AETIO-PATHOLOGICAL INVESTIGATIONS AMONG FISH SPECIES PRESENTING EPIZOOTIC ULCERATIVE SYNDROME (EUS) IN THE ZAMBEZI RIVER BASIN IN SESHEKE DISTRICT OF ZAMBIA

BY

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A dissertation submitted to the University of Zambia in partial fulfillment of the requirements of the Degree of Master of Science in Veterinary Pathology

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DECLARATION

I, **Mwansa Mathilda Songe** do hereby declare that the contents of the dissertation being submitted herein are my original work and they have not been previously submitted to any University for the award of a degree or any other qualification.

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CERTIFICATE OF APPROVAL

This dissertation submitted by **Mwansa Mathilda Songe**, is approved as fulfilling the requirements for the award of the degree of Masters of Science in Veterinary Pathology of the University of Zambia.

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ABSTRACT

This study was conducted in Sesheke district of Zambia to investigate the etio pathological changes that occur in fish species presenting with Epizootic Ulcerative Syndrome in the Zambezi River Basin. The disease is endemic in Asia, but it is being reported for the first time in Africa.

A total of two hundred and seventy (270) fish belonging to sixteen species were sampled from seven (7) major fishing camps in Sesheke district of Zambia during the study period. These species were: *Clarias ngamensis, C. gariepinus, Barbus poechii, Tilapia sparmanii, Seranochromis angusticeps, Brycinus lateralis, Micralestes acutidens, Sargochromis carlottae, Hydrocinus vittatus, Phryngochromis acuticeps, Schilbe intermedius, Hepsetus odoe, Labeo lunatus, Oreochromis andersonii, B. unitaeniatus* and *B. paludinosus.* Among the fish samples collected from the field, only *Tilapia sparmanii* did not show any macroscopic or microscopic lesions.

The disease was diagnosed by the histopathological technique. Hematoxylin and Eosin standard stains were used. Grocott stain was used to confirm the presence of fungal hyphae in the tissue sections. Furthermore, samples were cultured for isolation of *Aphanomyces invadans*, the causative agent of EUS.

Following isolation, the infectivity and role of *A. invadans* in the etiology of fish skin ulceration in *Oreochromis niloticus, Oreochromis andersonii* and *Barbus paludinosus* were investigated in the laboratory through the pathogenicity and challenge studies. Of these species, *O. niloticus* did not show any visible lesions, even after 32 days post-inoculation. The other two species, on the other hand, were successfully infected with the fungus after 15 days post-inoculation, upon exposure after disruption of the epidermis. The deeply penetrating ulcers observed in *O. andersonii* and *B. paludinosus* after the challenge were characterized by dermatitis, myofibrillar degeneration and necrotizing granulomatous myositis. The experimentally induced lesions also exhibited invasiveness, often involving the kidney, confirming Koch's postulates. Natural

infections were characterized by dermatitis, hemorrhages, severe muscular necrosis, necrotizing granulomatous inflammation and myofibrillar degeneration. Invasiveness involving internal organs was also observed. The study has demonstrated that EUS can be experimentally induced in *O. andersonii* and *B. paludinosus* after exposure to *A. invadans* zoospores isolated from the Zambezi river basin.

The challenge experiments are significant in establishing which fish species are susceptible to EUS. In this case *O. niloticus* would be an excellent culture species for the emerging fish farmers in Zambia, owing to its comparative resistance to the disease. It is, therefore, important that these studies are continued in a number of species. This will enhance the true understanding of which species can be affected by EUS in the Zambezi river basin.

DEDICATION

I dedicate this work to my wonderful husband, Kaampwe, and our lovely children, Shamsie, Muleba and Kaampwe Jr. A special dedication of this dissertation goes to my parents, Valentine and Winnie Songe, for their love, support and desire to see me improve academically.

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LIST OF ABBREVIATIONS

ASS	Acid sulfate soils
CAN	Chemoautotrophic nocardioform
ECP	Extra cellular products
EGA	Epizootic granulomatous aphanomycosis
EUS	Epizootic ulcerative syndrome
MG	Mycotic granulomatosis
UDRV	Ulcerative disease rhabdovirus
UM	Ulcerative mycosis
ppm	parts per million
psu	practical salinity units
ppt	parts per thousand
RSD	Red spot disease

CHAPTER ONE

INTRODUCTION

Fish serves as a very important cheap source of protein for the majority of Zambians, both directly and indirectly as a feedstock for their domestic animals. It accounts for approximately 47 percent of the average protein intake for the urban and rural communities (Hanyona 2004). Most local people in Zambia who live near lakes and rivers are part-time fishermen. They often buy their fish from full time fishermen, improving their nutritional requirements and social security. Due to low rainfall in some years, conventional agricultural activities do not fully support the food security situation of the local people, whose plight is then taken care of by the availability of fish. Even in good rainfall years, the food security situation in Zambia tends to be precarious, especially from November to March (Syampaku 1998). Fish trading provides an alternative source of income in most house holds both in rural and urban set ups. Therefore, the socio-economic importance of fish in this regard cannot be over-emphasized.

Fish protein is rich in essential amino acids required by the body for growth and maintenance of lean muscle tissue. It is of very high quality and, as such, does play a very important role in weight loss. This is due to the fact that it can be used to maintain an active metabolism, as opposed to low quality protein which does not contain all the essential amino acids required for use in protein synthesis. As a result, most people with diseases like hypertension are resorting to eating fish and cutting down on red meat. Higher intakes of fish protein are related to a lower risk of micro-albuminuria, as observed in young Swedish type 1 diabetic patients (Mollsten *et al.,* 2001). This further supports the fact that fish protein is indeed a much healthier option than red meat.

However, fish can be prone to a wide range of infectious diseases. Some of the more common diseases and parasites likely to infect fish in Zambia are the protozoan *Ichthyophthirius multifiliis,* copepods, helminthic diseases, leeches, free-living

crustacean ectoparasites, bacterial infections and fungal diseases. In Southern Africa, some of these infections, namely *Saprolegnia* and other phycomycete infections like *Achlya racemosa, Aphanomyces laevis, Dictyuchus sterile, Saprolegnia ferax, S. litoralis* and *S. parasitica* are a very common finding (Ogbonna and Alabi, 1991). Ectoparasitic protozoa, coccidioses like *Eimerine coccidian*, haemoprotozoa, helminthic infections and leeches, have had a great impact on the aquaculture industry, causing enormous losses for fish keepers and aquarium traders (Skelton 1993).

The present study focused on investigating the etiology and pathological alterations in fish species presenting Epizootic Ulcerative Syndrome (EUS) in the Zambezi River Basin, considering that the disease is being documented for the first time in Africa. EUS is a seasonal Epizootic condition of great importance in wild, farmed freshwater and estuarine fish characterized by the presence of invasive *Aphanomyces* infection and necrotising ulcerative lesions typically leading to a granulomatous response (Roberts *et al.*, 1994). It is caused by *Aphanomyces invadans* (Saprolegniaceae), a peronosporomycete fungus, and often affects stressed and injured fish in the wild (Skelton, 1993).

Recent evidence indicates that EUS does not always occur in seasonal outbreaks nor does it always cause high mortalities. It may be prevalent at only low levels throughout the year. EUS may, therefore, have an effect on productivity that cannot be measured in terms of mortalities alone, especially in wild fish. Communities that are heavily dependent on local fisheries have been affected by outbreaks of EUS. Social impacts may extend beyond persons directly affected by fish losses (Lilley *et al.*, 2002).

This study was designed to investigate the pathology of fish presenting with EUS in the Zambezi river basin in Sesheke district of Zambia, with the specific objectives of this study being:

1. To isolate, confirm and document the presence of *A. invadans* in fish samples collected from the Zambezi River Basin.
2. To identify fish species which are more susceptible to EUS.

3. To establish the pathogenecity of *A. invadans* in selected species of fish found in the Zambezi River.
4. To compare the lesions in fish infected in the wild to those that may be observed in laboratory-infected fish.

Justification of the Study

EUS has never been previously reported in Africa. However, in the later months of the year 2006, fish caught in the Chobe and Zambezi Rivers in the vicinity of the Chobe Game Reserve, Botswana, and Caprivi Strip, Namibia, as well as on the Zambian side of the Zambezi River were found with clinical signs similar to those of EUS (ulcers and focal areas of skin inflammation) (Andrew *et al.,* 2008). The disease was earlier designated as a 'mysterious disease'. A wide variety of fish species were recorded with lesions, including significant fisheries species. Amongst the larger species, Barbel (catfish) and Tiger fish were recorded with large skin ulcerations. Diagnostic investigations confirmed that the so-called 'mysterious disease' was in fact EUS (Andrew *et al.,* 2008). Very little or no work has been done on Epizootic Ulcerative Syndrome in Zambia, Southern Africa and Africa as a whole, making it a newly identified risk area. In view of this fact, it was considered necessary that more detailed work be conducted as to the pathological alterations involved in this new disease to this part of Africa. This will positively give a clear picture which should be compared with the reports from other parts of the world where the disease has been endemic for a considerate amount of time.

