishes to infection (Bunch and Bejerno 1997). Plumb et al. (1976) showed that low oxygen

resulted in hemorrhaging in the dermis and hypodermis and necrosis in underlying striated muscle bundles of channel catfish (*Channa striatus*). Such skin responses in fishes may play an important role in the frequent and devastating occurrence of opportunistic infections in fish culture operations and the increasing observations of dermatological pathologies in wild fishes.

Hypoxia as a stressor

Estuaries, such as those inhabited by menhaden, are often characterized by large fluctuations in dissolved oxygen and are particularly noted for their development of hypoxia (Burnett 1997). Hypoxia can occur for several reasons including ice cover, pollution, poor mixing, presence of a pycnocline and high primary productivity (Heath 1995). Time scales of variation in oxygen levels range from seasonal to hourly to daily with large diurnal oxygen fluctuations occurring in warmer months. In the Chesapeake Bay, extensive summer oxygen depletions occur, decreasing the ability of the bay to support fisheries resources. From June 16 - August 21, 1998, oxygen levels in the bay fell below 4 mg/L on 81% of the days and below 2 mg/L on 45% of the days. These oxygen depletions can come up rapidly, with levels dropping as much as 6mg/L in 4 hours (Breitburg 1990). The Neuse River, NC, has been a site of numerous fish kills involving menhaden over the last 10 to 15 years. Paerl et al. (1998) reported dissolved oxygen levels below 5mg/L occurring over a 40km stretch of the Neuse River, NC, between May and November from 1994-1996 with levels below 2mg/L occurring over 25km stretches of the river from July to September of those years. Indeed, the Neuse River experienced significant declines in dissolved oxygen over several summers and several of these hypoxic events resulted in large fish mortalities (Burkholder et al. 1995,

Paerl et al. 1998). The Great Wicomico River, Virginia, has been the site of regular monitoring and has been known to experience hypoxic events as well as occurrences of lesioned menhaden (Kator, personal communication, January 29, 2002). In September 2001, a fish kill occurred in the river. This was preceded in the week before by constant hypoxia in the bottom waters and some surface waters. The role of hypoxia in fish mortalities is certain however its role as an inducer of stress to fish skin is less clear.

Dissolved oxygen is essential for respiration and can play a role in fish diseases if present in either too high or too low a concentration. Fry (1969) believed that the reduction of the oxygen content of water is the most pressing source of stress for fish (in eutrophic lakes) and that almost all other stresses would be incidental to, or aggravated by that one primary stressor. The presumption is that at levels of oxygen below that required, fish will be expending excess energy to maintain homeostasis and will thus be experiencing physiological stress. Fish will adapt in any way they can to avoid hypoxia in their tissues (Heath 1995). Indeed, in Chesapeake Bay, fish actively avoid or flee from hypoxic areas and those caught in anoxic waters often breath air at the surface in an attempt to survive (Diaz et al. 1992).

The first line of defense against hypoxia is behavioral. Fish are mobile creatures and if possible, will move from the hypoxic area. If this is not possible, they will first try to adapt by lowering their energy demands. In order to maintain aerobic respiration, fish increase both their heart and ventilation rates (Burnett 1997) and this can lead to the GAS, as discussed previously. Plasma corticosteroid levels will increase (Tomasso et al. 1981, Carmichael 1984), which can lead to (among other effects) decreased inflammatory response, ulcers, poor wound healing, increased blood glucose and poor antibody

response to antigens (Neish 1980). Because the diffusion of oxygen into the blood stream is dependent on differences in partial pressures, there will be a concentration at which the fish will not be able to sustain uptake and the blood will cease to be fully oxygenated.

Hypoxia is an important and common cause of cell injury (Cotran et al. 1999). The exact cellular response to hypoxia depends on the cell and the severity and duration of exposure. As the oxygen within cells decrease, a loss of oxidative phosphorylation will occur, decreasing generation of ATP. This lack of ATP will result in cell swelling by allowing an influx of sodium into the cell, a switch to anaerobic glycolisis reducing glycogen stores, and disruption of protein synthesis. If the hypoxia continues, the cell will begin to lose its ultrastructural features and lysosome membranes will lose integrity causing leakage of enzymes into the cell leading to digestion of the cell and cell death (Cotran et al. 1999). Cellular injury can also occur when oxygen levels rise after a period of hypoxia, a phenomenon termed reperfusion injury. The sudden influx of oxygen into cells can result in oxygen free radical formation, further injuring cells that otherwise may have recovered from the hypoxia (Cotran et al. 1999, Law 2001).

Environmental hypoxia often is accompanied by high carbon dioxide levels, leading to low pH resulting in additional deleterious effects on the fish (Davis 1975). Daye and Garside (1976) reported sloughing of squamous epithelial cells and hypertrophy and distortion of mucous cells due to low pH. Kiryu and Wakabayashi (1999) immersed fish in fluorescent microspheres for 5 min and found that they adhered to areas where microscopic injuries had occurred. This included swollen cells, necrotic or dead cells and cells with damaged membranes, all of which have been described as

possible effects of hypoxia. These injuries represent the breakdown of the skin as a protective organ, allowing potential invasion of pathogens.

Structure of fish skin

Fish skin consists of four layers, the mucus coat, the epidermis, the dermis and the hypodermis (Gaines and Rogers 1975). It differs from vertebrate skin in that living epidermal cells are in direct contact with the environment, which subjects it to two types of stressors. The first is osmotic pressure gradients and the second is physical and chemical forces from the water itself and from other environmental hazards. The skin also represents the first line of defense against invasion by infectious microbial agents (Hawkes 1974).

The epidermis is a stratified squamous epithelium (Henrikson and Matolsty 1968a), which contains many types of secretory glands such as goblet cells, club cells, and chloride cells, the most common of which are goblet cells. Underlying the epidermis is the dermis, which is composed of two layers of connective tissue, a loosely organized stratum spongiosum (or laxum) comprised of loose areolar connective tissue and a stratum compactum composed of dense connective tissue. The thickness and structure of the epidermis and dermis can vary with season, age and location on the body. Often a hypodermis of adipose tissue will underlie the dermis (Henrikson and Matolsty 1968b). When scales are present they are embedded in the dermis and extend towards the surface of the fish and are covered only by the epidermis (Gaines and Rogers 1975). Unless lost, the scale is a permanent structure that grows by the secretion of collagen and mineral accretion (Hawkes 1974).

Fish skin performs three basic functions. It forms a smooth frictionless surface reducing drag for locomotion, provides an impermeable physiological barrier to the movement of fluids and salts, and represents the first line of defense against invasion by infectious microbial agents. Hence, it is critically important in maintaining internal homeostasis and a major reason why skin damage in fishes often results in disease development and death, irrespective of other organ involvement (Noga et al. 1996).

The skin is one of the largest organs in any organism and in fish can account for up to 10% of the total body weight. Because it is unkeratinized and unhydrated it is very sensitive and a major target of acute and chronic damage (McKim and Lien 2001). Law (2001) has evaluated the response of teleost epidermal cells in relation to injury in light of the lesions seen in menhaden, reporting a limited repertoire of morphological responses to injury. The four most vulnerable intracellular systems are cell membrane cohesion, mitochondrial respiration, protein synthesis, and genetic repair. Once irreversibly injured, the affected cells undergo death, including breakdown of the nuclei, lysis of endoplasmic reticula, membrane defects, and swollen mitochondria. The necrotic tissue will take on a different color and consistency and slough away to become an ulcer. Hypoxia can cause cellular injury leading to cellular death (Law 2001).

Summary

The lesions seen in menhaden are identical to those seen in EUS, which is caused by *A. invadans*. To date, EUS lesions have not been reproduced through aqueous exposures without an initial stressor. Callinan et al. (1994) reproduced lesions after exposure to acid runoff and lesions have been reproduced in numerous species through inoculation (Hatai et al. 1994, Chinabut et al. 1995, Wada et al. 1996, Catap and Munday

1998, 2001). *Aphanomyces invadans* plays an integral role in the development of certain lesions in Atlantic menhaden (eg. Blazer et al. 2002, Kiryu et al. 2002, 2003), however, further investigation is needed to elucidate whether *Aphanomyces* can be considered a primary pathogen.

Chapter 2 – Hypoxia and skin damage: Fast green studies

Introduction

Biological stains are commonly used to stain internal structures in cells or tissues (Boon and Drijver 1986) as well as indicators of external features. For example, Kiryu and Wakabayashi (1999) used trypan blue to stain epidermal injury sites on rainbow trout. This dye has also been used by others (Elliot et al. 2001), but is not usually a first choice due to its carcinogenic nature. In experiments to document epidermal damage by various agents (*Pfiesteria*, abrasion, hypoxia), we have found trypan blue stains the skin of fish too lightly and thus exposure results are difficult to interpret (unpublished data). Another dye, fast green FCF, which is commonly used as a food dye has been used as an exclusion dye testing for viability of mammalian cells (Weisenthal et al. 1983, Glavin et al. 1996). Elliot et al. (2001) documented the use of fast green to indicate areas of epidermal damage on chinook salmon (*Oncorhynchus tshawytscha*). Fish with descaling injuries were exposed to 0.1% Fast green for 1-2 min, then rinsed for 1 min. The areas of injury were dyed green and were readily observable without the aid of microscopy. Elliot et al. (2001) stated that the fast green appeared to stain dead cells, as the color would disappear after closure of the epidermis and the sloughing of dead cells (12-96 h later). Regardless, these results indicate that fast green may be a useful indicator for recent skin damage.

Fast green is widely used in histology as a counterstain (Conn 1953). It is known to stain proteins and plasma (among other components) and is related to the dye light green SF yellowish. Fast green is an acidic di-amino-triphenyl-methane derivative with a quinoid chromophore unit (Figure 5). The dye is soluble in both water and alcohol, with a maximum absorbency at 625nm (Conn 1953).

The goal of my study was to investigate the role of sublethal levels of oxygen as a stressor leading to epithelial damage. The null hypothesis (H_o) was that hypoxia has no effect on the epidermal integrity of fishes. The alternative hypothesis (H_1) was that hypoxia does affect the epidermal integrity of fishes. This was analyzed using vital dyes following exposure to hypoxic conditions.

Methods

Fish collection and maintenance

Atlantic menhaden were collected by cast net from local tributaries of the York River and held in a flow-through system consisting of 950 L fiberglass troughs and filtered (35 μm) water from the York River (salinity 20-24 psu, temperature 25-28°C). Fish were fed daily with an algal paste (*Nannochloropsis,* ~68 million/mL, 5 mL diluted in 1 L deionized water) mixture and several grams of HiPro 0.5GR Debut Corey Starter (Corey Feel Mills Ltd., New Brunswick, Canada).

Fast green studies

Fish (15-20 per tank) were held for 1 week in two 76L or two 206L glass aquaria containing artificial seawater (Marinemix Forty Fathoms, Marine Enterprises International, Inc., Baltimore, Maryland, 12 psu) at room temperature. Each tank was

equipped with two Whisper filters (size C, Tetra/Second Nature, Tetra Sales USA, Blacksburg, Virginia) containing a filter bag filled with crushed coral (Bed Rock, Marine Enterprises International, Inc., Baltimore, Maryland) (biological filtration) and activated carbon. The larger tanks contained one canister filter (H.O.T. Magnum) with activated carbon. For each experiment, water filtration was suspended for 36 hours in both tanks. The water level was dropped to approximately ¾ to ½ of the tank volume and the surface of one covered with styrofoam slabs to minimize air exchange. In this tank, dissolved oxygen was regulated at 30% air saturation using a Sable Systems Data Acquisition System (Datacan V) for 36 hours (Figure 6). The other tank was aerated continuously to remain at 90-100% saturation. The experiment was terminated by killing all fish with an overdose of MS-222.

Fish were carefully removed with forceps grasping either the tail or mouth and placed in 0.1% Fast Green FCF for 90 seconds then rinsed for 90 seconds. Areas that stained green indicated places where the epidermis of the fish had been compromised. The lateral side of each fish was photographed with a 35mm camera using Kodak 200 ASA color film, scanned and analyzed using an image analysis program (Image Pro Plus, Media Cybernetics, L.P.). After being photographed, a subsample of fish were processed for scanning electron microscopy (SEM) or histology (see below).

This was repeated four times on separate occasions.

Image analysis

The two dimensional surface area of the lateral side of each fish (including fins) was measured by tracing the fish's profile using the Image Pro software. Color hues that were considered "positive" for fast green were visually selected, encircled with the

software and assessed (Figure 7). Positive areas on the fins and head (defined as from the mouth to the edge of the gill operculum) were excluded from the count as these areas took up the stain regardless of treatment. The total area staining green was summed and a percentage of the affected surface area determined. This was done for each side of each experimental and control fish. Data for the two sides of the fish were then used to calculate a total percentage of the surface of the fish staining with fast green. Data were transformed using the square root -arcsin and evaluated for significance using a Student's t-test ($\alpha = 0.05$ significance level).

Fish were examined as to body region stained, using the method of Noga et al. (1988). Region E (Figure 8) was not used, as this was the area of body not included in the data (mouth to edge of operculum). This analysis was done only with fish in the hypoxia treatments (n=48). Separate categories were not made for regions of stain encompassing more than one body region. For example, if a fish showed green staining in areas B and D and it seemed to be one large, continuous area, the fish was counted as staining in B and D rather that creating a separate category of B/D as did Noga et al. (1988).

SEM

Tissue from 19 fish in each treatment was fixed in 4% paraformaldehyde/5% glutaraldehyde in 0.1M sodium cacodylate buffer overnight at 4°C then washed 3 times for 30 min in 0.1M sodium cacodylate buffer and stored at 4°C. Tissues were post-fixed in 1% osmium tetraoxide in 0.1M sodium cacodylate for 1 hour then rinsed 3 times for 30 minutes each in 0.1M sodium cacodylate. Tissues were dehydrated in an ethanol series, critical point dried with carbon dioxide (Polaron E3100), sputter coated (Anatech

Hummer VII) with gold palladium, and mounted on studs prior to storage in a dessicator. Both a Leo 435VP and an Amray 1810 scanning electron microscope were used to view the samples. A subsample of fish was fixed as above but 2% (w/v) alcian blue was added to the primary fixative to preserve the mucous coat (Powell et al. 1992).

In order to minimize damage in obtaining a tissue sample, a large section of the fish fixed in the primary fixative and a smaller sample of the epidermal layer excised with care prior to secondary fixation.

Histology

Seventeen fish from each treatment were fixed in 10% neutral buffered formalin. Tissues were decalcified with formic acid-sodium citrate solution, dehydrated with ethanol, embedded in paraffin, and blocks were sectioned transversely at 5μm with a rotary microtome. All slides were stained with Harris hematoxylin and eosin.

Other stains

The utility of other biological stains, Bismark brown and Alcian blue, was also examined. Bismark brown is a cytoplasmic dye while alcian blue stains mucopolysaccharides (Boon and Drijver 1986). The stains were tried in a small study with few fish. Fish were treated identically to the studies above. During one experiment, 6 fish from each treatment were selected and placed in 0.1% bismark brown for 3 minutes then rinsed for 60 seconds. A second subset of 5 fish was removed from each treatment and placed in 0.05% alcian blue for 3 minutes and rinsed for 60 seconds. Each side of every fish was photographed.

Results

Image analysis

In experiments 1, 2 and 4, the percentage of surface area that was stained by fast green was significantly greater in fish exposed to hypoxia than in controls (individual t-tests, $p<0.01$ for all). The only experiment in which this was not the case was the third experiment (t-test, $p>0.05$), which had a small sample size ($n=6$ per treatment). When data from all 4 experiments were combined, those fish exposed to hypoxia were stained significantly more than control fish (t-test, $p<0.001$). Between experiments, control treatments were not significantly different from one another (ANOVA, $p>0.05$) demonstrating that regardless of the experiment, all control fish did not stain or stained very slightly. On the other hand, the proportion of fish taking up the stain in the hypoxia treatment were found significantly different from each other indicating significant inter experimental variability (ANOVA, $p<0.01$). This was not size related (linear regression, $r^2 = 0.21$, $n=48$). Fish exposed to hypoxia most often stained in area B, medial ventral and area D, posterior ventral (Figure 9).

Figure 1. Average percentage of surface area staining with fast green FCF in both treatments for all experiments. Error bars are standard deviations.

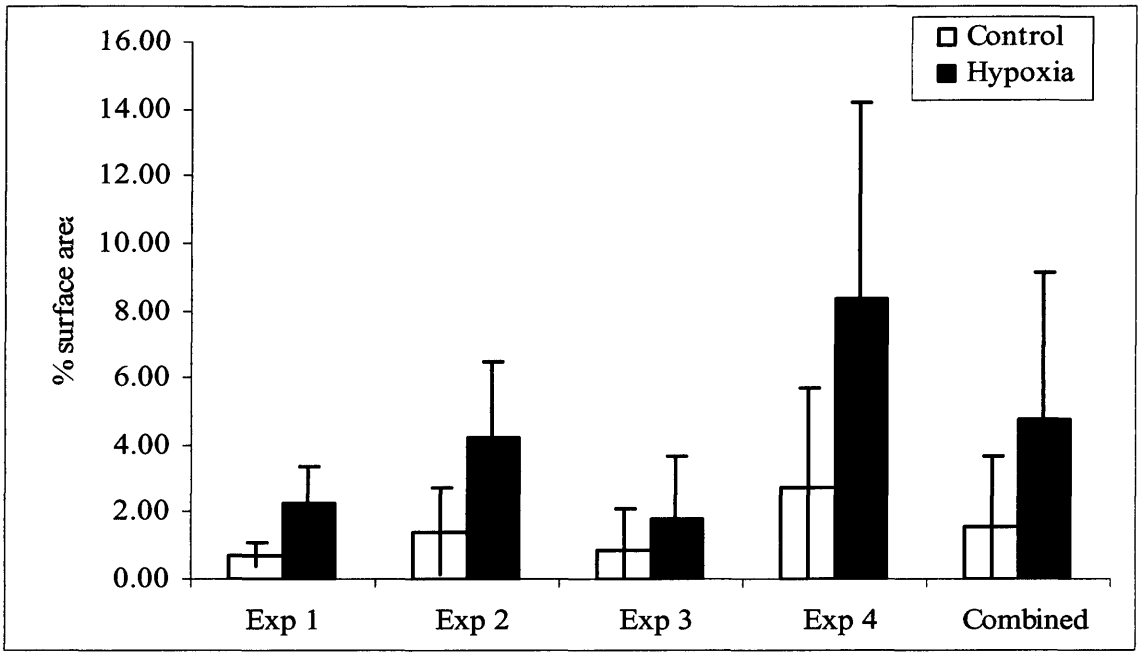


Table 1. Area of body staining with fast green in fish exposed to hypoxia

Area of body	Percentage of fish showing staining in that area
A	27%
B	58%
C	19%
D	75%
E	Not included

SEM

Of the 38 fish (19 each treatment) examined, no ultrastructural differences were seen in the epidermis of fish from control and hypoxia treatments. The specimens were

often difficult to handle because of their fragility and size, which created an uneven charging. Addition of alcian blue to the primary fixative did not aid in preservation of the surface ultrastructure.

Histology

No differences were seen histologically in the epidermis of fish in control and hypoxia treatments (17 fish per treatment). In most slides, the epidermis pulled away from the tissue during sectioning and artifacts were too numerous to fully realize any subtle changes in the epidermis.

Other stains

No obvious visual differences were seen between fish in control and hypoxia treatments when stained with bismark brown (Figure 10). Fish stained with alcian blue did appear to stain differently. Control fish had a greater surface area stained then did those exposed to hypoxic conditions (Figure 10), possibly indicating a loss of mucous coat in fish in the hypoxia treatment. Image analysis was not done on the photographs due to the small sample size (n = 5).

Discussion

Despite the lack of ultrastuctural evidence, the results obtained with fast green staining alone indicated an effect of hypoxia on the skin of menhaden. Since fast green stains recently dead skin cells (Weisenthal et al. 1983), the results indicate that the epidermis of menhaden is compromised by exposure to hypoxia. Elliot et al. (2001) reported that once dead skin cells are sloughed and the damage repaired, the area no longer stains with fast green. This may indicated a breach of epidermal integrity, and

could provide a means for oomycete penetration and may also aid zoospores of *A. invadans* in locating host fish through chemotaxis (Andersson 2001, Kiryu et al. 2003).

Menhaden in hypoxia treatments stained most often in the lower body and posterior flank region (Table 1). This pattern is consistent or reflected in lesionous fish from the field. Noga and Dykstra (1988) examined 424 menhaden with lesions and found 79% of the lesions occurring in these same areas. The cause behind the majority of menhaden developing lesions in this body region is unknown (Dykstra and Kane 2000). The thickness and structure of the epidermal layer can vary seasonally, with age and with location on the body (Gaines and Rogers 1975) and this area may be more susceptible to damage. Lesion prevalence has been shown to increase in menhaden when abraded with a net prior to zoospore exposure (100% vs. 34% for unstressed fish, Kiryu et al. 2002) and Kiryu et al. (2002) indicated that other mechanisms, such as external parasites, *P. piscicida*, or environmental stressors such as hypoxia could mimic this effect, enhancing the infectivity and lesion severity in menhaden exposed to *A. invadans*. The staining pattern of fish exposed to hypoxia suggests that hypoxia may cause a subtle portal of entry. In the wild, this may enhance infectivity by *A. invadans*, allowing initiation of lesions.

Environmental stress increases susceptibility to disease (Snieszko 1974), however little is known of the exact pathological consequences of possible stressors, particularly with hypoxia. Scott and Rogers (1980) found hemorrhaging and hyperemia throughout the liver, spleen and kidney of channel catfish exposed to hypoxia, but did not examine the epidermis. Plumb et al. (1976) also examined channel catfish after exposure to anoxic conditions and reported hemorrhaging in the dermis and hypodermis while

Drewett and Abel (1983) reported breaks in the gill epithelium from brown trout exposed to hypoxic conditions. Plumb et al. (1976) also reported the occurrence of epidermal lesions with no apparent etiologic agent.

Preliminary evidence from alcian blue staining suggests that fish exposed to hypoxic conditions may also experience a depletion of their mucous coat. This could further aid zoospores in attachment and penetration of the epidermis. The mucous layer of fish serves three functions: to reduce body friction, to regulate osmostic action at the surface of the skin and to protect the body from attack by pathogens. The mucous coat provides protection by preventing the attachment of microbes through continuous sloughing (Oosten 1957), acting as a barrier if attachment of a microbe occurs and through a variety a humoral immune factors (Bole et al. 2001) such as agglutins and lysins (Ingram 1980). The staining pattern of the fast green, coupled with the potential loss of mucous coat indicated by alcian blue staining suggests that hypoxia may be enough of a stressor to cause a loss of the initial defense mechanisms of Atlantic menhaden.

The mucous coat, along with the epidermal layer, provide the first line of defense against pathogens and the amount of mucous and epidermal thickness may play a role in the ability of the fish to resist disease (Fast et al. 2002). Fish have the ability to alter the amount of mucous secreted (Ingram 1980) and this, along with epidermal thickness may be altered by exposure to stressors. Iger et al. (1994) reported thinning epidermis in trout exposed to increased temperatures and depressed areas were present where pavement cells had been shed. Because the epidermis of fish is unkeratinized and hydrated, it is very susceptible to any stressor. The first response of the skin is to release copious

amounts of mucous. However, as this continues, the number of mucous cells in the epidermis decreases and the epidermis becomes thinner (Iger and Abraham 1990). If enough energy is available, the skin will compensate, cycling new mucous cells and thickening the epidermal layer (McKim and Lien 2001).

Under stressful conditions, especially hypoxia, available energy is reduced and fish may not be able to compensate for the epidermal changes, increasing susceptibility to pathogens. Decreased mucous had been thought to contribute to the attachment and germination of *Saprolegnia* spp. on channel catfish exposed to low temperatures (Quiniou et al. 1997). The staining results of this study indicate that during exposure to hypoxia, Atlantic menhaden may experience a loss of their mucous coat and possible hypoxic injuries to the epidermal cells. This may play a role in increased prevalence of disease, including ulcerative mycosis by providing an easy means for zoospore attachment, as well as providing a portal of entry into the dermis of the fish.

Chapter 3 – Hypoxia and zoospore bath exposures

Introduction

As recently as 2001, *Pfiesteria*, was considered the cause of lesions in menhaden and *Aphanomyces* an opportunistic invader of these lesions (Burkholder and Glasgow 1997, Burkholder et al. 2001). Superficial loss of epidermis has been reported in laboratory exposures of fish to *Pfiesteria*, however, the deeply penetrating lesions observed in wild menhaden have not been reproduced by experimental exposure to *Pfiesteria* (Noga et al. 1996, Vogelbein et al. 2001). This brings into question the role of *Pfiesteria* as an etiologic agent. Fish with lesions and massive fish kills have also been observed in water where no *Pfiesteria* has been reported (Dykstra and Kane 2000) and these lesions consistently show an intense granulomatous inflammation associated with invasive hyphae of *Aphanomyces* (Blazer et al. 1999).

The lesions seen in menhaden are identical to those seen in EUS, which have been successfully reproduced in laboratory injection studies in snakehead (Roberts et al. 1993, Chinabut et al. 1995, Lilley and Roberts 1997), gourami (*Colisa Ialia*) (Hatai et al. 1994), rosy barbs (*Puntius schwanefeldi*) (Khan et al. 1998) and ayu (Wada et al. 1996). However, early attempts to isolate the water mold from and reproduce lesions in menhaden were inconclusive. Kiryu et al. (2002, 2003) have recently shown that lesions can be induced by injection of fish with hyphae or secondary zoospores and bath exposure to zoospores of *Aphanomyces*. In addition, fish that experienced trauma (net

stress) before the bath exposure to the water mold developed a much higher prevalence of lesions than untraumatized fish (100% vs. 22%, respectively). While the trauma due to the net stress is unlikely to be seen in the environment, it demonstrates that damage to the epithelium facilitates infection by the oomycete. Similar cellular damage may occur from other sources of environmental stress, such as hypoxia, thus, creating a portal of entry for the zoospores and their further development into lesions in fish.

The goal of this chapter was to assess the relationship between hypoxia and the water mold *Aphanomyces* in the etiology of the lesions seen in menhaden. The null hypothesis (H_o) was that hypoxia was not involved in the etiology of lesions (ie. did not create a portal of entry). The alternate hypothesis (H_1) was that hypoxia is involved in the etiology of lesions, presumably by effecting skin damage and, thus, allowing a portal of entry for *A. invadans*. This was evaluated through infection trials with menhaden exposed to zoospores of *A. invadans* and low dissolved oxygen levels in different combinations of exposures.

Methods

Fish collection and maintenance

Atlantic menhaden were collected by cast net from local tributaries of the York River and held in a flow-through system consisting of 950 L fiberglass troughs and filtered (35 μm) water from the York River (salinity 20-24 psu, temperature 25-28°C). Fish were fed daily with an algal paste (*Nannochloropsis,* ~68 million/mL, 5 mL diluted in 1 L deionized water) mixture and several grams of HiPro 0.5GR Debut Corey Starter (Corey Feel Mills Ltd., New Brunswick, Canada).

Aphanomyces invadans culture and sporulation

An endemic isolate of *Aphanomyces invadans* from an isolated menhaden in Maryland (WIC strain, Blazer et al. 1999) was used in all infection trials. This isolate was routinely maintained on glucose peptone-pencillin-oxolinic acid agar (GP-POX agar, Willoughby and Roberts 1994, Lilley et al. 1998) for 5-7 days and sub-cultured into GP-POX broth for 3-4 weeks at room temperature.

For zoospore production, batch cultures were generated by taking 8 pieces of agar (6 mm in diameter) containing hyphae from the growing edge of a colony and placing them in GP-POX broth in a 250 mL culture flask (Becton Dickinson Labware, Flankline Lakes, New Jersey). These cultures were grown for 10-14 days and used to inoculate glucose-peptone-yeast (GPY) agar plates (Lilley et al. 1998). The plates were grown for 4, 5 or 6 days at 23°C in complete darkness. Plugs of agar (6mm in diameter) were then taken and each plug inoculated into a 25 mL culture flask with 25mL GPY broth. These cultures were allowed to grow for 5, 6 or 7 days at 23°C in complete darkness. To induce sporulation, cultures were washed three times then suspended and incubated in autoclaved water from the Poropotank River, Virginia, augmented to 1 psu at room temperature (~23°C). Cultures were then placed at 23°C in complete darkness for 36 hours. Numerous (150-200) flasks were prepared. After induction of sporulation, cultures and water containing zoospores were combined in a large culture flask for aid of counting and exposure. Zoospore densities were estimated with the aid of a hemacytometer (Neubauer/Bright-Line, Buffalo, New York). Briefly, an aliquot of culture was preserved in 10% neutral buffered formalin (1 culture:5 fixative), centrifuged

for 10 minutes at 850 rcf, 1.8 mL of the supernatant removed, the pellet resuspended and a 10 µL aliquot counted with the hemacytometer.