CHAPTER TWO

LITERATURE REVIEW

2.0.0. General Overview

EUS is a serious fish disease which has swept across Japan, Australia, Thailand, Philippines, Malaysia, India, Pakistan and the United States of America since the first outbreaks were reported in the early 1970's (Anonymous 2008). This has caused significant losses of income to fisheries and fish farmers. It has also resulted into a negative biodiversity and social impact. The first report of the disease came from Japan in 1971, where it is known as mycotic granulomatosis (MG) (Egusa and Masuda 1971). In Australia, where it was first reported in 1972 and primarily affected estuarine mullet (McKenzie and Hall 1976., Callinun *et al.*, 1989), it was called red spot disease (RSD). The first case of EUS in the United States of America (USA) occurred in 1978. There, outbreaks of ulcerative disease in menhaden, *Brevoortia tyrannus,* have been shown to be very similar to EUS in Asia (Blazer *et al.,* 1999). Since the early 1980s, EUS has spread westwards across the South-East and South Asia, affecting over 100 species of freshwater fish (Roberts *et al.,* 1994., Vishwanath *et al.,* 1997). The first reported typical EUS outbreaks in Malaysia were in December 1980, in rice-field fishes (Jothy 1981). Significant, well-documented epizootics have occurred annually in Thailand since 1981 (Anonymous 1983). Ulcerated *Labeo rohita* were first observed at the Pearl River Fisheries Institute in Guangzhou, South China in 1982 (Lian 1990). The first report of ulcerated snakeheads in Vietnam, and therefore the most likely first occurrence of EUS in that country, came from the Mekong delta in 1983 (Xuan 1990). Myanmar, Lao PDR and Cambodia first reported major outbreaks of EUS in 1983 or 1984 (Lilley *et al.*, 1992). Laguna de Bay in the Philippines experienced a serious outbreak of EUS in December 1985 (Llobrera and Gacutan 1987). A major outbreak of EUS in freshwater and estuarine fish in western Sri Lanka occurred in December 1987, prior to any outbreaks on the subcontinent mainland (Costa and Wijeyaratne 1989). The disease was first reported in Bangladesh in 1988 (Barua *et al.*, 1991). In India, the

North-Eastern states were the first to report losses due to EUS in 1988. The disease appeared to spread through rivers, reservoirs and paddy fields to most states, affecting some Indian major carp farms as well (Lilley *et. al.*, 1998). The country to be affected most recently by EUS was Pakistan, where EUS was confirmed in snakeheads from Punjab Province in April 1996 (Anonymous 1998a). The pattern of spread between and within countries was consistent with progressive dissemination of a single infectious agent (Baldock *et al.*, 2005). On the basis of findings from studies conducted by Lilley *et al.* (1997b; 2003), the authors suggested that *A. invadans* achieved its colonisation of Australia, Asia and USA in one relatively rapid episode. More recently, scientists proposed that EUS should be re-named as epizootic granulomatous aphanomycosis (EGA) (Baldock *et al.*, 2005). At present, red spot disease, mycotic granulomatosis, ulcerative mycosis and epizootic granulomatous aphanomycosis are all synonyms for EUS.

Currently over 50 species of fish have been confirmed by histological diagnosis to be naturally affected by EUS (Lilley *et al.*, 1998). Some of these species, which can be found in Zambia as well, include: *Clarias gariepinus*, *Clarias ngamensis*, *Hepsetus odoe*, *Pharyngochromis acuticeps*, *Labeo lunatus*, *Schilbe intermedius*, *Oreochromis andersonii*, *Barbus paludinosus*, *Barbus poechii*, *Sargochromis carlottae*, *Brycinus lateralis*, *Micraletes acutidens* and *Hydrocinus vittatus*. Other culture species like *Chanos chanos*, *Oreochromis niloticus*, *Ctenopharyngodon idella* and *Cyprinus carpio* have been shown to be naturally resistant to EUS (Wada *et al.*, 1996). Experimental infections have demonstrated that goldfish are very susceptible to EUS (Hatai *et al.*, 1977; 1994).

A. invadans was originally documented as the cause of mycotic granulomatosis of ayu (*Plecoglossus altivelis altivelis*) in Japan (Hatai *et al.*, 1977) and later as the cause of epizootic ulcerative syndrome or red spot disease of striped snakehead (*Channa striata*) and striped mullet (*Mugil cephalus*) from southern Asia and Australia (Frazer *et al.*, 1992, Roberts *et al.*, 1993; Callinan *et al.*, 1995 Nandeesha 2001; Karunasagar and Otta 2003). These skin diseases (mycotic granulomatosis, epizootic ulcerative syndrome, red

spot disease, and ulcerative mycosis) are clinically identical and occur in either freshwater or estuarine fish.

Bacterial genera such as *Aeromonas* and *Vibrio* are very commonly incriminated with EUS infections in fish. They have been implicated in several ulcerative disease syndromes and are undoubtedly involved in worsening the lesions of many of the fish specimens presented with typical EUS. Furthermore, bacteria which are ubiquitous in the aquatic environment have been considered to be primary pathogens in some cases (Law 2001). However, to date, no single species has been consistently isolated to suggest it as an initiating pathogen or major cause of EUS (Law 2001). *A. invadans* should not be considered the sole etiology for all ulcerative lesions. Stress, poor nutrition, and certain parasitic, bacterial and viral infections are all capable of producing similar gross lesions (Kane *et al.*, 2000; Noga 2000a; Law 2001; Reimschuessel *et al.*, 2003).

2.1.0. Epidemiology of Epizootic Ulcerative Syndrome (EUS)

The spread of EUS is thought to be due to flooding and movement of affected carrier fish. The disease usually occurs at about the same time every year in endemic areas, mostly after periods of heavy rainfall and when temperatures are low between 18°C and 22°C (Bondad-Reantaso *et al.*, 1992). These conditions favour sporulation of *Aphanomyces invadans* (Lumanlan-Mayo *et al.*, 1997). Low temperature delays the inflammatory response of fish to oomycete infection (Chinabut *et al.*, 1995: Catap and Munday, 1998). In some countries like Bangladesh, Papua New Guinea and Australia, EUS outbreaks were first reported in wild fish spreading into fish ponds (Anonymous 2006). This is likely to happen if infected fish is thrown into a healthy fish pond. Khan and Lilley (2002) showed that there is a significantly higher relative risk of EUS occurring in farmed fish when wild fish are present in the pond. EUS outbreaks have been reported to cause high mortalities in various freshwater fish in the wild (including rice-fields, estuaries, lakes and rivers) and, on fish farms during periods of low temperatures and/or after heavy rainfall. A number of comprehensive reviews of EUS were published in the 1990's (Roberts *et al.*, 1993; Noga 1993; Chinabut *et al.*, 1995;

1998; Roberts 1997; Lilley *et al.*, 1998). It is likely that EUS is spread geographically, and persists locally, by affected or carrier fish, most probably in wild fish populations.

An important question concerning *A. invadans* pathogenesis that yet has to be answered is how natural infections are initiated. Although hundreds of ulcerative mycosis (UM) lesions have been examined, no oomycete reproductive structures have been observed (Noga unpublished). This observation implies that although *A. invadans* readily infects fish and often proliferates rapidly, fish are probably a dead end host. Either the infections are transferred directly between fish (less likely) or an alternate source of infection is present in the environment. Laboratory exposure studies suggest that secondary zoospores are the most likely source of UM infection (Kiryu *et al.*, 2002; 2003; Johnson *et al.*, 2004). Changes in the aquatic environment (such as a change in salinity or water temperature) cause *A. invadans* to undergo asexual reproduction. During sporulation (asexual reproduction) thousands of swimming zoospores are released into the water. Infection occurs when these motile spores are chemotactically attracted to the skin of fish. They penetrate the fish skin and germinate, forming fungal hyphae. The latter invade widely into the surrounding skin and deeply into underlying muscle tissues, resulting in extensive, bloody ulceration and destruction of tissues. Initially, the lesions may be characterised by the appearance of raised areas of induration and erythema. Subsequent skin erosion results in the formation of ulcerative lesions on the body. If a host is not found, the zoospores become cysts and sink to the mud or sediment.

A. invadans occurs worldwide and infects both wild and cultured fish, often leading to mass fish mortality (Blazer *et al.*, 1999). In the United Kingdom, Lilley and Roberts (1997) provided convincing evidence that *A. invadans*, and not one or more other fungi, is responsible for much of the characteristic pathology of EUS. They injected zoospores from 58 fungal isolates intramuscularly into snakehead fish, *Channa striata.* These fungi comprised of *Aphanomyces* strains isolated in Asian countries and Australia from EUS-affected fish, saprophytic *Aphanomyces*, *Achlya* and *Saprolegnia* spp. from infected waters and further *Saprolegniaceous* fungi involved in other diseases of aquatic

animals. Only the *Aphanomyces* strains isolated from fish affected by EUS, RSD or MG were able to grow invasively through the fish muscle and produce the distinctive EUS lesions. The snakehead-pathogenic strains were further distinguished from all the other fungi under comparison by their characteristic temperature-growth profile and inability to grow on certain selective fungal media. *A. invadans* can utilize oxygen in tissues, where as *Saprolegnia* cannot (Chinabut 1994).

As with other *Saprolegniacean* fungi, *A. invadans* is aseptate and produces two zoospore forms, the secondary form being free-swimming and laterally biflagellate. No sexual reproductive structures have been observed in any of the isolates from EUS, MG or RSD outbreaks. The lack of sexual structures is considered to be a particularly common phenomenon among the more pathogenic members of the *Saprolegniaceae* (Alderman and Polglase 1988).

2.2.0. Risk factors associated with outbreaks of Epizootic Ulcerative Syndrome

A. invadans is a slow-growing monoclonal species of fungus which, if provided with suitable conditions, can invade the tissues of susceptible fish. Initiating factors which facilitate the entry of the fungus are varied. In some locations infection is predisposed by cooler water temperatures, while in others higher temperatures appear to trigger it (Chinabut and Roberts 1999). EUS has been associated with low temperature, and has often occurred after periods of heavy rain. Phillips and Keddie (1990) observed that EUS outbreaks occurred during months when the mean daily temperature was below the annual mean temperature (18.9°C, 20°C, 24°C, 15°C to 26°C) in Bangladesh, China, India and Lao-PDR respectively. However, EUS outbreaks in the Philippines and Thailand were also recorded in warmer months. Chinabut *et al.* (1995) challenged striped snakehead (*Channa striata*) by injecting with zoospores of *A. invadans* and found a weaker inflammatory response, higher mortality rate and more extensive fungal invasion in fish held at 19°C compared to fish held at 26°C and 31°C. This confirms that lower temperatures stress the fish, making them susceptible.

Studies in the Philippines (Lumanlan-Mayo *et al.,* 1997) suggested that outbreaks in rice-fish plots will not occur when maximum diurnal water temperatures remain above 30°C. *A. invadans* is substantially inactive at these temperatures. *A. invadans* hyphae grow only poorly at temperatures above 31°C and do not grow at 37°C (Hatai and Egusa 1978; Fraser *et al.,* 1992; Roberts *et al.,* 1993). Zoospores are more sensitive than hyphae to temperature effects and zoospore production is inhibited at 35°C (Campbell unpublished).

From laboratory observations, low salinities appear crucial to the transmission of the pathogen (Kiryu and Blazer 2005). Environmental parameters have also been studied. These abiotic factors are believed to cause sublethal stress to the fish, initiating disease outbreaks. Potential causes of stressful environmental conditions include: temperature, eutrophication, sewage, metabolic products of fishes, industrial pollution and pesticides. The quality of water also appears to be significant from an aetiological point of view. Parameters like salinity, alkalinity, temperature, hardness, turbidity and chloride concentration (many of which are seasonally variable) are known to predispose fish to attacks of EUS. Infected fish showed signs of improvement when transferred to clean freshwater ponds.