Zoospore bath exposure

Fish (~20 per tank), averaging 7.3 cm standard length (4.2-10.0 cm) and 6.8 g (1.1-15.5 g), were held for 2 weeks in ten 76 L glass aquaria containing artificial seawater (Marinemix Forty Fathoms, Marine Enterprises International, Inc., Baltimore, Maryland) at room temperature. Each tank was equipped with two Whisper filters (size C, Tetra/Second Nature, Tetra Sales USA, Blacksburg, Virginia) containing a filter bag filled with crushed coral (Bed Rock, Marine Enterprises International, Inc., Baltimore, Maryland) (biological filtration) and activated carbon. For all tanks, salinity was lowered from 16 psu to 6 psu over one week and the fish were held at 6 psu for at least one week prior to bath exposures. Water quality was checked weekly (NH_3, NO_2 and pH) and 25% water changes were made as needed to keep water quality parameters within acceptable limits.

The ten tanks were divided into 5 treatments (2 replicate tanks per treatment): 1) no hypoxia, no oomycetes, 2) no hypoxia, oomycetes, 3) hypoxia, no oomycetes, 4) hypoxia, oomycetes and 5) net stress, oomycetes (Table 1). Treatments 2 and 4 were experimental treatments to elucidate whether hypoxia played a role in the development of lesions. All other treatments were controls. Treatment 1 served as an overall control while treatment 3 was a negative control, demonstrating the effect of hypoxia alone and treatment 5 served as a positive control to confirm that the oomycetes were indeed infectious, as fish in treatment 5 should develop lesions after Kiryu et al. (2002).

Table 2. Experimental design for bath challenges with hypoxia

Treatment	Stressor	Oomycete zoospores?	Type of treatement
1	None	No	Control
2	None	Yes	Experimental
3	Hypoxia	No	Negative control
4	Hypoxia	Yes	Experimental
5	Net	Yes	Positive control

In all treatments, water filtration was suspended 36 hours prior to bath exposure and the water level dropped to ½ tank volume. In hypoxia treatments, the water surface was covered with styrofoam slabs to minimize air exchange. In hypoxia tanks, dissolved oxygen was regulated at 30% air saturation (~2-3 mg/L) using a Sable Systems Data Acquisition System (Datacan V, Figure 6) for 36 hours prior to bath exposure. All other treatments were continuously aerated to remain at 90-100% saturation. For the exposure, the water level of all tanks was augmented to 30 L at a salinity of 1 psu with continuous aeration. For treatment 5 (net stress), the fish were captured 3-4 at a time and held in a net for 20 seconds then moved to a new aquarium under identical conditions prior to zoospores being added. For all oomycete exposures, zoospores were added to the aquaria to give an estimated concentration of 100 zoospores mL^{-1}. For control treatments with no zoospores, sterile Poropotank River water (1 psu) was added. Fish were exposed to zoospores for 6 hours. Exposure was terminated by adding 12 psu ASW to each tank to raise the salinity to 6 psu, which inhibited secondary zoospore motility (Kiryu et al. unpublished data, Blazer et al. 2002). Fish that died during the exposure were eliminated from the experiment and the remaining fish in all treatments were monitored for 28 days.

Plate recovery was done on flasks of zoospores added to confirm viability. GP-POX agar plates were inoculated with 0.1 mL of the zoospore suspension and monitored for 5 days for colony growth.

Gross examination and data collection

Aquaria were monitored daily and all dead and moribund fish removed. These fish were weighed, measured, examined for gross pathology and some were photographed. For those fish displaying lesions, the size and location of the lesions was recorded and photographs taken. While the development of lesions was the primary endpoint, mortality data were also taken.

Results

The fungus-hypoxia experiment was attempted a total of eight times. The first 6 attempts failed due to unexplained fish mortalities, error regulating dissolved oxygen concentrations or failed sporulation events. Most recently, the experiment was conducted twice with minimal results (Table 3). In attempt 7, lesions were observed on fish in each zoospore treatment but lesions prevalence was very low, even in positive control treatments with no difference between experimental treatments (x^2, $p=0.99$). Plate recovery for both experiments was good, confirming zoospore viability.

Table 3. Results from aqueous exposures to zoospores of *A. invadans* in combination with hypoxia.

Treatment	Attempt 7			Attempt 8		
	lesions	**mortality w/out lesions**	**mortality w/lesions**	**lesions**	**mortality w/out lesions**	**mortality w/lesions**
Net stress	(3/20) 15.0%	(4/20) 20.0%	(2/20) 10.0%	(1/4) 25.0%	(2/4) 50.0%	(1/4) 25.0%
Oomycete only	(2/26) 7.7%	(9/26) 34.6%	(2/26) 7.7%	(0/32) 0.0%	(9/32) 28.1%	(0/32) 0.0%
Control	(0/24) 0.0%	(5/24) 20.8%	(0/24) 0.0%	(0/31) 0.0%	(9/31) 29.0%	(0/31) 0.0%
Hypoxia only	(0/49) 0.0%	(24/49) 49.0%	(0/49) 0.0%	(0/43) 0.0%	(7/43) 16.3%	(0/43) 0.0%
Oomycete and Hypoxia	(1/39) 2.6%	(12/39) 30.7%	(1/39) 2.6%	(0/40) 0.0%	(12/40) 30%	(0/40) 0.0%

Discussion

The WIC strain of *A. invadans* was pathogenic, and capable of infecting seemingly healthy menhaden, but the stress of hypoxia did not increase the prevalence of lesions in experimental exposures. Kiryu et al. (2002) has reported 100% prevalence of lesions on net stressed fish exposed to a zoospore concentration of 70/mL for 2 hours and 32% prevalence on untraumatized fish exposed to zoospore concentrations of 110/mL for 5.5 hours. I was unable to confirm significant development of lesions in bath challenges. The failure of the net stress treatment as the positive control did not allow the data to be evaluated in light of the hypothesis made. *A. invadans* was capable of initiating lesions in Atlantic menhaden (Figure 11) but not at levels reported recently (Kiryu et al. 2002, 2003).

Factors such as temperature, pH and salinity have been reported to affect EUS outbreaks. Low temperatures are thought to result in stress, inhibiting the ability of the fish to contain and inactivate *A. invadans*. Acidified water run off and organic matter resulting in low oxygen are both thought to cause areas of epidermal necrosis in fish, allowing colonization by *A. invadans*, though this has not been demonstrating conclusively. EUS and UM can only occur when susceptible fish, infective forms of the fungus and suitable environmental conditions are all present (Lilley et al. 1998). The epidermal damage resulting from hypoxia exposure, as shown in Chapter 2, should provide a portal of entry for oomycete zoospores to penetrate into the dermis, initiating lesions when all other conditions are conducive to lesion development.

WIC was induced to sporulate and plate recovery confirmed zoospore viability, however, the ability to produce zoospores may not be related to the ability of an isolate to infect hosts. Bratner and Windels (2000) isolated sixteen strains of *Aphanomyces cochlioides* and assayed them for zoospore production and ability to infect sugar beets (*Beta vulgaris*). Isolates varied widely in zoospore production but more surprisingly, no correlation was found between amount of zoospore production and pathogenicity or transmission. Those isolates producing large numbers of zoospores were not always able to initiate root rot in sugar beets, even with large doses of 200,000 zoospores/plant. Confirming zoospore viability of an isolate or selecting flasks with greater sporulation may not yield consistent results in lesion initiation. This could account for variability seen between experimental challenges with the same strain.

Andersson (2001) described the steps that must be taken by a fungal or oomycete pathogen for infection to occur in the host. Relating these steps to secondary zoospores

of *A. invadans*, the zoospore must find and adhere to the surface of the host, then germinate and penetrate host barriers. This may be aided by the presence of a wound as in crayfish (Dieguez-Uribeondo et al. 1994 in Andersson 2001); invasion by *A. euteiches* occurs most often at joints, wounds and body openings (Nylen and Unestam 1980). The WIC strain of *A. invadans* may have lost its infectivity. This has been reported in *A. euteiches* after a year in culture (Fitzpatrick et al. 1998) and has been hypothesized to occur in *A. invadans* by Lilley et al. (2001). The WIC strain of *A. invadans* has been in culture for 3 years now with these exposures occurring 1-2 years after those by Kiryu et al. (2002), who used the same culture. Other aquatic fungi have been reported to secrete a self-staling substance after repeated sub-culturing in liquid media (Willoughby and Chinabut 1996). A similar phenomenon may occur in *A. invadans*, affecting the oomycete's infectivity. Repeated sub-culturing has also been reported to reduce virulence in *Aphanomyces* spp. (Blazer et al. 2002) and given the length of time in culture and repeated sub-culturing, reduced virulence may explain our results.

Chapter 4 - Investigations into the sporulation of *A. invadans*

Introduction

Ulcerative lesions have been reproduced in fish through injection of both the hyphae (Roberts et al. 1993, Blazer et al. 2002) and zoospores (Fraser et al. 1992, Lilley and Roberts 1997, Catap and Munday 1998, Kiryu et al. 2002) of *Aphanomyces* spp. Callinan et al. (1996) reproduced lesions through bath challenges following exposure to acidified water and more recently, Kiryu et al. (2002, 2003) reproduced lesions in Atlantic menhaden though bath challenges in 38 L aquaria. The free-swimming infectious stage of *Aphanomyces* spp. is the secondary zoospore. After finding a suitable host or medium, the zoospores encyst. A hypha will penetrate into the dermis of the fish, allowing the oomycete to invade into deeper tissue.

Sporulation methods have been published for *Aphanomyces invadans* (Chinabut et al. 1995, Lilley et al. 1998, Catap and Munday 1998, 2002, Sihalath 1999) though almost always on a small scale designed for injection trials. Most methods involve growing the fungus on agar for a few days then transferring the culture to a broth medium for a few days. The culture is then washed with autoclaved pond water and sporulation occurs after 24 to 36 hours. Most cultures are grown in small petri dishes with no more than 25 mL of media. The method of washing and number of washes with water usually varies between authors, as does the water source. For bath challenges with menhaden, I needed to produce large numbers of zoospores and followed the methods of Kiryu et al.

(2002) who were able to produce zoospores in 500 mL volumes. However, attempts to "batch" sporulate large numbers of zoospores of *Aphanomyces* yielded highly variable results. Thus, the purpose of the following sets of experiments was to elucidate the optimal conditions for the batch sporulation of *Aphanomyces invadans*; that is, to derive a method to consistently produce large numbers of zoospores in larger volumes than 25 ml. The factors investigated included (1) water source used to induce sporulation, (2) volume of media (in effect surface area to volume), (3) number of rinses with the water, (4) temperature, (5) type of growth media, (6) number of agar plugs as a crowding effect and (7) light cycle.

Methods

Three separate oomycete strains were used: WIC (an endemic isolate of *Aphanomyces invadans* from a menhaden in Maryland; Blazer et al. 1999), ATCC (American Type Culture Collection 62427) and PA7 (an isolate of *A. invadans* from striped snakehead from Nonthaburi, Thailand, Lilley and Roberts 1997). The ATCC strain was once thought to be *A. invadans*, but is now known to be different based on 16S rDNA sequence analysis (Kator, personal communication, January 29, 2002). All strains were routinely maintained on glucose-peptone-oxolinic acid agar (GP-POX agar, Willoughby and Roberts 1994, Lilley et al. 1998) for 5-7 days and subcultured into GP-POX broth for 3-4 weeks at room temperature. Sporulation of zoospores was evaluated on a semi-quantitative scale of 0-3 (Sihalath 1999) using an inverted microscope (Olympus IX50, Tokyo, Japan). A score of 0 indicated no zoospores were seen in the microscope field, a score of 1indicated 1-10 zoospores were seen, a score of 2 indicated

11-100 zoospores were seen and a score of 3 indicated greater than 100 zoospores were seen.

For all experiments described below, agar plates containing specified media were inoculated from the routine cultures and allowed to grow for 5 days in the conditions previously described. Plugs of agar, 6 mm in diameter, were taken from the growing edge of the colony and placed in culture flasks with broth and allowed to grow for 4 days before undergoing sporulation as previously described.

Experiment 1 - Effect of sporulation media

The WIC strain of the water mold was grown with GP-POX media at room temperature. One agar plug was placed into each of fifteen 25 mL culture flasks (Becton Dickinson Labware, Flankline Lakes, New Jersey) and the flasks were divided into three groups of 5. Group 1 was sporulated using 0.45 μm filtered, autoclaved water from the Poropotank River, Virginia, augmented to 1 psu at room temperature. Group 2 was sporulated using 0.45 μm filtered, autoclaved water from the Beaverdam Reservoir, Virginia, augmented to 1 psu at room temperature. Group 3 was sporulated using a sporulation medium of sterilized 0.25 mM $CaCl_2$ and 0.25 mM KCl in deionized water (Griffin 1978). To induce sporulation, cultures were washed by carefully pouring out all growth media so not to lose the hyphal mat and pipetting 20-25 mL of the appropriate water into the flask. The flasks were carefully inverted and moved side to side to wash all growth media out of the flask and the water was carefully poured out. This was done three times, the hyphae were resuspended and incubated in the water at room temperature. Cultures were evaluated at 24, 36, 60, 72 and 96 hours after sporulation. The null hypothesis (H_o) was that no difference would be seen in sporulation between

water sources, the alternate hypothesis (H_1) was that a difference would be seen in sporulation between water sources.

Experiment 2 - Effect of volume of media

The WIC strain of the water mold was grown using GP-POX media at room temperature. A total of 20 flasks were prepared in 5 groups of 4 as shown below.

Table 4. Sporulation experiment 2 - Effect of media volume and plug number on sporulation

Group	Surface area	Volume	# plugs	Volume per
1	12.5 cm^2	25 mL	1	25 mL
2	25 cm^2	70 mL	2	35 mL
3	75 cm^2	250 mL	8	31.3 mL
4	150 cm^2	600 mL	10	60 mL
5	150 cm^2	600 mL	20	30 mL

All groups were sporulated by washing cultures 3 times then resuspending and incubating them using 0.45 μm-filtered, autoclaved water from the Poropotank River, Virginia, augmented to 1 psu at room temperature. Cultures were evaluated at 24, 36, 60, 72 and 96 hours after sporulation. The null hypothesis (H_o) was that volume of media would not affect sporulation. The alternate hypothesis (H_1) was that volume of media would affect sporulation. The ratio between the volume of the media was kept relatively constant in relation to the number of inoculum plugs so to control for potential differences in staling factors that could stall sporulation.

Experiment 3 - Effect of number of washings

The WIC strain of the water mold was grown with GP-POX media at room temperature. One agar plug was placed into each of twelve 25 mL culture flasks and the

flasks divided into three groups of 4 replicates. Sporulation was induced by washing cultures then suspending and incubating them using 0.45-μm filtered, autoclaved water from the Poropotank River, Virginia, augmented to 1 psu at room temperature. Group 1 was washed once, group 2 twice and group 3 three times before being resuspended in water. Flasks were evaluated at 24, 36, 60, 72 and 96 hours after sporulation. The null hypothesis (H_o) was that sporulation would not be affected by the number of washes in sporulation medium. The alternate hypothesis (H_1) was that sporulation would be affected by the number of washes in sporulation medium.

Experiment 4 - Effect of temperature on sporulation between strains

WIC, ATCC and PA7 were grown using GP-POX media at room temperature and 1agar plug was placed into each of 152 culture flasks (25 mL) and the flasks divided into 19 groups of 8 replicates. Sporulation was induced by washing cultures two times then suspending and incubating them using 0.45-μm filtered, autoclaved water from the Poropotank River, Virginia, augmented to 1 psu at room temperature. Each strain was incubated at 10°C, 15°C, 20°C, room temp (23°C), 30°C and 35°C, with ATCC being placed at 4°C as well. Flasks were evaluated at 24, 48, 72 and 96 hours after sporulation. The null hypothesis (H_o) was that temperature would have no affect on sporulation and that sporulation would not differ between strains. The alternate hypothesis (H_i) was that temperature would affect sporulation and that sporulation would differ between strains.

Experiment 5 - Effect of growth media

WIC and PA7 strains were grown with either GP-POX, glucose-peptone-yeast (GPY) or glucose-peptone (GP) (Lilley 1998) media at room temperature. Each strain was grown using each media with 1 plug placed into 25 mL of media. Each media/strain

combination contained 4 replicates. Sporulation was induced by washing cultures two times then suspending and incubating them using 0.45 μm-filtered, autoclaved water from the Poropotank River, Virginia, augmented to 1 psu at room temperature. Cultures were evaluated every 24 hours for 5 days after sporulation. The null hypothesis (H_o) was that sporulation would not be affected by growth media. The alternate hypothesis (H_i) that sporulation would be affected by growth media.

Experiment 6 - Effect of growth media and crowding

WIC strain fungus was grown using both GP-POX and GPY media at room temperature. Flasks were prepared as shown below. The ratio between the volume of the media and the number of inoculum plugs was varied to examine for potential differences in staling factors that could stall sporulation.

Table 5. Sporulation experiment 6 – The effect of growth media and crowding on sporulation in various size flasks.

Surface area	Media volume	# plugs	volume per plug
12.5 cm^2	25 mL	1	25 mL
12.5 cm^2	25 mL	3	8.3 mL
25 cm^2	70 mL	1	70.0 mL
25 cm^2	70 mL	6	11.6 mL
75 cm^2	250 mL	1	250 mL
75 cm^2	250 mL	6	25 mL
75 cm^2	250 mL	9	27.8 mL

Each treatment consisted of four replicates for each medium. Sporulation was induced by washing cultures two times then suspending and incubating them in 0.45 μm filtered, autoclaved water from the Poropotank River, Virginia, augmented to 1 psu at room

temperature. Flasks were evaluated at 24, 48 and 72 hours after sporulation. The null hypothesis (H_o) was that growth media, media volume and plug number would have no affect on sporulation. The alternate hypothesis (H_1) was that growth media, media volume and plug number would affect sporulation.

Experiment 7 - Effect of light cycle

The WIC strain of the water mold was grown using GP-POX and GPY media at room temperature. Both 25 mL and 250 mL volumes of media were used; with 1 plug of agar being placed in the 25 mL flasks and 6 in the 250 mL flasks. Each media/volume combination was sporulated then placed in either complete darkness or on a 12h light:12h dark schedule at 23°C. Each treatment contained 4 flasks. Sporulation was induced by washing cultures three times then suspending and incubating them in 0.45 μm filtered, autoclaved water from the Poropotank River, Virginia, augmented to 1 psu at room temperature. Flasks were evaluated at 24, 36, 48 and 60 hours after sporulation. The null hypothesis (H_o) was that light cycle would play no role in sporulation with the alternate hypothesis (H_i) that sporulation would be affected by the light cycle.

Results

Experiment 1- Effect of sporulation media

Water from both the Poropotank River and Beaverdam Reservoir yielded good sporulation. After 36 hours, all cultures had a score of 3. The sporulation medium induced poor sporulation, with few to no zoospores seen in each flask.

Experiment 2 - Effect of volume of media

Large volumes of media significantly decreased sporulation (ANOVA, $p<0.01$) with no sporulation occurring in 250 mL and 600 mL volume (Table 6). Mycelial growth was not quantified, but dense hyphal mats were observed in all cultures prior to sporulation. Smaller volumes supported better sporulation.

Table 6. Results from experiment 2 – Effect of media volume on sporulation.

Experimental treatment	**Average sporulation score at 36 hours with SD**
25 mL, 1 plug	2.5 ± 0.6
70 mL, 2 plugs	1.5 ± 1.2
250 mL, 8 plugs	0
600 mL, 10 plugs	0
600 mL, 20 plugs	0

Experiment 3 - Effect of number of washings

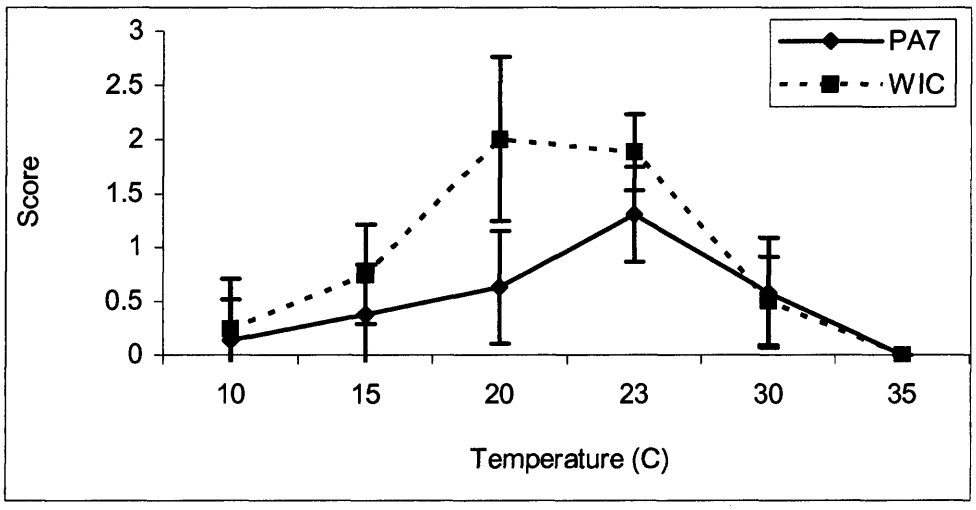
The number of washes significantly affected sporulation of cultures (ANOVA, $p=0.036$). Cultures that received either 2 or 3 washes sporulated with scores of 3 after 36 hours. Cultures receiving 3 washes sporulated early, reaching a score of 3 after only 24 hours and continuing throughout the duration of the experiment. Cultures washed once sporulated significantly less than other treatments with an average score of 2 after 36 hours.

Experiment 4 - Effect of temperature on sporulation between strains

As expected, temperature affected sporulation and differentially affected each strain. The ATCC strain sporulated poorly and only at 20°C with an average score of 0.5.

WIC sporulated well at 20°C and 23°C whereas the PA7 culture did not produce copious numbers of zoospores except at 23° (Figure 2).

Figure 2. Results showing effect of temperature on sporulation. Data shown is average sporulation score of flasks at 48 hours. Error bars are standard deviation.



Experiment 5 - Effect of growth media

Both strain and media significantly affected sporulation (ANOVA, p=0.005) with WIC sporulating better than PA7. Two one-way ANOVAs were done within each strain to compare growth media. Growth media did not have a significant effect on sporulation of PA7 (p=0.64), but did significantly effect sporulation of the WIC strain (p=0.036) with GP-POX supporting significantly more sporulation than GP media alone (Tukey's HSD, p=0.032).

Table 7. Effect of growth media on the sporulation of PA7 and WIC.

Strain	Average sporulation score at 48 hours with SD		
	GP-POX	GPY	GP
PA7	0.3 ± 0.5	0.3 ± 0.5	0
WIC	1.8 ± 1.0	0.8 ± 0.5	0.3 ± 0.5

Experiment 6 – Effect of growth media and crowding

Growth media, media volume and the number of inoculum plugs all had significant effects on sporulation (ANOVA, $p<0.05$, Table 9). The treatments supporting the greatest sporulation occurred in 25 mL of GPY media and 70 mL of GP-POX, both with only one plug (Table 8). Paradoxically, sporulation occurred in the 250 mL flasks, whereas in Experiment 2, no sporulation occurred in what was essentially an identical treatment. The number of inoculum plugs was a signficant factor when analyzed in relation to the volume of media (Table 9, nested ANOVA), but the volume of the media appeared to be the largest factor affecting sporulation, with smaller volumes supporting greater spoulation.

Table 8. Effect of growth media and crowding on sporulation

Treatment	Average sporulation score at 48 hours with SD	
	GP-POX	GPY
25 mL, 1 plug	1.0 ± 0.8	2.5 ± 0.6
25 mL, 3 plugs	1.0 ± 0.0	1.5 ± 1.0
70 mL, 1 plug	3.0 ± 0.0	1.3 ± 0.5
70 mL, 6 plugs	1.3 ± 0.5	1.5 ± 0.6
250 mL, normal 1 plug	0	0
250 mL, normal 6 plugs	0.3 ± 0.5	2.0 ± 0.8
250 mL, normal, 9 plugs	0.6 ± 0.6	1.8 ± 0.5

Table 9. ANOVA table showing the effect of growth, media and crowding on sporulation

Source	Sum of Squares	df	Mean-Square	F-ratio	P
Medium	2.302	1	2.302	5.217	0.027
Volume*Medium	7.193	2	3.596	8.152	0.001
Volume (Plugs)	18.902	3	6.301	14.281	0.000
Error	20.763	47	0.441		

Experiment 7 - Effect of light cycle

A three way ANOVA was done comparing media volume, media and light cycle. Media volume did not have a significant effect on sporulation ($p=0.797$) nor did media ($p=0.062$). Light cycle was highly significant ($p=0.001$) with darkness supporting greater sporulation that a 12 hour cycle (Table 10). No interactions occurred between the light cycle and media volume ($p=0.077$), nor the light cycle and media ($p=0.330$).

Table 10. Effect of light cycle on sporulation of WIC grown in two different media.

Volume	Average sporulation score after 36 hours with SD			
	GP-POX		GPY	
	12L:12D	Darkness	12L:12D	Darkness
25 mL	1.8 ± 0.8	2.5 ± 0.6	0.7 ± 0.5	3.0 ± 0.0
250 mL	0.8 ± 0.8	1.6 ± 0.8	2.5 ± 1.2	2.7 ± 0.52

Table 11. ANOVA table showing the effect of light cycle on sporulation

Source	Sum of Squares	df	Mean - Square	F-ratio	P
Volume	0.055	1	0.055	0.067	0.797
Medium	3.002	1	3.002	3.690	0.062
Cycle	11.055	1	11.055	13.589	0.001
Cycle*Volume	2.686	1	2.686	3.302	0.077
Cylce*Medium	0.792	1	0.792	0.973	0.330
Error	32.542	40	0.814		

Discussion

Water source was a key factor in inducing sporulation in *A. invadans.* Poropotank water was chosen for most experiments as it gave consistent results in the past (unpublished data). River water is not a constant; nutrient and ion concentrations are dependent on rainfall, productivity and salinity. Other authors simply report using autoclaved pond water to induce sporulation (Roberts et al. 1993, Willoughby and Roberts 1994, Catap and Munday 1998), which can differ dramatically between sources and temporally at one source. Mitchell and Yang (1996) did a series of experiments using *Aphanomyces euteiches* and found that many ions or other chemicals in high concentrations could affect zoospore production. Both cysteine and thioglycollic acid reportedly inhibited the motility of secondary zoospores along with Mg^{2+}. Iron and zinc both inhibited secondary zoospore development above concentrations of 10^{-4}M and 10^{-5}M respectively, while Ca^{2+} caused significant alteration in the pattern of sporulation and was essential for primary secondary zoospore differentiation. Willoughby and Roberts

(1994) reported Ca^{2+} to cause a loss of zoospore motility for *A. invadans* above concentrations of 100-200 mg/L.

The effect of various ions and metals may explain some of the inconsistency with sporulation. The sporulation medium in experiment 1 failed to induce sporulation. Lilley et al. (2002) also could not induce sporulation in *A. invadans* using the same sporulation medium though Dykstra et al. (1986) reported sporulation of *Aphanomyces spp.* after 20 hours with this sporulation medium.

To date, Kiryu et al. (2002) is the only one that has reported the ability to sporulate *Aphanomyces invadans* in large quantities. In this study, no sporulation occurred in any 600 mL of media and sporulation was highly variable in 70 mL and 250 mL. This could be due to interactions that may occur when more than one plug is placed in the same flask. This is unlikely, though, as sporulation still occurred, albeit poorly, when three plugs were placed in 25 mL of media and sporulation was not achieved with only 1 plug in a larger volume. Fowles (1976) reported a volume effect of media on the sporulation of *A. stellatus* over a short period of time. Water volume may play a role in the turgor pressure of the sporangium and therefore influence zoospore release (Johnson et al. 2002). Increased water volumes have also been reported to result in decreased sporangial development and reduced sporulation in water molds due to reduced oxygen tension (Johnson et al. 2002), which may be a feature of a low SA:V ratio. Schneider (1963, in Johnson et al. 2002) believed the optimal ratio of hyphal mat to water to be 1:3 or 1:4 for *A. cochioides* and believed that greater sporulation could be achieved if the water mold was grown in a small volume of broth then placed in a large volume of water.