A. invadans requires some predisposing condition of the host, such as debilitation or breach of the normal mucosal barriers along with favorable growth conditions before they can become established. Such conditions would include compromise of the animal's normal immune status (i.e., systemic and/or mucosal immunity). Some tank trials have been used to show that healthy, intact fish in aquaria exposed to *A. invadans* zoospores in water would not develop EUS lesions and that prior damage to skin was necessary before lesions could be induced in fish (Jones and Hunt 1983).

Ultrastructural examination of fish gills and skin showed that the low pH and elevated concentrations of monomeric aluminium, representative of estuarine acidification, induces significant lesions in fish (Sammut *et al.*, 1996). In aquarium trials, RSD was subsequently induced in fish exposed sublethally to artificially acidified water (at both

pH 3 and pH 5) and pathogenic *Aphanomyces* spores, even at low concentrations of monomeric aluminium (Callinan *et al.*, 1996a).

Severe epidermal necrosis has been shown to be induced in fingerling sand whiting *Sillago ciliata* sublethally exposed to very acidic water from Acid Sulfate Soil (ASS) areas (Callinan *et al.*, 1996b). Mild to moderate epidermal necrosis has been induced in fish sublethally exposed to less acidic ASS runoff (pH 5.1). Typical EUS lesions, i.e. necrotizing granulomatous dermatitis and myositis associated with invasive non-septate fungal hyphae, were induced significantly more often than in controls when these fish were subsequently exposed to *A. invadans* zoospores. The fungus was recovered on culture from affected fish. There was no evidence from these studies that other biotic agents, such as viruses or bacteria, were necessary causes. Injection of spores under the dermis consistently reproduced the clinical and pathological features of the disease in India (Mohan *et al.*, 1999). There is experimental evidence of variation in resistance to *A. invadans* infection among species and age classes. The fry and fingerlings of Indian major carp appear to be susceptible to *A. invadans* infection whereas yearlings appear to be resistant. In Indian major carp, there is also evidence for increased resistance with age but this does not appear to be the case for puntius and snakeheads. The cellular defense mechanisms against the fungus appear to be better developed in yearlings of Indian major carp and advanced fingerlings and yearlings of common carp when compared to snakeheads and puntius of a similar age. In the more resistant species and age classes, few spores appear to germinate and the resulting fungal hyphae are confined and killed by very well developed epithelioid granulomata (Mohan unpublished).

2.3.0. Pathogenesis of Epizootic Ulcerative Syndrome

The highly invasive, deeply penetrating pathogenic oomycete *A. invadans* (synonyms *Aphanomyces invaderis* and *Aphanomyces piscicid*), is the primary etiological agent in EUS (Blazer *et al.*, 2002). *A. invadans* has been shown to be slow-growing and thermo-labile in culture (Lilley *et al.*, 1997a). Changes in the aquatic environment (such as a change in salinity or water temperature) cause *A. invadans* to undergo asexual

reproduction. During sporulation (asexual reproduction) thousands of swimming zoospores are released into the water. Infection occurs when these motile spores are attracted to the skin of fish. They penetrate the fish skin and germinate, forming fungal hyphae. The latter invade widely into the surrounding skin and deeply into underlying muscle tissues, resulting in extensive, bloody ulceration and destruction of tissues. Initially, the lesions may be characterised by the appearance of raised areas of induration and erythema. Subsequent skin erosion results in the formation of ulcerative lesions on the body. If a host is not found, the zoospores become cysts and sink to the mud or sediment.

The presence of *A. invadans* is accompanied by an intense granulomatous inflammatory response. Once established in the tissues of susceptible fish, *A. invadans* migrates towards the central nervous system, and then throughout the body, producing proteolytic enzymes which destroy muscle and other tissues (Chinabut and Roberts 1999). *A. invadans* has a life cycle that consists of three different stages. These are: hyphae, zoospore, and cyst (Figures 2.1 and 2.2).

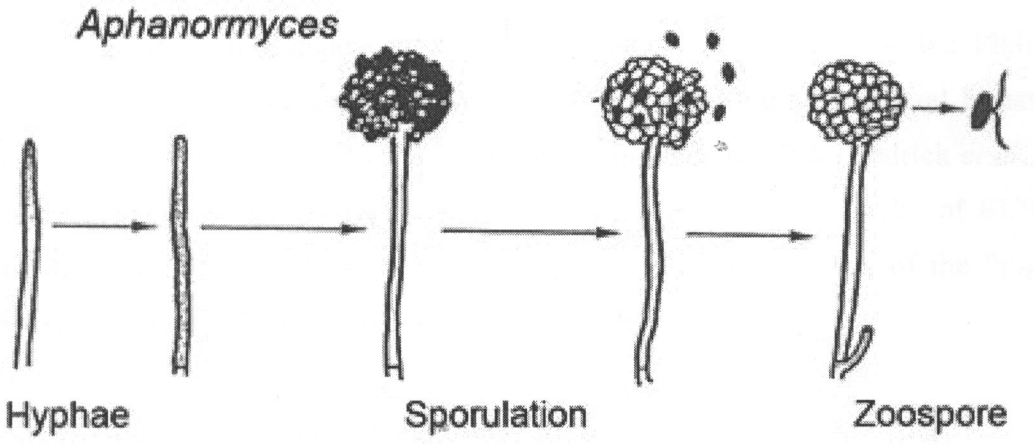


Fig. 2.1: Life cycle of *Aphanomyces invadans* (Lilley *et al.*, 1998).

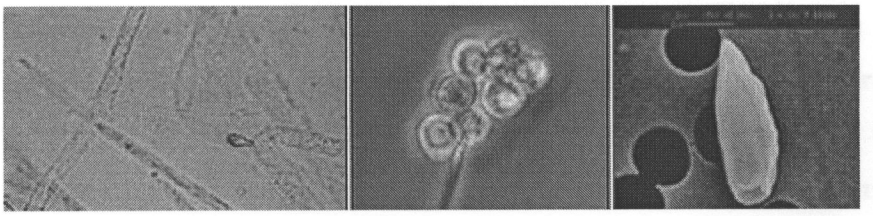


Fig. 2.2: Life cycle of *Aphanomyces invadans* showing hyphae, sporulation and zoospore stages. (Lilley *et al.*, 1998)

Secondary infections due to fungi, bacteria and parasites seem to play a role in pathogenesis, as they are generally observed in lesion scrapes. Fungal infections may include *Achlya bisexualis, Phialemonium dimorphosporum, Pythium spp* and saprophytic *Aphanomyces* strains, while bacterial infections may involve haemolytic strains of *Eschericha coli, Aeromonas hydrophila, Pseudomonas aeruginosa, Staphylococcus epidermidis* and *Klebsiella* species (Kar 1999). Parasites that have been found in EUS-affected fish include: species of *Palisentis, Triancloratus, Dactylogyrus, Gyrodactylus, Henneguya, Epistylis, Trichodina, Dactylogyrus, Gyrodactylus* and protozoan parasites such as *Chilodonella* spp., *Trichodina* spp., *Costia* spp., *Henneguya* spp. and *Ichthyophthirius* species (Reungprach *et al.*, 1983; Chinabut 1998). 6 rhabdovirus isolates have been obtained from Thailand, Myanmar, Sri Lanka and Australia (Frerichs *et al.*, 1986; 1989, Roberts *et al.*, 1989). These isolates have been named ulcerative disease rhabdovirus (UDRV) (Lilley and Frerichs 1994). No viruses were obtained from Bangladesh, Lao PDR, Malaysia, Indonesia or the Philippines during the same sampling period. Aside from the rhabdoviruses, several birnaviruses and a single reovirus have also been isolated from ulcerated fish (Hedrick *et al.*, 1986; Subramaniam *et al.*, 1993). If viruses have a role in the pathogenicity of EUS, their most likely effect is to cause skin lesions sufficient to allow entry of the fungus, *A. invadans* (Kanchanakhan 1996).

2.4.0. Diagnosis of Epizootic Ulcerative Syndrome

Suitable tissues for histopathological examination and for oomycete isolation are kidneys, livers and muscles. However, presumptive diagnosis is based on the clinical

findings which include loss of appetite; fish generally become darker, often found floating below the surface of the water, and becoming hyperactive with a jerky movement. Infected fish then develop red spots or small to large ulcerative lesions on the body surface, head operculum or caudal peduncle. Large red or grey shallow ulcers, often with brown necrotic areas, are observed in the latter stages. Large superficial lesions occur on the flank or dorsum. Most species will die at this stage. In highly susceptible species, such as snakehead, the lesions are more extensive and can lead to complete erosion of the posterior part of the body, or to necrosis of both soft and hard tissues of the cranium, so that the brain is exposed in the living fish (Anonymous 2006).

As sexual stages have not been identified for this fungus, further diagnosis relies in particular on asexual morphology characters (Willoughby *et al.*, 1995; Lilley *et al.*, 1997b), and on its ability to invade the internal tissues of EUS susceptible fish (Lilley and Roberts 1997). Histopathology is currently the recommended method for confirmatory diagnosis of EUS (Lilley *et al.*, 1998; OIE 2003). Definitive diagnosis of EUS is achieved by demonstrating the presence of mycotic granulomas in histological sections, or by isolating *Aphanomyces invadans* from internal tissues. Early EUS lesions are erythematous dermatitis with no obvious oomycete involvement. *A. invadans* hyphae are observed growing in skeletal muscle as the lesion progresses from a mild chronic active dermatitis to a severe locally extensive necrotising granulomatous dermatitis with severe floccular degeneration of the muscle. The oomycete elicits a strong inflammatory response and granulomas are formed around the penetrating hyphae, as a result of the fish cellular immune response to quarantine or arrest the invading fungus (Chinabut 1994). Muscle squash preparations of the infected area around the lesion can show non-septate hyphae of *A. invadans* which are 12 to 25 μm in diameter.

2.5.0. EUS prevention and control

At present there are no systemic treatments for use against EUS in fish. The very nature of the disease, fungal hyphae penetrating deep muscle and tissue, results in the physical protection of the pathogen from the external environment. Research has therefore

mainly targeted prophylactic and preventative treatments or measures to prevent the transmission of the disease, rather than treatment of affected fish. The control of EUS in wild fish populations, in open water bodies, is most likely impossible. All research efforts have been directed at management techniques and therapeutics for aquaculture, notably pond culture systems. Khan and Lilley (2002) showed that there is a significantly higher relative risk (over 10 times more chance) of EUS occurring in farmed fish when wild fish are present in the pond. The following measures, if put in place, would help control or even prevent EUS on farms.

a) Exclude all wild fish from farm sites, as it is the wild fish themselves that are considered risk factors for EUS. Khan and Lilley (2002) also showed that sites that were artificially stocked showed no significant association with occurrence of EUS.

b) Collect seed and brood stock only from disease-free areas, to ensure EUS-free progeny are produced. A study on the prevalence of EUS in three floodplain areas was undertaken by Subasinghe and Hossain (1997) using a histological diagnosis of the disease. They showed that the prevalence of EUS was generally lower in artificially stocked fish sampled from natural waters than in wild fish.

c) Improvement of water quality. Poor water quality stresses the fish, resulting in failure by fish to mount an adequate immune response to fight disease (Rottmann *et al.*, 1992).

d) Ensuring that water salinities of 2 practical salinity units (psu) or less are avoided as they are ideal for sporulation, motility and infectivity of *A. invadans* (Blazer *et al.*, 2002).

e) Increase of dissolved oxygen to desirable levels. Dissolved oxygen values have been shown to decline prior to EUS outbreaks, and to have remained at lower levels (between 0.75 and 1.2 mg/L) during the outbreak period (Pathiratne and Jayasinghe 2001).

f) Lime addition to the water. Both prophylactic and therapeutic treatment, which usually involve the addition of quicklime, to raise the pH of the water.. This has reported satisfactory results (Anonymous 2006).

g) Prophylaxis revolves around good general husbandry practices including disinfection, opting for water from tube wells rather than irrigation canals or paddy fields, and ensuring disease-free stock and healthy fry. Apart from not overstocking ponds, other preventive measures include the use of antibiotics and chemicals, to take care of secondary bacterial invaders. Though these have brought some satisfactory results, they have many undesired side-effects (Areechon 1992). The side-effects include residues, cost increases, development of bacterial resistance, and negative impact on the environment.

h) Maintenance of good hygiene on all fisheries equipment. Disinfection of nets by sun-drying, boiling in water and restriction on transferring the use of nets and other potentially transmitting agents from infected to unaffected water columns. Fish showing signs of EUS must be removed as soon as possible from the pond.

2.6.0. Differential diagnosis of Epizootic Ulcerative Syndrome

A range of other skin disease conditions occur with great frequency, which may present some difficulty distinguishing them from EUS. Some of these diseases actually do excite a granulomatous response within the skin or the tissues and it is important that these can be differentiated from EUS (Chinabut and Roberts 1999).

The most frequent condition is mycobacteriosis, caused by *Mycobacterium fortuitum* or *Mycobacterium marinum.* This condition is common in many fish species and can present as a series of small haemorrhagic lesions on the skin, very similar to the earliest stages of EUS. Histology, however, shows a very different picture. If the lesion is relatively immature it may be possible to demonstrate acid-fast bacilli and cocco-bacilli within the centre of the tuberculous granulomas, but even if not, the centrum of the granuloma is filled with dark amorphous material. It does not have the clear centre, with

possible fold of hyphal material, seen in the EUS granuloma. Also in mycobacteriosis the granulomas are spherical rather than tubular in longitudinal section, and they generally have lymphoid involvement in the earlier stages of their development (Chinabut 1999).

One of the other conditions which, in its early stages has been confused with EUS, is the iridovirus infection of the skin, particularly common in estuarine fishes, known as lympocystis. In this disease, viral particles are introduced into dermal fibroblasts and induce massive enlargement of the cell and development of a hyaline capsule round it. Early lesions may resemble mycotic granulomas, but again there is a solid cytoplasmic centre in the infected lymphoblasts. By the time that the lesion is mature, infected fibroblasts will have grown to 100 times their original size and cannot be mistaken for any other lesion (Dukes and Lawler 1975).

Occasionally, estuarine fishes such as mullets and sand whitings, which are vulnerable to EUS, will be infected by *Ichthyophonus hoferi.* This unusual pseudofungus invades the musculature and will induce granulation tissue quite similar to the lesion caused by *A. invadans.* It is distinguished by the extensive granular cytoplasm normally found within the granuloma and by the very different nature of the fungal hyphae, which do not have the empty structure of the encased *Aphanomyces* (Kocan *et al.,* 1999).

Various granulomatous myxosporidian and microsporidian cysts can be found on occasion within the superficial tissues of farmed and wild fishes. These can sometimes look very similar to EUS but can be distinguished from EUS by the variable size of the cysts and the shiny refringent yellow brown spores, encased within them (Bruno *et al.,* 2006).

Other skin parasites such as leeches (*Hirudina spp.*) and fish lice such as *Argulus* and *Lernaea spp.* also cause surface ulceration which frequently becomes secondarily infected. These are readily distinguished from the ulcers of EUS, however, by the

absence of the typical granuloma formation and extension into the muscles (Chinabut and Roberts 1999).

Other water molds such as Leptomitales and Saprolegniales infections may also cause some confusion. However, these present as relatively superficial, cottony growths on the skin and gills and the molds rarely penetrate beyond the superficial muscle layers (Noga 2000b).

2.7.0. Public Health implications of Epizootic Ulcerative Syndrome

There is no scientific evidence that the EUS itself causes any human or animal illness. However, the potential public health risk derived from the presence of opportunistic infections like *A. hydrophila* should not be underestimated (González-Serrano *et al.*, 2002). These infections may cause gastroenteritis in healthy individuals or septicemia in persons with impaired immune systems, and can be fatal in the latter group. This is very important especially with the advent of Acquired Immuno Deficiency syndrome related ailments. Parasites and rhabdoviruses have also been associated with particular outbreaks of EUS. Secondary gram-negative bacteria such as *Aeromonas, Vibrio, Plesiomonas* and *Pseudomonas* exacerbate the EUS lesions (Blazer *et al,*. 1999; Mastan and Qureshi 2001). Toxins that may be present in EUS-affected fish could also cause human illness. Chemoautotrophic nocardioform (CAN) bacteria had been repeatedly isolated from fish with EUS from the massive epizootics that had repeatedly occurred since 1988 in eastern India as the major or only pathogenic agent in the background of distinctive environmental and epizootic data (Ganguly *et al.*, 2004).

The uncontrolled use of chemotherapeutants to treat EUS or other diseases in intensive culture systems is also a matter of public health concern. There is the danger that consumers may be exposed to drug residues in marketed fish that had been hurriedly harvested before the recommended withdrawal period had been completed.

2.8.0. Socio-economic significance of Epizootic Ulcerative Syndrome

It had been recorded that the fish mortality due to EUS resulted in economic losses in some countries of Asia-Pacific region of over US$ 124 millions in a 16 year period from 1980-1996. The country most affected was Thailand, which lost about US$ 5.5 millions in 1982-83 and about US$ 100 million in 1983-93 (Lilley *et al.,* 1998).

During and after EUS outbreaks, indirect economic loss has been encountered, due to market rejection of harvested ulcerated fish and the fear of disease transmission to consumers leading to a drastic decrease in market demand for all food fish. Potential investors and financial agencies have thus lost confidence in freshwater fish farming (Lilley *et al.,* 1998).

EUS outbreaks impact on local and national authorities due to high expenditures for diagnostic and control activities to eradicate the fungus from already infected sites, especially in the countries where the intensive culture of EUS-susceptible species has been practiced. The importance of convincing farmers, fishermen and consumers to have a better understanding of the wider effects of EUS has been recognized. For example, the eradication activities during EUS outbreaks resulted in problems of uncontrolled use of chemotherapeutics in intensive culture systems, leading to a risk of build up of bacterial resistance in treated fish and the possibility of severe allergic reactions in farm workers in contact with the drug. Consumers may also be exposed to drugs residues in marketed fish which have been hurriedly harvested before the recommended withdrawal period has been completed (Lilley *et al.,* 1992)

CHAPTER THREE

MATERIALS AND METHODS

3.0.0. Study area: Description of Study area

The present study focused on the fish species that had manifested EUS-like lesions in the Zambezi river basin during the period from October 2007 to July 2008. Sampling sites included the major fishing camps in Sesheke district of the Western Province, namely:

i. Kasaya Malo fishing camp on the Kasaya River, a tributary of the Zambezi River (17°27'33.80"S and 25°00'08.27"E).
ii. Mwanalisa fishing camp on the Zambezi River (17°28'36.38"S and 25°03'28.77"E).
iii. Mwandi fishing camp on the Zambezi River (17°29'07.45"S and 24°19'01.77"E).
iv. Mambova fishing camp on Kalata River, a tributary of the Zambezi River (17°46'35.13"S and 25°14'19.45"E).
v. Katimamulilo in Sesheke on the Zambezi River (17°28'30.18"S and 24°17'49.30"E).
vi. Lipumpu fishing camp on Lwanja River, a tributary of the Zambezi River (17°27'09.99"S and 24°22'55.58"E).
vii. Machile fishing camp on Machile River, a tributary of the Zambezi River (17°27'00.60"S and 24°22'46.61"E).

Fig. 3.1: Map showing the exact location (marked ♦) where sampling was done

3.1.0. Study Design

A purposive (biased) sampling design was employed, where the selection parameter was the lesions on the fish i.e. fish manifesting EUS-like lesions, as well as healthy looking fish of the same species were included in the sample. In most cases, fish was caught using gill nets and seine nets and/or purchased from fishermen in the study area. Individual fish randomly selected from among those with lesions were examined.

The sample size was calculated using **WinEpiScope**, the software for quantitative Veterinary Epidemiology. This study used 95 percent as the Level of Confidence. The minimum prevalence level was put at 5 percent, as observed from results obtained in the

Pathology laboratory from samples previously analysed. Maximum prevalence was 40 percent, coming up with an adjusted sample size, (n), of 270 assuming sampling was done from a population of 1000.

3.2.0. Sampling Procedure

Live or moribund specimens of fish with clinical lesions were sampled. The fish was killed humanely and without undue tissue damage, i.e., by a blow on the head (Kabata 1985). A detailed observation of the gross pathology and clinical signs of each species was critically examined and noted. The fish was then placed on a board with an easily cleaned surface. It was positioned on its right side, head pointing left and with the ventral side to the examiner, to allow for easy physical examination. Water pH and temperature at each sampling location was determined, using a pH-meter and thermometer respectively. Identification of the fish to species level was achieved by following the 'species accounts' as outlined by Paul Skelton (1993). The fish was then counted, followed by a thorough external examination of the fish. The following observations were to be noted: Status of the skin, Condition of the eyes (weather cloudy or hemorrhagic), were there visible pathogens or tufts of fungus (like soggy cotton wool)? Were the fins intact or were they split, eroded or ragged? Were there signs of ulcer healing?