It was believed that this diluted any residual nutrients around the mycelium, maximizing sporulation. I essentially simulated this by varying the number of washes in water.

At larger volumes, 3 washes may not have been enough to remove all nutrients from the flask and, therefore did not induce sporulation. Other authors have reported washing mycelia as much as 5 or 6 times (Willoughby and Roberts 1994, Catap and Munday 2002) and Cerenius (1998) incubated the mycelia in water for 1hour intervals to induce sporulation in *A. astaci.* I chose 3 washes as the maximum as obtaining and preparing large volumes of water was problematic at times. Water had to be obtained from the appropriate source then filtered at 0.45 μm. If the water contained excessive sediments, it was filtered through a series of filter sizes (ie. a 20 μm, then a 5 μm then a 0.45 μm), a time consuming process for the large volumes required (10-20 liters). All water then had to be augmented with artificial sea salts to 1 psu and autoclaved before use.

Temperature played an obvious and expected role in sporulation. WIC sporulated best at room temperature, which was expected as optimal growth is reported between 20°C and 30°C (Fraser et al. 1992) and our cultures have been grown at room temperature since isolation. Sihalath (1999) reported similar results, with optimum sporulation of *Aphanomyces invadans* occurring at 22°C, with little sporulation at 18°C and 27.5°C. This may help explain why outbreaks of EUS often occur in autumn when temperatures begin to drop (see Chapter #1 for review).

Fowles (1976) reported that *Aphanomyces cochloides* produced 4 times more oogonia when kept under dark conditions but that no detectable differences were seen in growth. This is similar to our results, as no detectable difference was seen in the

diameter of colonies when grown on agar plates in darkness (data not shown) however, sporulation was greater in cultures grown in complete darkness than those grown in a 12:12 light period. Little is known about the effect of light on sporulation. Direct light may have a negative effect on sporangial development by increasing temperature within flasks; however most literature suggests that sporulation is improved with lengthening of the light cycle (Johnson et al. 2002). Photooxidation may alter nutrients required for sporulation or produce inhibitors.

The conditions supporting optimal sporulation, or at least, the most consistent sporulation, occurred in 25 mL of media with 1 plug of agar grown in GPY broth. Sporulation should be done using at least 3 washes of water and flasks should be placed in total darkness. These optimal culture conditions may be the result of artificial laboratory selection or routine subcultures under similar conditions.

Chapter 5 - Transfection of estuarine fishes with *A. invadans*

Introduction

Epidemiological studies have shown that young-of-the-year menhaden are by far the most common estuarine species to exhibit ulcerative mycosis. Levine et al. (1990b) examined fish caught in pound and trawl nets in the Tar Pamlico Estuary from May 1985 through April 1987. Of those fish caught, Atlantic menhaden, silver perch, weakfish, gizzard shad (*Dorosoma cepedianum*), Atlantic croaker (*Micropogonias undulates*), spot (*Leistomus xanthurus*) and southern flounder (*Paralichthys lethostigma*) were found with ulcerative lesions. However, only Atlantic menhaden showed lesion prevalences greater than 2%.

Noga et al. (1991) did a similar study from July 1984 through 1988 using only pounds nets; however lesions were reported only for species other than Atlantic menhaden. This list included southern flounder, hickory shad (*Alosa mediocris*), striped bass, bluefish, Atlantic croaker, weakfish, spot, silver perch, hogchoker (*Trinectus maculatus*) and pinfish (*Lagodon rhomboides*). Most species had only one representative individual with a lesion with only a few fish of each species caught. Kane et al. (1998) examined species in the Chicamacomico River, Maryland using cast nets and found lesions on a majority of the menhaden caught. External lesions were also seen on one spot, one spotted sea trout (*Cynoscion nebulosus*) and one flounder. Neither of these studies attempted to isolate the water mold from any affected fish.

Numerous other studies have demonstrated the high prevalence of lesions seen on menhaden. Dykstra et al. (1989) collected menhaden from the Rappahanock River and found 69% to show external ulcerative lesions in November 1986 and 29% in January 1997. Noga et al. (1988) collected 424 affected menhaden from July 1984 through July 1986 using pound nets in the Pamlico River. Neither of these studies reported lesions on any other fish caught.

The purpose of this study was to explore the infectivity of *Aphanomyces invadans* when inoculated into five different host species: Atlantic menhaden, striped killifish (*Fundulus majalis*), mummichog (*Fundulus heteroclitus*), mullet and hogchoker. All five species are commonly found in estuaries where UM is prevalent in menhaden; however none of the species, other than Atlantic menhaden, have been observed with high prevalence of lesions. The objectives of this study were (1) to determine if menhaden are more susceptible to the oomycete than other estuarine fishes, (2) to identify a more robust model of infection for the laboratory setting, and (3) to examine possible host or ecological barriers to infection. The null hypothesis (H_o) was that there would be no difference in lesion development and prevalence between the species. The alternate hypothesis (H_1) was that there would be a difference in the lesion development and prevalence between the species.

Methods

Fish Collection and Maintenance

Juvenile Atlantic menhaden (estimated fork length 9-11 cm) were collected by cast net from local tributaries of the York River and held in a flow-through system consisting of 950-L fiberglass troughs and filtered (35 μm) water from the York River

(salinity 20-24 psu, temperature 25-28°C). Fish were fed daily with an algal paste (*Nannochloropsis,* ~68 million/mL, 5 mL diluted in 1 L deionized water) mixture and several grams of HiPro 0.5GR Debut Corey Starter (Corey Feel Mills Ltd., New Brunswick, Canada). For the experiments, fish were either kept in 76-L glass aquaria at 12 psu and room temperature (~23°C) or in 206 L glass aquaria with a flow through system (~22 psu and 23°C). The 76 L glass aquaria were equipped with two Whisper filters containing a filter bag filled with crushed coral and activated carbon. Water quality was monitored weekly and water changes made daily. The 206 L glass aquaria received water from the York River. The water was passed through a sand filter, an activated carbon filter followed by 10-μm and 1-μm canister filters before being distributed to the tanks. System filters were rinsed daily and replaced every few weeks as needed.

Striped killifish (average total length 90 mm) and mummichog (average total length 80 mm) were collected by seine nets and minnow traps baited with crab or squid from local tributaries of the York River and held in 76-L glass aquaria containing artificial seawater (Marinemix Forty Fathoms, Marine Enterprises International, Inc., Baltimore, Maryland, 12 psu) at room temperature. Each tank was equipped with two Whisper filters (size C, Tetra/Second Nature, Tetra Sales USA, Blacksburg, Virginia) containing a filter bag filled with crushed coral (Bed Rock, Marine Enterprises International, Inc., Baltimore, Maryland) (biological filtration) and activated carbon. Fish were fed every other day with TetraMarine Marine Fish Flakes, mortalities were removed daily and water quality monitored weekly. Water changes were made as necessary.

Mullet (not measured) were caught with cast nets by personnel in the Fisheries Science department, VIMS, and kept in 206-L glass aquaria with a flow-through system connected to the York River. The water was passed through a sand filter, an activated carbon filter followed by 10-µm and 1-µm canister filters before being distributed to the tanks. System filters were rinsed daily and replaced every few weeks as needed. Fish were fed daily with either Cory Hi Pro feed or TetraMarine Marine Fish Flakes.

Hogchoker (average total length 10.5 cm) were collected by trawl nets from local tributaries of the York River and held in 76-L glass aquaria containing artificial seawater (10 psu) at room temperature. The bottom of each tank was covered with a thin layer (~1/2") of autoclaved sand collected from the local beach. Each tank was equipped with two Whisper filters containing a filter bag filled with crushed coral (biological filtration) and activated carbon. Fish were fed numerous food items such as squid, blood worms, Tetra Marine Flakes, brine shrimp, and bait fish however they did not seem to eat anything offered to them. Mortalities were removed daily and water quality monitored weekly. Water changes were made as necessary.

Oomycete culture and sporulation

An endemic isolate of *Aphanomyces invadans*, WIC, was obtained from an Atlantic menhaden in Maryland (U.S. Geological Survey, Leetown, West Virginia; Blazer et al. 1999). The culture was routinely maintained in glucose-peptone-penicillin-oxolinic acid broth (GP-POX broth; Willoughby and Roberts 1994, Lilley et al. 1998) for 3-4 weeks at room temperature and sub-cultured onto GP-POX agar for 5d.

For zoospore production, a piece of agar containing hyphae (6.0 mm diameter) was excised from the growing edge of a colony on glucose-peptone yeast agar (GPY

agar; Lilley et al. 1998) and placed in 25 mL of GPY broth in a 25 mL culture flask (Becton Dickinson Labware, Franklin Lakes, New Jersey). Cultures were grown for 5d at 23°C in darkness and washed three times with 0.45 µm-filtered (Whatman 54; Whatman International Ltd., Maidstone, England), autoclaved river water from the Poropotank River, Virginia, augmented to 1psu. To induce sporulation, cultures were suspended in the water for 12-36 hours at 23°C in darkness. Zoospore densities were estimated with the aid of a hemacytometer (Neubauer/Bright-Line, Buffalo, New York). Briefly, an aliquot of culture was preserved in 10% neutral buffered formalin (1 culture:5 formalin), centrifuged for 10 minutes at 150 rcf, 1.8 mL of supernatent removed, the pellet resuspended and a 10 µL aliquot counted with the hemacytometer.

Zoospore injection study

Fish were removed from the tanks and anesthetized using MS-222. Each fish was injected with an estimated 0.1mL of an 800 zoospore/mL suspension (80 zoospores/fish) using a 27-gauge, 12.7 mm needle and a 1.0 mL syringe. Fish were injected in the right flank just below the dorsal fin, allowed to recover from the anesthesia in clean water and placed back in the aquaria in which they had been maintained. Control fish were treated in the same manner but were injected with 0.1mL of 1 psu sterile water. To confirm oomycete viability, triplicate samples of 0.1 mL of the suspension were repeatedly plated onto GP-POX agar.

Gross examination and histologic sampling

Aquaria were checked daily for 28 days and all dead and moribund fish removed. All fish were examined for gross pathology and those exhibiting lesions photographed. Live and moribund fish were killed with MS-222, the lesion excised and fixed in 10%

neutral buffered formalin. Tissues were decalcified with formic acid-sodium citrate solution, dehydrated with ethanol, embedded in paraffin, and blocks sectioned transversely at 5 μm with a rotary microtome. All slides were stained with Harris' hematoxylin and eosin.

Results

Lesion prevalence and mortality

Menhaden developed ulcerative lesions identical to those previously described (see Kiryu et al. 2002). Lesions appeared within 5 days post injection and by day 23 all menhaden were moribund or dead. No control menhaden developed lesions, though mortality was high (Table 12). Striped killifish developed ulcerative lesions similar to those in menhaden (Figure 12), but they appeared 7-10 days after those in menhaden. Killifish experienced similar mortality to menhaden, as all fish, except for one that developed lesions, were moribund or dead at the termination of the experiment. No control fish developed lesions.

Hogchokers had the highest prevalence of lesions. A reddened area around the site of injection appeared within 5 days with swelling of the area by day 7 (Figure 13). Mortality data were lost as all hogchokers (control and experimental) died on day 16. No hogchokers had been found moribund or dead before this date and water quality was within acceptable parameters.

Mummichogs experienced a lower prevalence of lesions compared to the other species. Lesions appeared as reddened/purple areas under the skin along the dorsal surface (Figure 13). At no time did lesions develop into open ulcers, as did those on the

menhaden and killifish. Mortality in mummichogs was low; less than half of those that developed lesions died. At the end of the experiment, many of the infected mummichogs appeared to be recovering from the lesions.

The mullet did not develop any lesions or experience any mortality.

Table 12. Lesion prevalence and mortality from transfection with *A. invadans*

Species/Treatment	Lesions	Mortality w/out lesions	Mortality w/lesions
Menhaden Experimental	(13/24) 54.2%	(8/24) 33.3%	(13/24) 54.2%
Menhaden Control	(0/24) 0.0%	(12/24) 50.0%	(0/24) 0.0%
Mummichog Experimental	(22/48) 45.8%	(2/48) 4.2%	(10/48) 20.8%
Mummichog Control	(0/30) 0.0%	(1/30) 3.3%	(0/30) 0.0%
Killifish Experimental	(29/34) 83.5%	(3/34) 8.8%	(28/34) 82.4%
Killifish Control	(0/23) 0.0%	(4/23) 17.4%	(0/23) 0.0%
Hogchoker Experimental	(28/30) 93.3%	(2/30) 6.7%	(28/30) 93.3%
Hogchoker Control	(0/14) 0.0%	(14/14) 100%	(0/14) 0.0%
Mullet Experimental	(0/7) 0.0%	(0/7) 0.0%	(0/7) 0.0%

Histology

No lesionous menhaden were processed for histology because the pathology of *A. invadans* infections in menhaden has been well documented (Blazer et al. 1999, 2002, Kiryu et al. 2002, 2003).

Moribund striped killifish showed similar lesion pathology as menhaden previously described by Kiryu et al. (2002). By day 14 post injection, immature, newly generated granulomas with a few layers of epitheliod cells were seen. Brown-pigmented hyphae were sporadically observed near surface skin tissue and sometimes within the core of the granuloma but never within the deeper infected areas of skeletal muscle. A few multi-nucleated giant cells were seen. At 18 days post injection, multi-nucleated giant cells were more abundant. At day 20, the numbers of multi-nucleated giant cells present dropped, and by day 25, they were completely absent (Table 13, Figure 14).

Lesions in mummichogs were characterized by aggregates of inflammatory cells including macrophages, fibrocytes, and granulomas at day 20 post injection (Table 13). The granulomas surrounded brown-colored hyphae that filled the necrotic spaces of the skeletal muscle. Near the surface of the skin surface, hemorrhaging and congestion were seen. Fungal invasion extended into the skeletal muscle of the opposite side from which zoospores were initially inoculated and granulomas were detected at the alveolar space, between the spinal cord and the vertebral column. Hyphae at remote sites stained blue to black as had been previously observed in menhaden by Kiryu et al. (2002).