3.3.0. Sample packaging and transportation to the laboratory

After initial examination to determine species, the selected fish was individually wrapped in sterile, extra heavy aluminum foil. Spines on the fish were sheared to minimize punctures in the aluminum foil packaging (Stober 1991). A sample identification label was taped to the outside of each aluminum foil package, each individual fish was placed into a waterproof plastic bag and sealed, and another label was attached to the outside of the plastic bag. Once packaged, the fish samples were cooled on ice immediately. The time the samples were collected and their time of arrival at the laboratory were recorded. Part of the fish was immediately fixed in 10 percent formalin for histopathology.

3.4.0. LABORATORY PROCEDURES

A number of procedures were carefully performed in the laboratory, to arrive at a definitive diagnosis, as well as to isolate the causative agent of the disease in the fish.

3.4.1. Specimen collection

Skin and muscle samples (<1 cm^3), including the leading edge of the lesion and the surrounding tissue were taken. The tissues were immediately fixed in 10 percent buffered formalin. The amount of formalin used was 10 times the volume of the tissue being fixed. Tissues were fixed for 4 to 5 days.

Samples were also collected for isolation of *Aphanomyces invadans* according to the OIE protocol and as described by Fraser *et al.,* (1992) and Willoughby and Roberts (1994).

3.4.2. Histopathology

This included histological processing: dehydration through ascending alcohol grades, clearing in xylene, impregnation with wax (Chinabut and Roberts 1999), cutting at about 5μm, mounting on a glass slide, complete de-waxing and staining in haematoxylin and eosin (Chinabut and Roberts 1999). The tissue sections were then examined under a light microscope at 40, 100, 200 and 400 magnifications.

Staining also included the use of Grocott's modification of Gomori's methenamine silver (GMS stain), to demonstrate the presence of the fungus (Grocott 1955). The grocott-stained tissue sections were then examined under a light microscope at 40, 100, 200 and 400 magnifications.

3.4.3. Preparation of special media for isolation and sporulation of *A. invadans* (Glucose Peptone Media)

The media was prepared according to the procedure described by Willoughby (1994). The media composition included; 3 g/litre glucose (Heigar; Oslo, Norway), 1 g/litre peptone (Oxoid; Hampshire, England), 0.128 g/litre $MgSO_4.7H_2O$, 0.014 g/litre

KH_2PO_4, 0.029 g/litre $CaCl_2.2H_2O$, 2.4 mg/litre $FeCl_3.6H_2O$, 1.8 mg/litre $MnCl_2.4H_2O$, 3.9 mg/litre $CuSO_4.5H_2O$, 0.4 mg/litre $ZnSO_4.7H_2O$ and pond water from the site where moribund and dead fish were collected. This was used at one third of the total volume of the water required. Bacteriological agar (Unipath Ltd; Basingstoke, Hampshire, England) was added at 1 percent. The mixture was then heated until all the ingredients dissolved completely. This was then followed by autoclaving at 121°C for 15 min. When the mixture cooled down to 50°C, 100 units/ml of penicillin-G and 10 µg/ml streptomycin were added to take care of gram positive and gram negative bacteria respectively. Finally, 20 ml of the media was dispensed into Petri dishes, ready for use. The media was stored at 4°C before use.

3.4.4. Isolation and growth of *A. invadans* from internal tissues

The present study used the following method of isolation of *A. invadans*, as described by Fraser *et al.,* (1992) and Willoughby and Roberts (1994). Briefly the growth and isolation of *A. invadans* was done as described below.

The fish was aseptically dissected as shown in figure 3.2. Lesions located on the flank or tails of fish were sampled by cutting the fish in two using a sterile scalpel and slicing a cross-section through the fish at the edge of the lesion. The scalpel was flamed until red hot to sterilize the exposed surface of the muscle. A circular block of muscle (2-4 mm^3) from beneath the lesion was cut and placed on a petri dish of GP media with penicillin-G and streptomycin. Other sample specimens such as the spleen, kidney and liver were also placed on the media (Fig 3.3). The inoculated media was then incubated at approximately 25°C. The plates were checked continuously after inoculation. When there was some growth, part of the fungus was transferred to a new plate. The emerging hyphal tips were repeatedly transferred to plates of GP agar until axenic cultures were obtained. They were then maintained at 10°C on GP agar and sub-cultured at intervals of 6 days. The oomycete isolate was also maintained at 25°C on GY agar (1 percent glucose, 0.25 percent yeast extract and 1.5 percent agar) and transferred to fresh GY agar once every month (Hatai *et al.,* 1977)

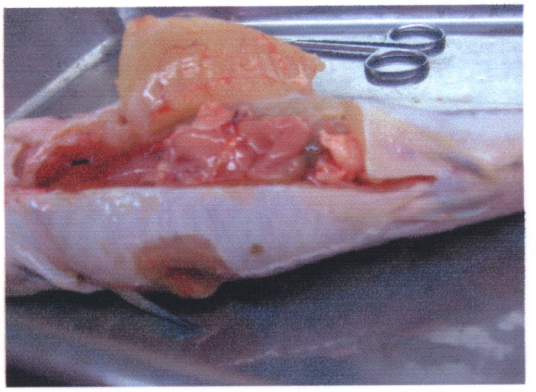


Fig. 3.2: Aseptic dissection of fish that showed gross lesions, so as to collect sections of muscle, spleen, kidney and liver.

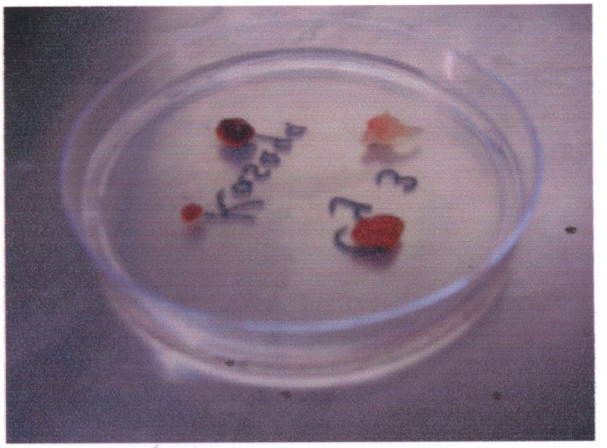


Fig. 3.3: Samples of muscle, spleen, kidney and liver, placed on GP Media, for isolation of *A. invadans*.

3.4.5. Identification of *Aphanomyces invadans* cultures

The induction of asexual reproductive structures is necessary for identifying oomycete cultures as members of the genus *Aphanomyces.* The sporulation was induced as briefly described below.

The agar plugs of 3 mm in diameter resulting from the actively growing mycelium mat was washed by sequential transfer through five Petri dishes containing autoclaved pond water, and was left overnight at 20°C in autoclaved pond water. After about 12 h, the formation of achlyoid clusters of primary cysts and the release of motile secondary zoospores were apparent under the microscope.

3.5.0. Experimental infections of fish

3.5.1. Source of fish for artificial infection

Healthy fingerlings of *Oreochromis andersonii, Barbus paludinosus* and *Oreochromis niloticus* were acquired from the Department of fisheries (Ministry of Agriculture and Cooperatives) in Chilanga. The decision to use *O. niloticus* was influenced by the fact that it was the only one available at the Department of fisheries among the species of fish that have been shown to be EUS-resistant by researchers in other parts of the world. *O. andersonii* and *B. paludinosus* were randomly selected. The age of the fish was taken note of, and recorded upon arrival. 30 fingerlings per species of fish were placed in different aquaria of 100 liters capacity, along with the sediment and gravel, purchased along with the aquaria. The fish were gradually acclimated to a salinity of 7 ppt (pH 8.0) at 23°C to 27°C for 2 weeks prior to use. They were fed once a day. A 25 percent water change was carried out at the end of the first week to maintain proper water chemistry. Care was taken to refill the aquaria with water of the same temperature. After the two-week acclimatization period, five fish were transferred to smaller aquaria for infection and challenge experiments. The fish were challenged in the months of June, July and August when temperatures fall to an average of 20°C. This is because temperatures play an important role in the growth of the fungus.

3.5.2. Preparation of fungal inoculums

Before injection zoospores were counted using a hemacytometer (Neubauer, Buffalo, New York). The medium was concentrated by centrifuging for 15 min at 3,000 *xg* to remove the any excess of it. A 10 µl aliquot was used in a hemacytometer after which the total zoospore estimation per ml was calculated. The viability of each inoculum was confirmed by plating 100 µl on GP media. No growth was observed in controls.

3.5.3. Infection of fish

The fish were slightly anesthetized with azaperone (Tokyo, Japan) had part of their epidermis interrupted and then inoculated with 0.1 ml of zoospore suspension containing about 8 X 10^3 spores using a 27-gauge needle. Each fish was injected on the

flank, just below the dorsal fin and above the lateral line. Control fish were treated in exactly the same manner but received 0.1 ml of sterile pond water as proposed by Kiryu *et al.*, (2002). Sterile pond water was used as it had also served as the diluent for the zoospore suspension. Some fish were inoculated with the fungus without prior interruption of the epidermis. Fish were allowed to recover from the anaesthesia in clean water before being returned to their aquaria. No water changes took place in the infection tanks, and the quantity of feed was increased. This resulted in excessive algae growth and deteriorating water quality, creating very stressful conditions for the fish. The fish were then monitored daily, for 4 weeks, for signs of morbidity, lesions and mortality. All dead or moribund fish were promptly removed from the tank and processed for histopathology and microbiology. In addition, one fish was removed from each of the experimental tanks and processed for histopathology every week, to allow for the monitoring of sequential histopathology. At the end of every 4 week period any fish surviving from the first group of 5 were replaced by 5 freshly inoculated subjects. Ultimately, a total of 15 fish were challenged per species.

Following infection, the experimental tank was monitored and the fish were fed daily until experiment termination. All dead or moribund fish were removed and processed by use of routine diagnostic techniques. Representative photographs of gross external conditions were taken. Skin, skeletal muscle, posterior kidney, liver and spleen scrapings of moribund fish were examined for the presence of fungal hyphae. Fungal isolation was also attempted by using the methods of Lilley *et al.*, (1998). All inoculation experiments were terminated between 28 and 32 days post-injection.

Ulcerated skin and muscle samples (1 cm^3) or tissue samples from the area of injection were fixed in 10 percent neutral buffered formalin, dehydrated in a graded ethanol series, and embedded in paraffin wax. Additionally, gill, liver, spleen, and posterior kidney tissues from challenged fish were examined histologically. Histological sections were cut to a thickness of 5 μm on a microtome and then stained routinely with hematoxylin and eosin. Stained slides were then examined for the presence of fungal hyphae as earlier described. For confirmation of fungal growth in the experimentally

hyphae as earlier described. For confirmation of fungal growth in the experimentally infected fish, *A. invadans* isolation was done following the procedure described in section 3.4.4.

3.5.4. Data analysis

The results were analysed as positive following the determination of lesions as being caused by *A. invadans*. The percent positives were calculated as follows:

$$\% \text{ positives} = r/n \times 100$$

Where percent positive is the percentage of fish exhibiting lesions or culturing positive for *A. invadans*.

r is the total number of positive samples.

n is the total sample population.

CHAPTER FOUR

RESULTS

4.0.0. Field Observations

A total of two hundred and seventy (270) fish belonging to sixteen species were sampled during the study period. These species were; *Clarias ngamensis, C. gariepinus, Barbus poechii, Tilapia sparmanii, Seranochromis angusticeps, Brycinus lateralis, Micralestes acutidens, Sargochromis carlottae, Hydrocinus vittatus, Phryngochromis acuticeps, Schilbe intermedius, Hepsetus odoe, Labeo lunatus, Oreochromis andersonii, Barbus unitaeniatus* and *Barbus paludinosus.* The fish species were examined from the seven sampling points of Sesheke district. At each sampling point the temperature and pH were recorded as indicated in Table 1. Furthermore, the water turbidity was noted. In some areas the water was very turbid. It was full of organic debris and algae blooms.

4.1.0 Water quality parameters and fish species by sampling site

Of the 270 fish samples collected, 156 (57.8 percent) showed gross lesions. From the various sites, occurrence of lesions varied from 45 percent to 64.7 percent. *Clarias gariepinus* was the most abundant, representing 20.4 percent. *Brycinus lateralis, Micralestes acutidens* and *Hydrocinus vittatus* on the other hand, accounted for the least numbers at only 2.2 percent of the sample size while 57.8 percent of the whole catch showed lesions grossly. The fact that most of the fish were caught in lagoons may support the assumption that the water quality parameters at the sampling sites had an effect on the fish.

4.2.1. Prevalence by clinical or gross examination

Of the 270 fish samples, 156 (57.8 percent) showed gross lesions and occurrence of these lesions at the various sampling sites varied from 45.0 percent to 64.7 percent. A total of 56 fish samples were examined at Mambova, representing the highest, followed by Machile with 40 fish. However, Katimamulilo, in spite of recording much fewer samples than the above-mentioned two locations, recorded the highest percentage of

fish showing gross lesions (64.7 percent). In fact, the lowest percentage of affected fish was found at Machile (45.0 percent). Mwandi had the lowest number of fish but a higher percentage of affected fish than Kasaya-Malo and Machile. Lipumpu had fewer fish samples examined than Mwanalisa but a higher percentage of affected fish.

Table 1: Temperature and pH measurements for each Location

Fishing camp	**Fish species sampled at each location**	**Water pH**	**Temperature (^{o}C)**
Kasaya Malo	*Hepsetus odoe, Labeo lunatus, Oreochromis andersonii*	6.0	24
Mwanalisa	*Clarias ngamensis, Serranochromis angusticeps, Pharyngochromis acuticeps*	6.2	24
Mwandi	*Claria gariepinus, Barbus poechii*	6.1	24
Mambova	*Hydrocinus vittatus, Schilbe intermedius, Barbus unitaniatus*	6.5	24
Katimamulilo	*Barbus poechii, Serranochromis angusticeps, Labeo lunatus, Barbus paludinosus*	6.6	24
Lipumpu	*Barbus poechii, Tilapia sparmanii, Serranochromis angusticeps, Brycinus lateralis*	6.3	24
Machile	*Clarias gariepinus, Micralestes acutidens, Sargochromis carlottae*	6.1	24
Mean		6.2	24

Table 2: Fish examined at each sampling site

Sampling site	No. of fish examined	No. of fish showing lesions	% affected
Kasaya-Malo	33	17	51.5
Mwanalisa	39	23	59.0
Mwandi	32	18	56.2
Mambova	56	35	62.5
Katimamulilo	34	22	64.7
Lipumpu	36	23	63.9
Machile	40	18	45.0
Total	270	156	57.8

Clarias ngamensis, the next most abundant species only accounted for 14.1 percent of the total sample size. Although *Tilapia sparmanii* represented 7.0 percent of the sample, none of the fish belonging to this species showed any gross lesions. Among the *Barbus* species, *Barbus paludinosus* had the lowest percentage of fish showing lesions, at 3.3 percent of the total sample size. A detailed analysis on the frequency of fish samples exhibiting gross lesions in the field per species is shown in Table 3.

A detailed observation on the gross pathology and clinical signs of each species was critically examined and noted. Pictures of some of gross lesions in some of the fish species follow below, while Table 4 shows a description of gross pathology and clinical signs of all the fish species sampled from the field.

a) *Barbus* species

There were 3 *Barbus* species, namely *B. paludinosus, B. poechii* and *B. unitaeniatus*, accounting for 22.2 percent of the total examination. Most of the fish belonging to this species generally showed very mild lesions, presenting only as petechial hemorrhages. A few showed more advanced lesions, ranging from small circular wounds to large necrotic ulcers. The sores were noticed on different parts of the body such as head, tails

and the mid section. The most interesting observation in this group was that the fish continued to swim very actively, even in cases where they had very advanced lesions (Fig. 4.1).

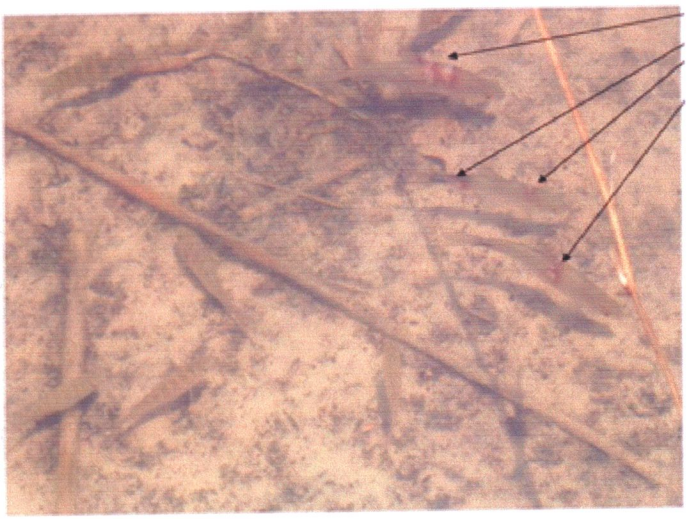


Fig. 4.1: *Barbus paludinosus* showing gross lesions (arrows), but still able to swim actively

b) *Serranochromis angusticeps*

12 fish of this species were analysed with 66.7 percent showing lesions. This species of fish was mostly found to have very advanced lesions, ranging from large, circular bruises (Fig. 4.2a) to extensive ulcers. In some cases, the infection could even lead to necrosis of part of the dorsal fin (Fig. 4.2b). Worm-like parasites were observed in advanced lesions, leaving deep holes owing to the fact that the parasites burrowed deeply into the muscle of the infected fish (Fig. 4.3).

Table 3: Frequency of fish samples exhibiting gross lesions in the field per species

Species		No. examined	No. showing Lesions grossly	% affected within species	% examined out of total sample
1	*Serranochromis angusticeps*	12	8	66.7	4.4
2	*Barbus unitaeniatus*	20	12	60.0	7.4
3	*Barbus paludinosus*	18	9	50.0	6.7
4	*Barbus poechii*	22	13	59.1	8.1
5	*Brycinus lateralis*	6	2	33.3	2.2
6	*Clarius gariepinus*	55	47	85.4	20.4
7	*Micralestes acutidens*	6	3	50.0	2.2
8	*Sargochromis carlottae*	13	5	38.5	4.8
9	*Hydrocinus vittatus*	6	4	66.7	2.2
10	*Tilapia sparmanii*	19	0	0.0	0.0
11	*Pharyngochromis acuticeps*	11	6	54.6	4.1
12	*Hepsetus odoe*	9	4	44.4	3.3
13	*Labeo lunatus*	7	4	57.1	2.6
14	*Oreochromis andersonii*	20	14	70.0	7.4
15	*Clarius ngamensis*	38	23	60.5	14.1
16	*Schilbe intermedius*	8	2	25.0	3.0
	TOTAL NUMBER	**270**	**156**	57.8	

a. b.

Fig. 4.2: *Serranochromis angusticeps* showing an ulcerated lesion (arrow) while (b) shows where the infection led to loss of the dorsal fin part. The ulcer involved the skin and the underlying lateral musculature.

Fig. 4.3: Deep hole from where a parasite was removed (arrow)

c) *Clarius gariepinus* and *Clarius ngamensis*

A total of 91 catfish were examined, with 70 exhibiting lesions grossly. In catfish, the primary lesion was a simple blister, which progressed into a more bruise-like lesion in the secondary stage. In the later stages, the infection progressed into a sore. Big, round ulcers, like those shown in Figs. 4.4 and 4.5, were common findings in *Clarias gariepinus*. Sometimes the infection was so severe that the caudal fin would almost be severed off (Fig. 4.6). In some cases, advanced ulcerous lesions exhibited a zone of

superficial tissue necrosis that contained abundant saprophytic fungal contaminants. The lesions were also apparent as fuzzy outgrowths.