At 27 days post injection, the observed hyphae were swollen and prominently brown in color and the number of eosinophilic granular cells (EGCs) increased in comparison to those at day 20. EGCs were consistently observed along with elongate-

shaped granulomas surrounding hyphae. Inflammatory cells, such as macrophages, were still present along the myosepta. Some of the fish exhibited a healing response characterized by regenerating myocytes (Figure 15).

Mullet did not show any evidence of fungal invasion or immune response. No damage to the tissue was seen in any of the seven fish inoculated at the end of the experiment.

Table 13. Characteristic histopathological findings compared among three fish species. - absent, + mild, ++ moderate, +++ severe.

Histopathological findings	Fish species		
	Killifish	Mummichog	Menhaden[1]
Granulomas	immature	developed	developed
Eosinophilic granulocytes	+	+++	+++
Giant cells	++	-	-
Brown hyphae	+	+++	-
Wound healing	-	+++	++

[1] Data from Kiryu et al. (2002, in press)

Discussion

Clearly other estuarine species are susceptible to infection by *A. invadans* when inoculated with secondary zoospores. All of the species inoculated inhabit similar estuarine environments, yet Atlantic menhaden is the only species that is consistently found with ulcerative lesions. This suggests that barriers to infection must exist, preventing other species from developing lesions. The increased prevalence in Atlantic

menhaden could be due to the behavior of the species. Juvenile menhaden form large filter feeding schools (thousands of fish) that frequent shallow, low salinity areas. It is in these low salinity areas that zoospore attachment must occur since motility of the zoospores ceases above 2 psu (Blazer et al. 1999). Once sporulation is induced in *A. invadans*, it will continue for 12-60 hours (unpublished data). A school of menhaden that enters a low salinity region when *Aphanomyces* is sporulating would all be exposed to secondary zoospores at the same time, potentially resulting in an entire school being exposed to infection. Mummichogs and killifish have a much more territorial life style than menhaden. During the summer months, mummichogs maintain a home range of 36-38 meters and rarely migrate out of this area (Abraham 1985). Schools sometimes form, but are smaller, numbering only in the hundreds at rare times. Mummichogs also prefer higher salinity areas, above 8 psu, though killifish are often found in fresh waters (Abraham 1985).

Environmental factors such as pH, hypoxia, and temperature have been hypothesized to play a role in the development of UM and EUS (Dykstra and Kane 2000). Menhaden have a thin epidermal layer and therefore may be more susceptible to environmental stressors. Zoospores may have an easier time penetrating the epidermis of menhaden than that of other species. Menhaden tissue has also been reported to support increased growth of hyphae of *A. invadans* when compared with agar (Dykstra et al. 1989). Thus, the predilection of *A. invadans* for Atlantic menhaden may be in part determined by the susceptibility of the host to stress and in part by the nature of the oily flesh serving as an easy and highly supportive food for the oomycete. Mummichogs and killifish are both fairly resistant to stressors. They are able to tolerate abrupt salinity

changes, a wide temperature range and low dissolved oxygen levels (Abraham 1985). Because they are hardier species, they may not be as susceptible to disease as Atlantic menhaden. Mummichogs were only mildly affected by the oomycete. The histology showed that mummichogs were able to eliminate the penetrating hyphae and regenerate damaged muscle tissue, healing from the infection. The response of the mummichog was similar to that of the rosy barb reported by Khan et al. (1998). After injection with zoospores of *A. invadans*, rosy barbs developed macrophage infiltration around the injection site and by day 20, there was evidence of muscle regeneration in the tissues. However, rosy barbs experienced a 100% mortality rate after 22 days as opposed to the 20.8% mortality in mummichogs. Resistance to infection by *A. invadans* has been reported to occur in tilapia, stickleback and roach (Khan et al. 1998). Carp (*Cyprinus carpio*) inoculated with *A. piscicida* showed no gross signs of inflammation and mycotic lesions only occurred around the injection site (Wada et al. 1996). Surprisingly mullet in our inoculation experiment did not develop lesions. They have been reported to be susceptible to *A invadans* species in Australia (Sinderman 1988, Virgona 1992, Lilley et al. 1998, Shaheen 1999) and Florida. However, this study had only a small sample size with no control treatments.

Menhaden are hard to keep in the laboratory and we routinely lose 25-50% of fish 24 hours after being caught. In a pilot study, killifish were exposed in an aqueous challenge to 330 zoospores/mL for 5.5 hours. Only 1 fish out of 8 developed a lesion, which was on day 28 and there were no control treatments (unpublished data). Killifish developed lesions similar to menhaden, a promising sign that they may provide a more robust laboratory model for future studies

CONCLUSIONS

Environmental stressors are hypothesized to play a role in the initiation of lesions in EUS and UM, both of which are caused by *Aphanomyces invadans*. Using fast green, these studies suggest that a common environmental stressor, hypoxia, may cause subtle epidermal damage in areas commonly affected by lesions in Atlantic menhaden. This is promising in establishing a link between the initiation of lesions and environmental conditions. Despite this, we were not able to show that exposure to hypoxia increases lesion prevalence by providing a portal of entry for zoospores. This was most likely due to a loss of infectivity in our strain of *A. invadans,* as few fish in any treatments developed lesions. It is clear, however, that *A. invadans* is capable of reproducing lesions in Atlantic menhaden identical to those seen in the wild, as previously reported by other investigators. *A. invadans* was also shown to be capable of initiating lesions (through inoculation trials) in striped killifish, hogchoker and mummichog, species that are not observed with lesions in the environment. The lack of lesions seen in the wild is most likely due to differing lifestyles between the species and differing resistance of the species. Mummichogs were able to isolate fungal hyphae with an effective granulomatous response and began to show muscle regeneration 4 weeks after inoculation. Killifish developed lesions similar to menhaden and show promise as a future laboratory model. Additional research is needed to further investigate species

responses to zoospores of *Aphanomyces invadans* and to elucidate optimal conditions for initiation of lesions in the wild.

Figure 3. Characteristic lesions seen on Atlantic menhaden


USGS
A

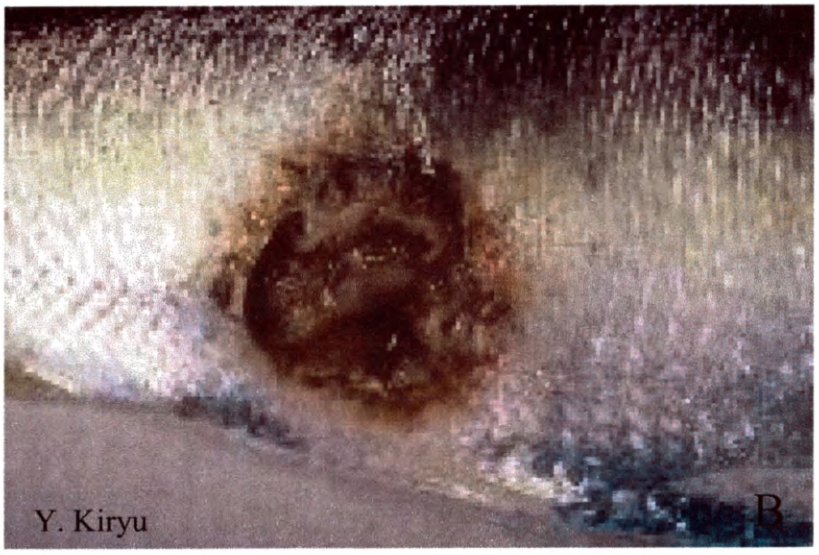
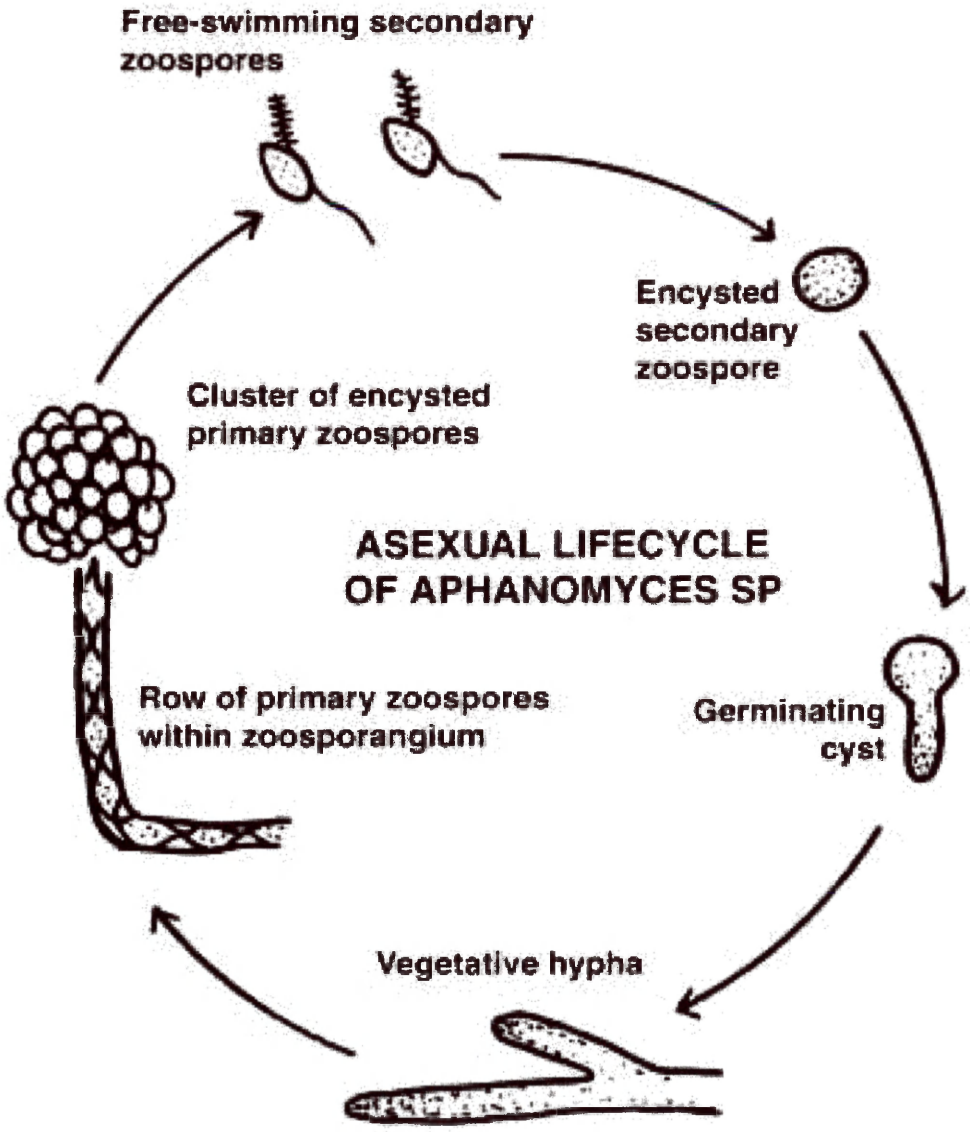
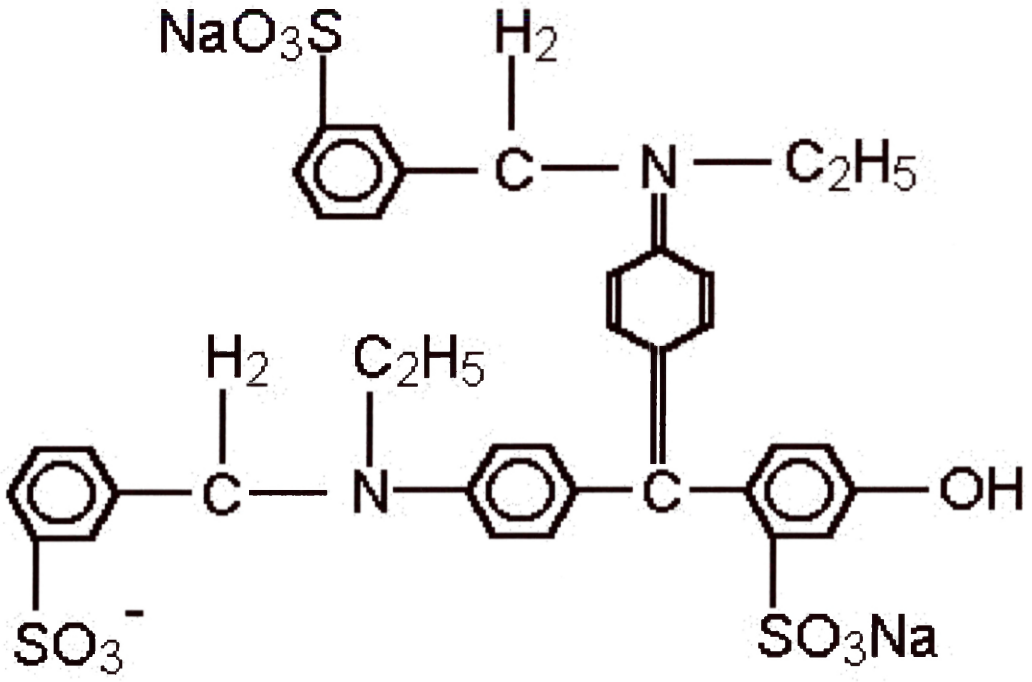

Y. Kiryu
B

Figure 4. Life cycle of *Aphanomyces* sp


Free-swimming secondary zoospores
Encysted secondary zoospore
Germinating cyst
Vegetative hypha
Row of primary zoospores within zoosporangium
Cluster of encysted primary zoospores
ASEXUAL LIFECYCLE OF APHANOMYCES SP