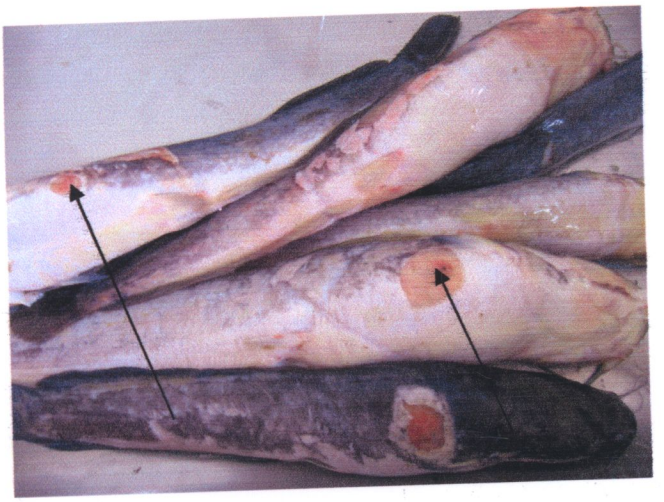


Fig. 4.4: Progression of the lesion from a simple blister to a sore

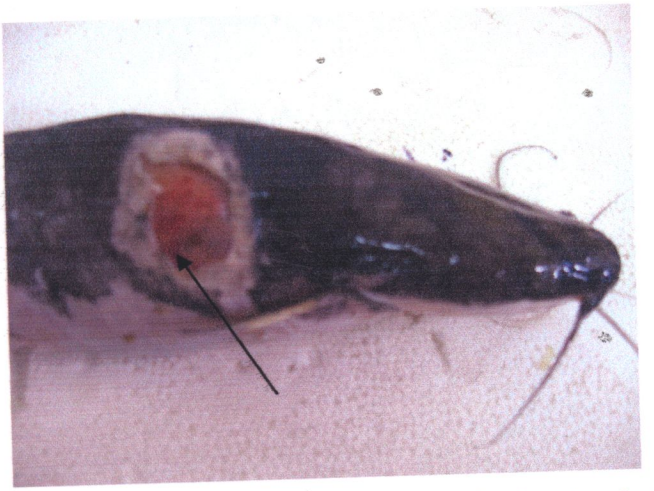
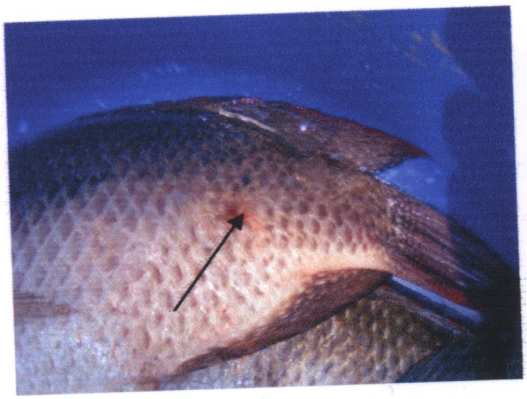


Fig. 4.5: Catfish showing a severely ulcerated lesion on the dorsal surface

Fig. 4.6: Catfish exhibiting partial mutilation of the caudal fin (Arrow)

d) *Sargochromis carlottae*

13 fish of this species were analysed with 5 showing gross lesions. This species of fish mostly showed brightly red-colored lesions, presenting as hemorrhages whose shape varied from pinpoint, circular lesions (Fig. 4.7) to extensive ulcers, covering large areas of the fish body (Fig. 4.8). The characteristic skin lesions in *S. carlottae* were located on different positions of the trunk.



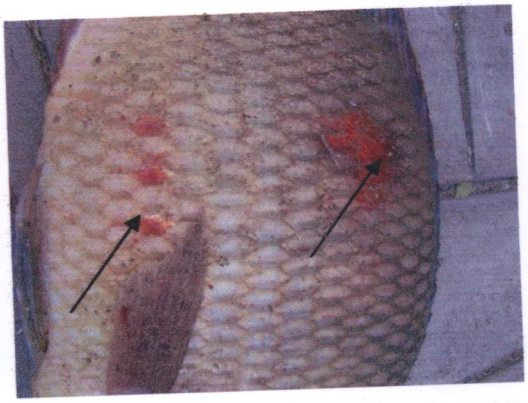
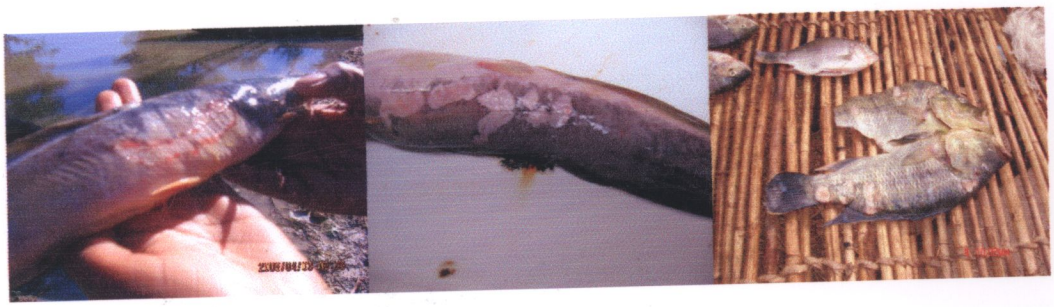


Fig. 4.7 & Fig. 4.8 respectively: ***S. carlottae*** **showing ulcerated lesions near the tail and the flank** respectively.

Some of the fish that were caught in areas of fast-running, deeper waters were actually showing signs of healing, as observed (Fig 4.9 a, b & c).



a. b. c.

Fig. 4.9: Signs of healing were observed in these fish. a. *Clarias gariepinus*; b. *Clarias ngamensis;* c. *Sargochromis. carlottae*

e) Other species

The other fish species generally exhibited similar clinical and gross lesions as described above. The gross pathology and clinical signs per species are summarized in Table 4.

Table 4: Gross pathology and clinical signs per species

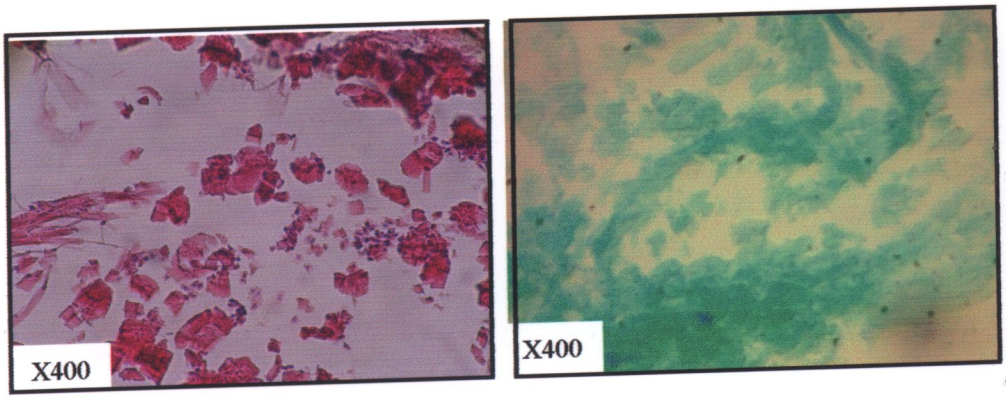
SPECIES	CLINICAL SIGNS
Barbus paludinosus	Localized swelling of the body surface, petechial hemorrhages and skin erosion. In more advanced cases, the center of the lesion was necrotic.
Serranochromis. Angusticeps	Protruding scales, hemorrhage, scale loss, skin disintegration, exposure of underlying musculature and ulceration. Ulcers spread over a broad area and developed into a wide ulcer. Very advanced lesions, ranging from large, circular bruises to extensive ulcers and necrosis of part of the fins.
Clarias gariepinus	Initial stage characterized by localized hemorrhages, advanced stage showing degeneration of epidermal tissue and ulceration, final stage characterized by deep and large ulcers on various parts of the body.

Table 4: Continued

SPECIES	CLINICAL SIGNS
Clarias ngamensis	Simple blister, progressing into a more bruise-like lesion in the secondary stage. In the later stages, the infection progressed into a sore. Big, round ulcers, large, hemorrhagic dermatitis near the caudal peduncle
Sargochromis carlottae	In the initial stages, the disease was characterized by tiny red spots on the skin surface, which gradually grew in size until finally a circular to oval deep hemorrhagic ulcer exposing the skeletal musculature was visible.
Tilapia sparmanii	No gross lesions observed.
Hydrocinus vittatus	Red spots, blackish burn-like marks, deeper ulcers with red centers and white rims.
Pharyngochromis acuticeps	Large hemorrhagic dermatitis.
Hepsetus odoe	Hemorrhagic ulcerative lesions on the body with varying degrees of necrosis.
Labeo lunatus	Small area of reddening (red spot), large ulcer, sometimes extending into abdominal cavity, exposed skeletal muscle due to missing overlying skin and scales.
Oreochromis andersonii	This fish species showed more advanced lesions, ranging from small circular wounds to large necrotic ulcers.
Barbus poechii	Fish exhibited large hemorrhagic dermatitis in the area immediately posterior to anus and towards the caudal peduncle. The lesion was covered with fungal-like mycelia.

Fig. 4.10: Muscle tissue stained with H&E (a) and Grocotts'silver stain (b). Granulomatous lesion and enclosed branching hyphae (arrows) in the lateral musculature, in which muscle fibers have been fragmented and liquefied and replaced with granulation tissue

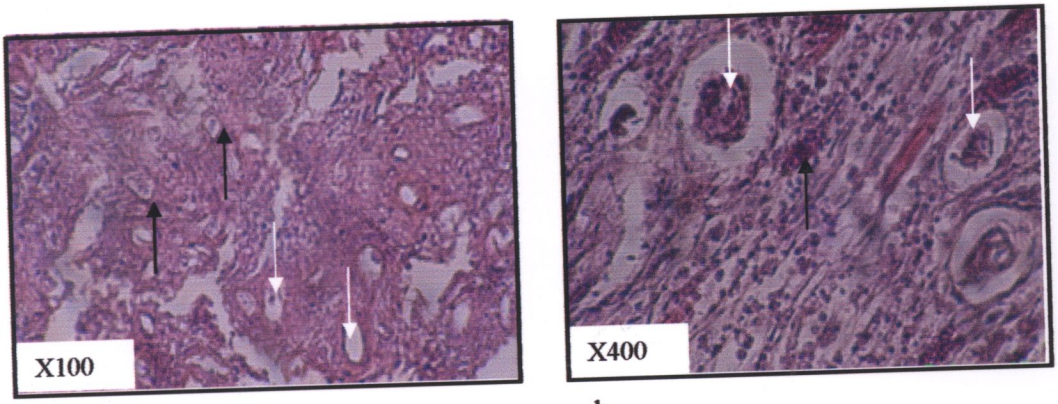
Some lesions were also observed that were not associated with granulomatous inflammation, as shown in Figs. 4.11 a and b.



a b

Fig. 4.11: Lesions not associated with granulonatous inflammation. (a) H &E, (b) Grocotti.

In other cases, granulomas were well organized and composed largely of macrophages. Fungal hyphae were readily discerned within granulomas in tissue sections stained routinely with H & E (Figs. 4.12a & b).



a b

Fig. 4.12: Detail of granulomatous lesions (white arrows) with hyphae (black arrows) in the lateral musculature. Invaded hyphae are enclosed within granulomas (H & E). (b). Granulomatous lesions surrounded by macrophages (black arrow). The granulomas at the centre containing fungal hyphae (white arrows).