Lilley 1997

Figure 5. Molecular structure of fast green FCF



http://193.51.164.11/htocs/monographs/col116/fastgreenfcf.html

Figure 6. Schematic showing experimental set-up and oxygen regulation

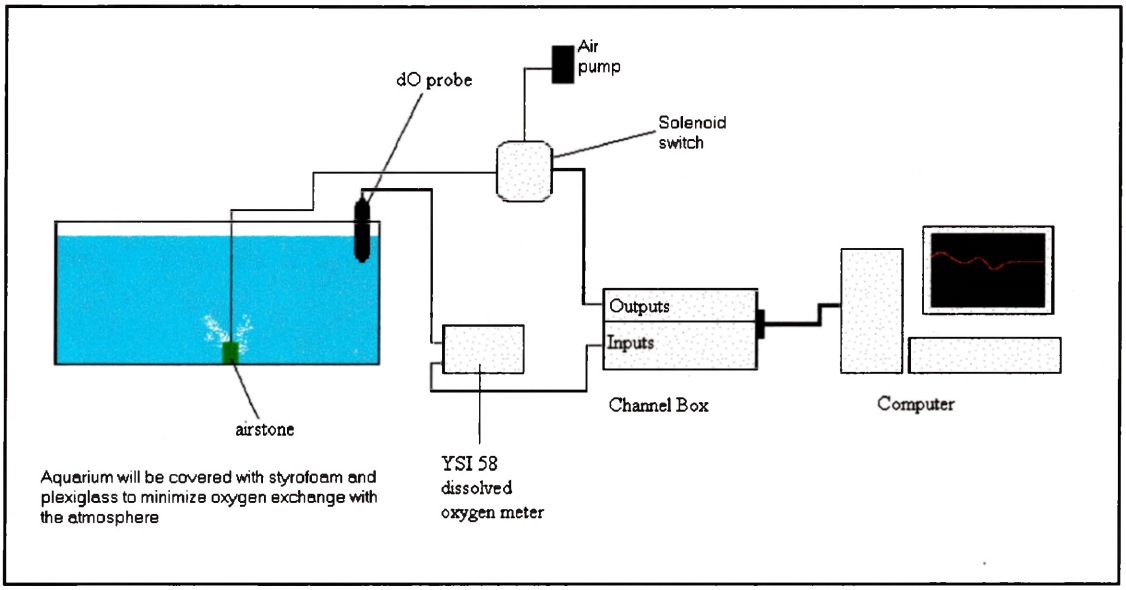
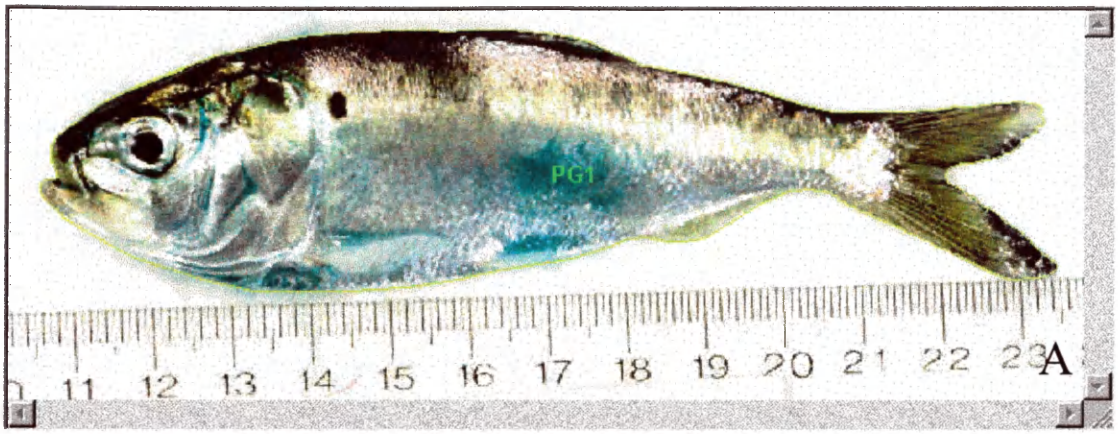
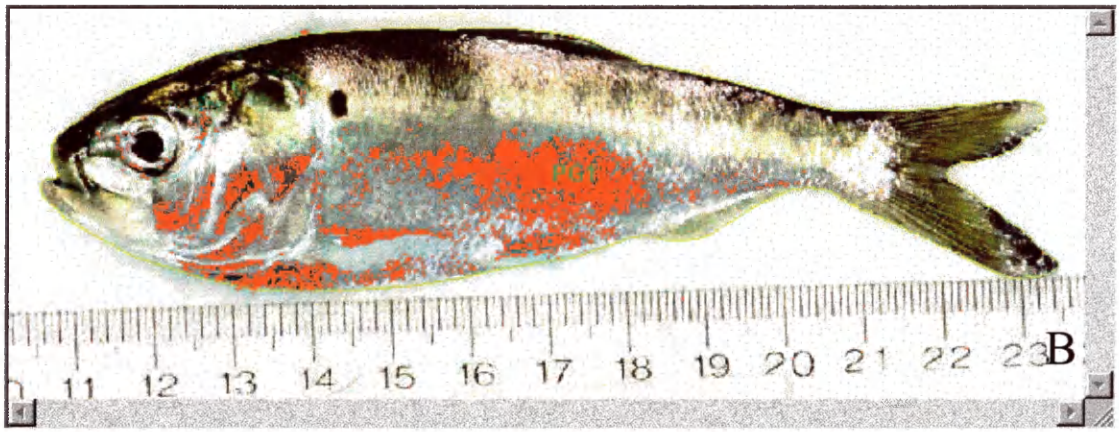

Air
pump
dO probe
Solenoid
switch
Outputs
Inputs
Channel Box
Computer
airstone
Aquarium will be covered with styrofoam and
plexiglass to minimize oxygen exchange with
the atmosphere
YSI 58
dissolved
oxygen meter

Figure 7. Series of photographs demonstrating the process used for image analysis. A) the fish was outlined (shown in yellow) and the surface area of the measured B) those areas "positive" for green were selected (shown highlighted in red) C) the areas circled and the surface area dyed measured


PG1
11 12 13 14 15 16 17 18 19 20 21 22 23
A


PG1
11 12 13 14 15 16 17 18 19 20 21 22 23
B

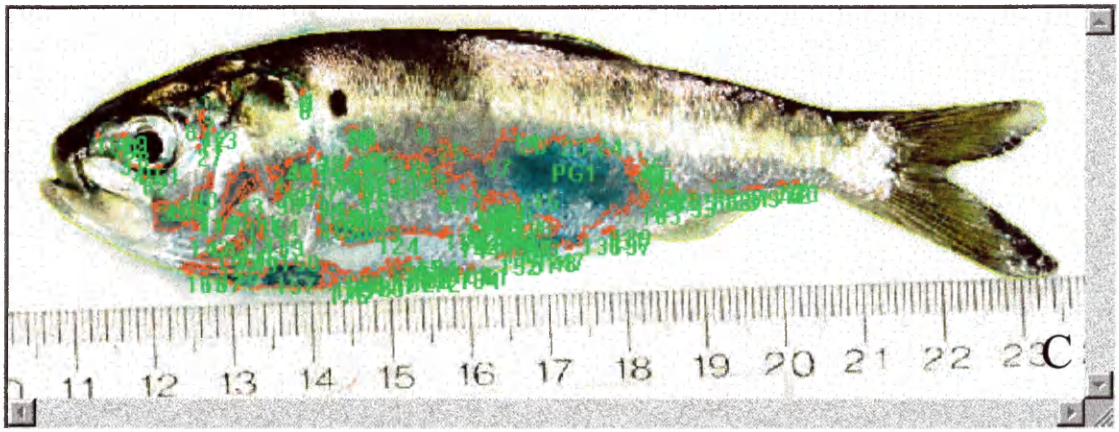

PG1
11 12 13 14 15 16 17 18 19 20 21 22 23
C

Figure 8. Body regions used for analysis of staining

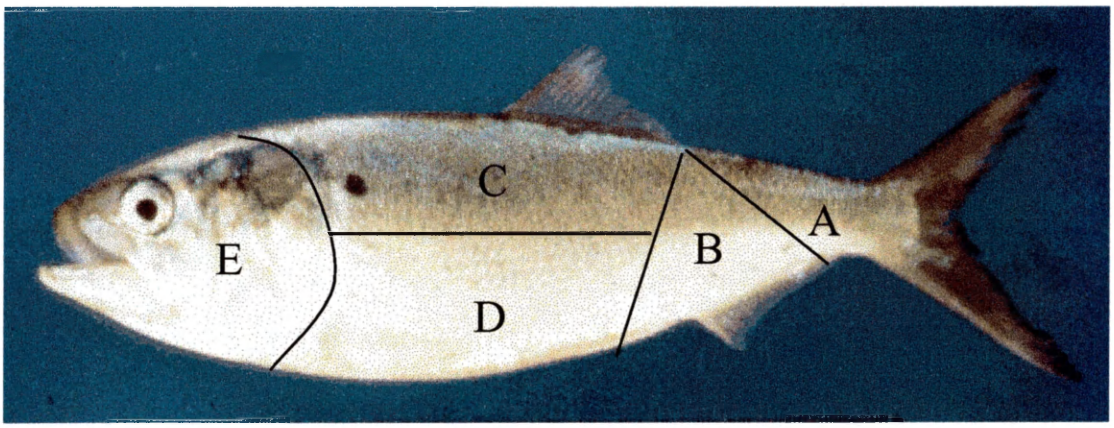
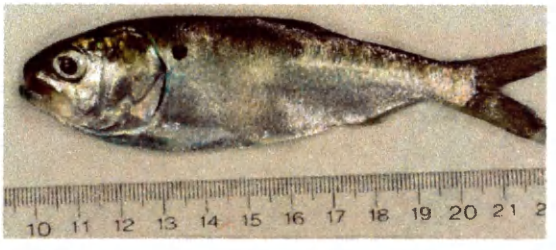

C
A
E
B
D

Figure 9. Series of photographs selected from all fast green experiments. Those in the left hand column are all control fish while those in the right hand column were exposed to 30% oxygen saturation for 36 hours. All fish have been dipped in fast green for 90 seconds and rinsed for 90 seconds

CONTROL



HYPOXIA

Figure 10. Photographs representing other biological stains used. A) control fish dyed with bismark brown B) fish exposed to 30% oxygen saturation dyed with bismark brown C) control fish dyed with alcian blue D) fish exposed to 30% oxygen saturation dyed with alcian blue

CONTROL

HYPOXIA

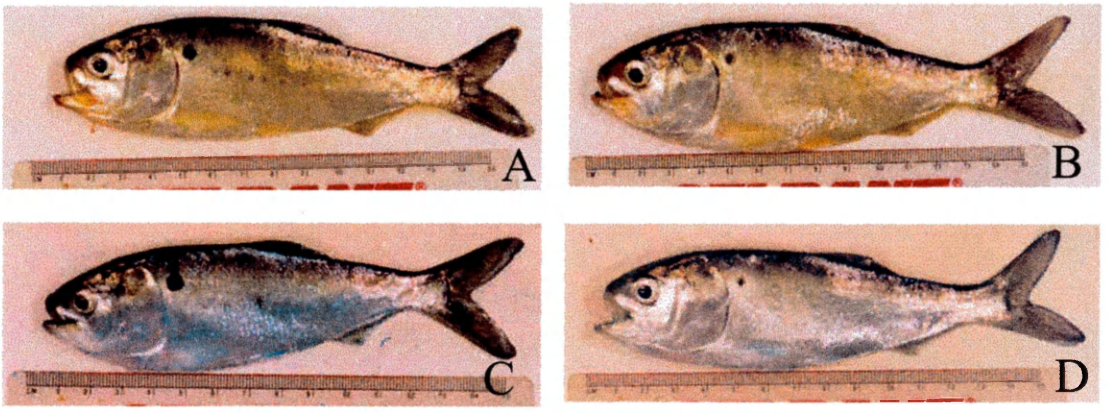


Figure 11. Lesions on Atlantic menhaden from net stress treatments at day 13 (A) and day 20 (B) after aqueous exposure to *A. invadans* zoospores.

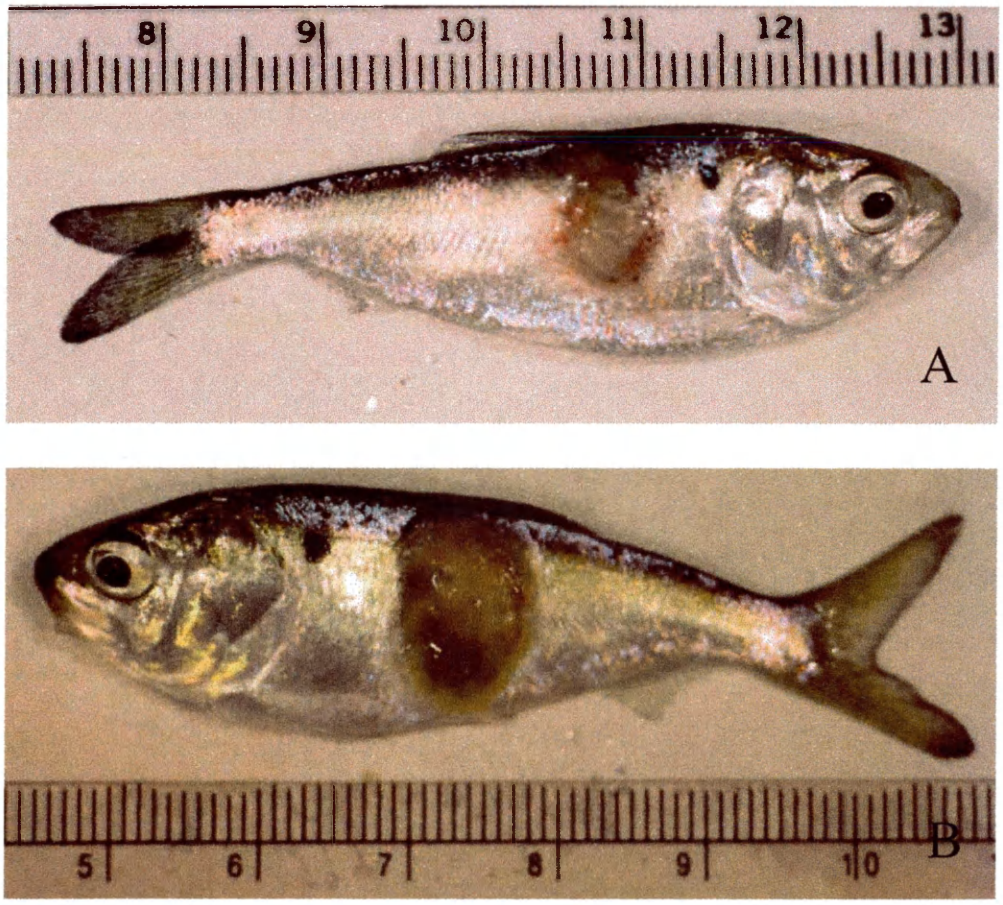

8
9
10
11
12
13
A
5
6
7
8
9
10
B

Figure 12. Lesions on striped killifish at 14 (A, B) and 20 (C) days post injection with *A.invadans* zoospores


J. Zabrecky
A


J. Zabrecky
B

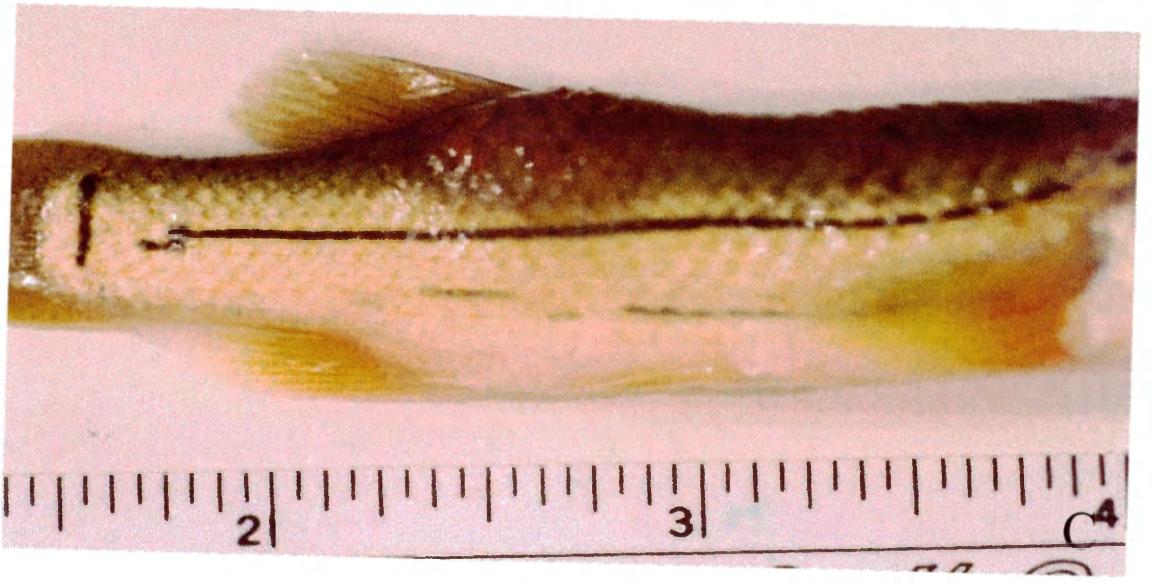
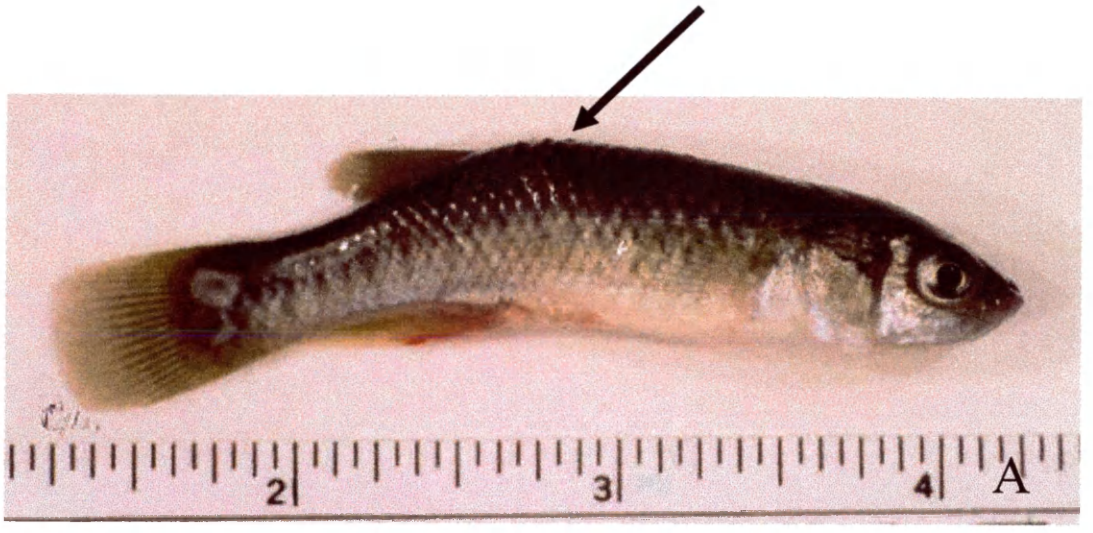

2
3
4

Figure 13. A) Mummichog at 20 days post injection with *A. invadans zoospores*. Arrow indicates reddened area. B) Hogchoker at 6 Days post injection with *A. invadans zoospores*. Arrowhead indicates reddened area.


2
3
4
A

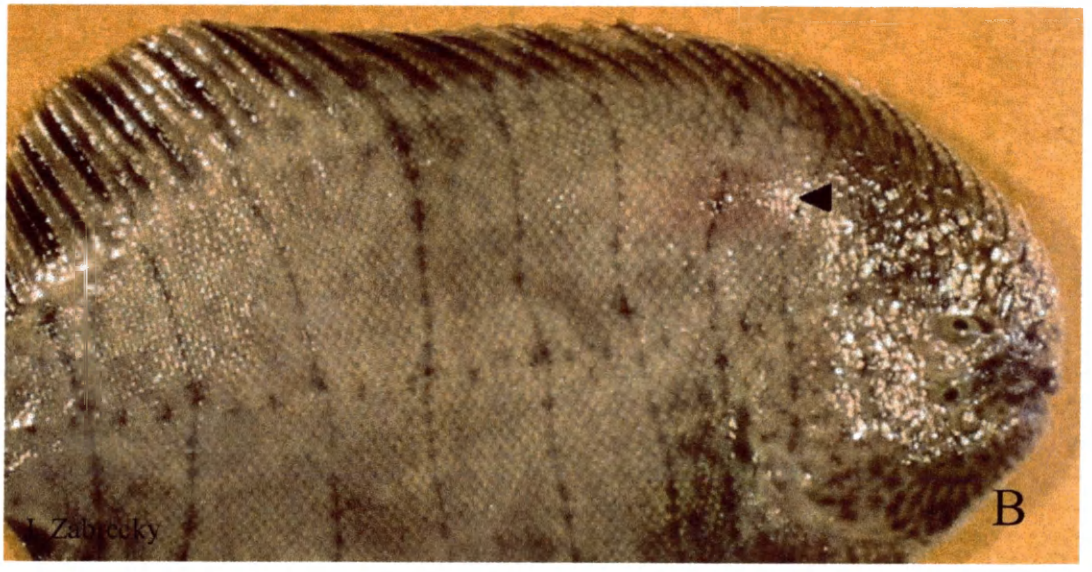
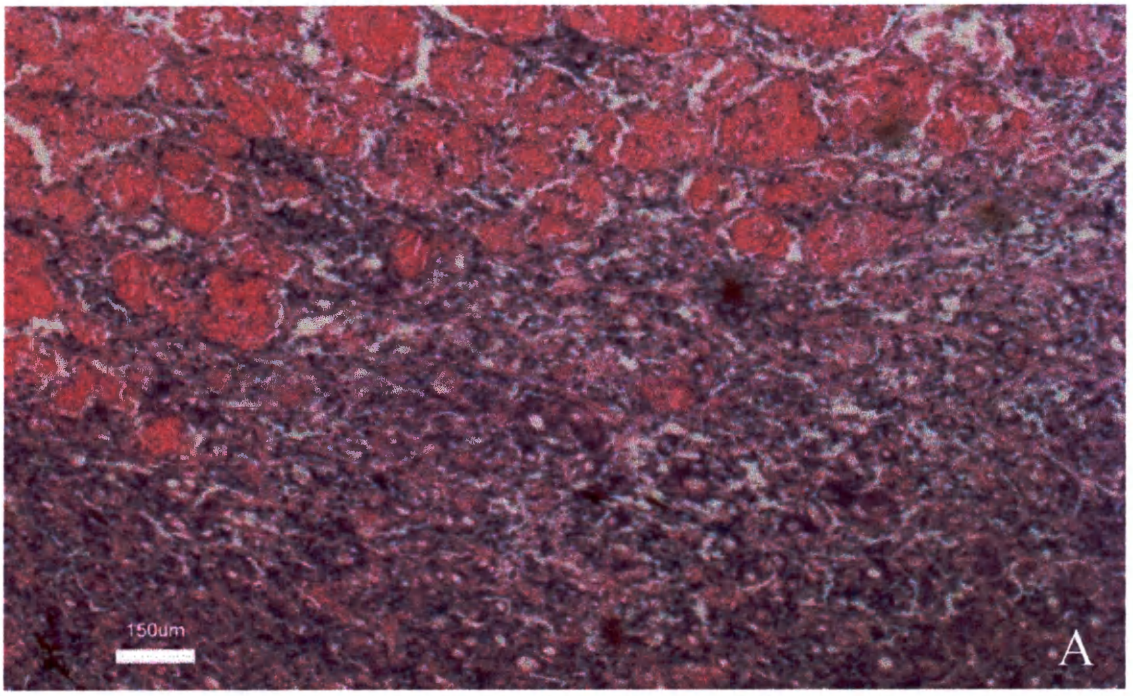
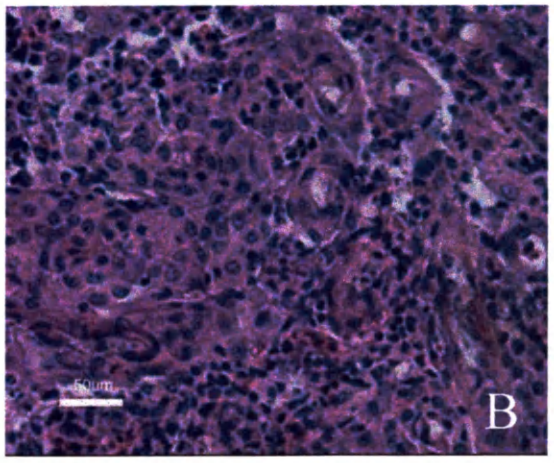

Zabrecky
B

Figure 14. A) General histology of killifish lesions at 14 days post injection. Photograph shows the border between muscle tissue and the extensive granulomatous response B) Closer view of granulomas of striped killifish at 14 days post injection and at C) 18 days post injection.


150um
A


B

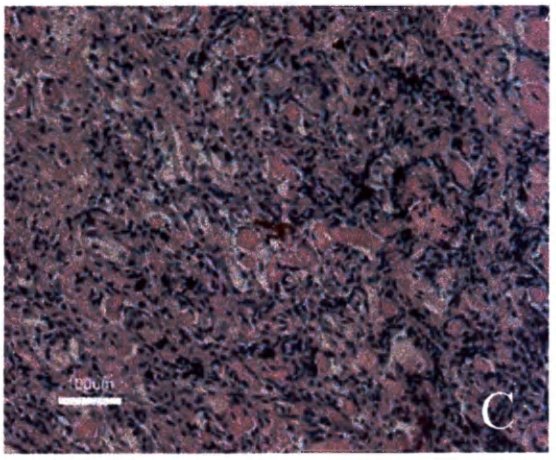
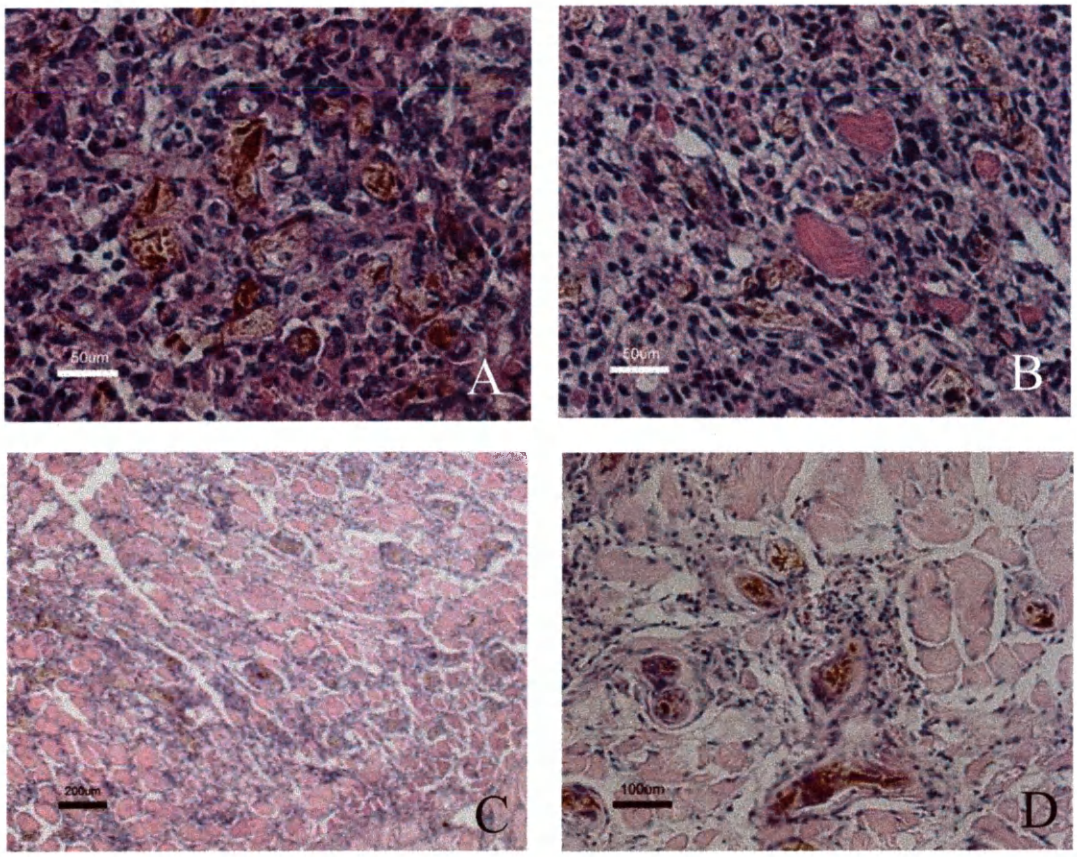

C

Figure 15. A) Histologic section from an experimental mummichog at 20 days post injection. Note the brown pigmented oomycete hyphae. B, C, D) Histologic section of experimental mummichog at 27 days post injection. Note the isolation of oomycete hyphae by granulomas and the regeneration of muscle tissue.


50um
A
50um
B
200um
C
100um
D

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