Fig. 4.10: Muscle tissue stained with H&E (a) and Grocotts'silver stain (b). Granulomatous lesion and enclosed branching hyphae (arrows) in the lateral musculature, in which muscle fibers have been fragmented and liquefied and replaced with granulation tissue

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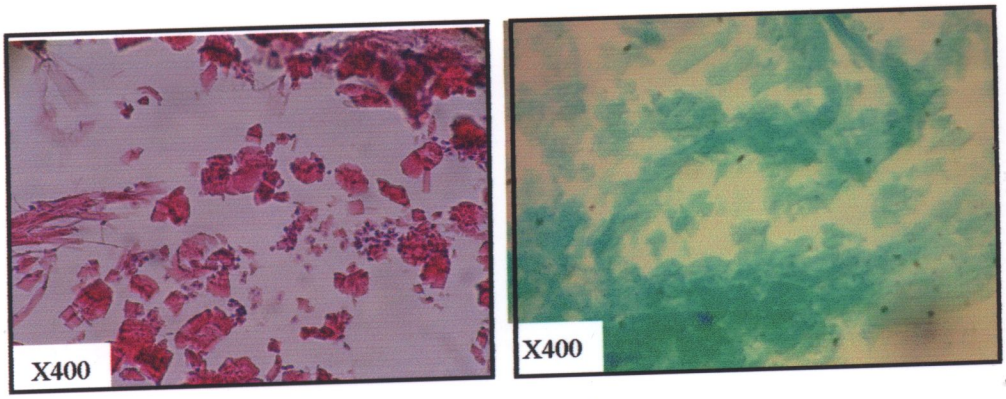

a b

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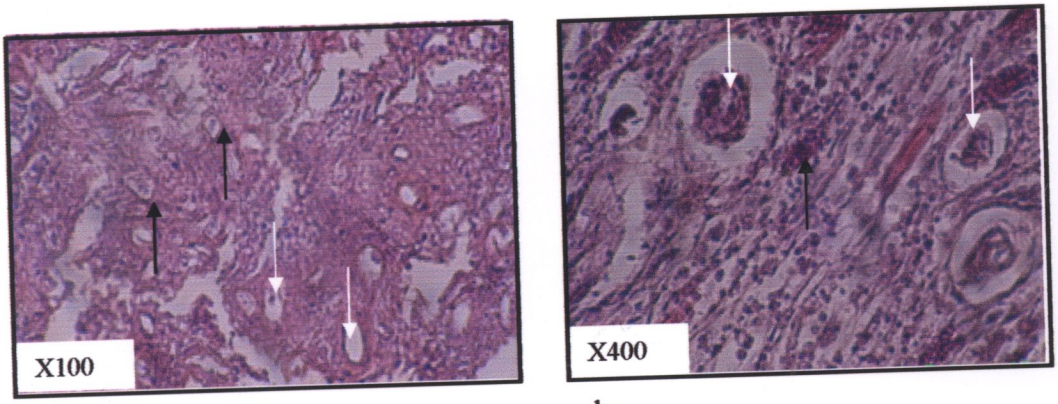

a b

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b) Specific Observations

In young catfish (3 months old) which were found floating on the surface gasping, or even already dead with a highly distinctive dark red to brown lesion on the flank, a severe invasive myositis was evident (Fig. 4.13). Very delicate, sparsely located fungal hyphae of varying diameter could also be seen in areas of severe myonecrosis. Virtually no host inflammatory response was noted in such cases.

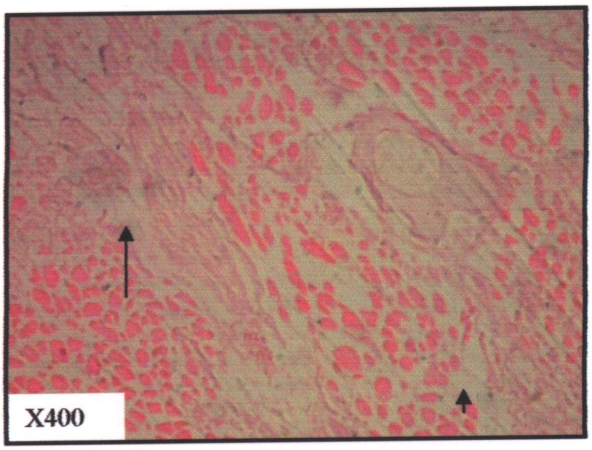

Fig. 4.13: Young *C. gariepinus* (3 months old) early peracute infection demonstrating fungal hyphae (arrows) within small areas of necrosis (H & E).

In older catfish which managed to survive the per-acute phase but did not survive long enough for more chronic and extensive lesions to develop, a slightly different picture was observed. This was characterized by an active inflammatory response, where an organized tube of macrophages encased the growing fungus as it extended through the tissues. In transverse section they appeared as small granulomas with a clear centre within which is located a section of deformed and apparently dead mycelium (Fig. 4.14).

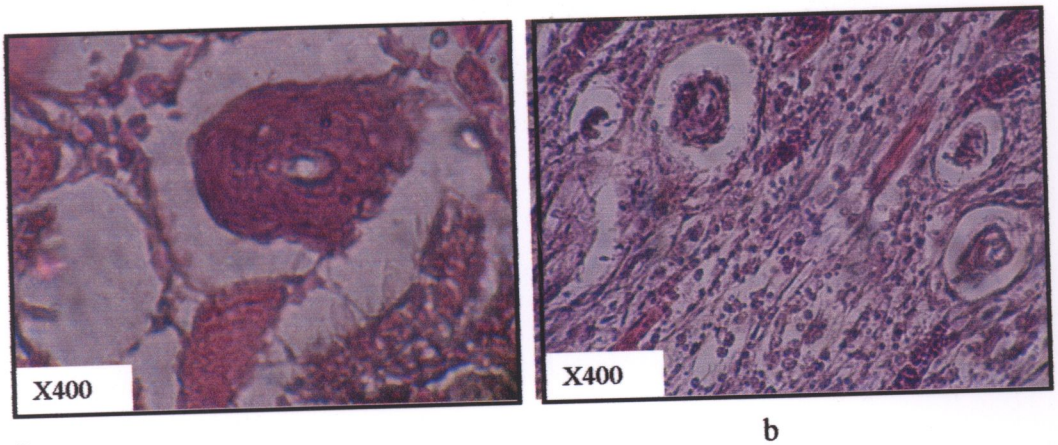

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Fig. 4.14: a) Muscle of mature *C. gariepinus* showing enveloping fungal hyphae and b) an inflammatory focus of macrophages around the hyphae (H & E).

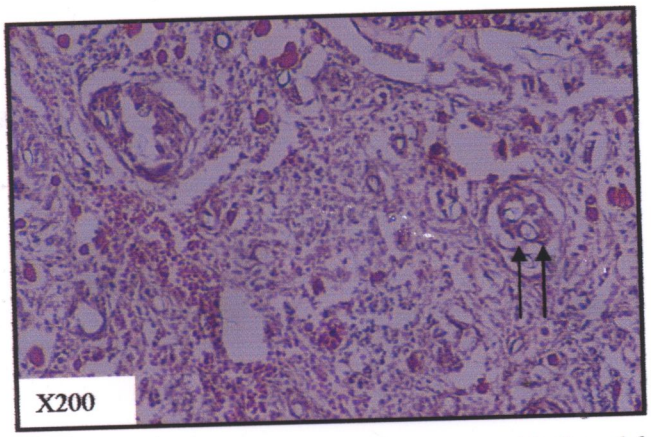
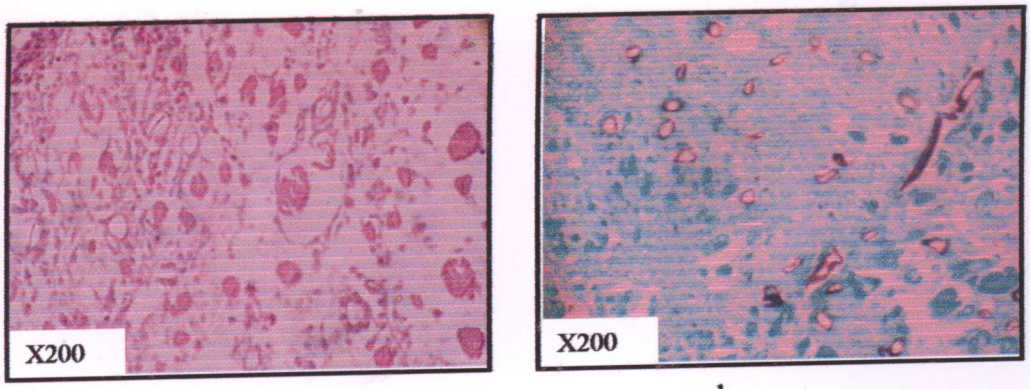


Fig. 4.15: Kidney of older *C. gariepinus*, with fungal hyphae growing along renal tubule of kidney. Granulomatous lesions in the kidney. Granulomas enclose hyphae penetrated into the kidney with severe renal vacuolation due to necrosis (H & E).

On histopathology, one fish belonging to the species *S. carlottae* showed an area of extensive hyperaemia and inflammation with fungal hyphae surrounded by macrophages. In this case, the granulation tissue was thick, condensed and the dead fungal hyphae appeared as empty outline shapes within the granuloma (Figs. 4.16 a & b).



a b

Fig. 4.16: Note that the fungus is well encapsulated appearing, as empty outline shapes within the granulomata, in both H&E (a) and Grocott sections (b).

A summary of the histopathology results in some selected individual specimens are shown in Table 5, with the locations where samples were obtained.

Table 5: Histopathology results in some selected individual specimens

FISHING CAMP	FISH SPECIES	HISTOPATHOLOGICAL FINDINGS
Kasaya-Malo	*Hepsetus odoe*	Muscular degeneration, localised inflammatory reaction in the skin.
	Oreochromis andersonii	Moderate muscular necrosis and inflammatory reaction, early granulomas, haemorrhage.
Mwandi	*Barbus poechii*	Desquamation of the epithelium and mild inflammation, no granulomas observed.
	Clarius gariepinus	Dermatitis, haemorrhages, early epidermal granulomas.

Table 5: Continued

FISHING CAMP	FISH SPECIES	HISTOPATHOLOGICAL FINDINGS
Katimamulilo	*Barbus poechii*	Severe muscular necrosis, severe inflammatory reaction, granulomas with well circumscribed hollow centers with central mass.
	Seranochromis angusticeps	Moderate muscle necrosis, mild inflammatory reaction, small diffuse (spaced out) granulomatous lesions.
Mwanalisa	*Seranochromis angusticeps*	Severe muscle necrosis, islands of inflammatory cell aggregates in vacuoles a non-granulomatous reaction.
Lipumpu	*Seranochromis angusticeps*	Severe muscular necrosis, severe inflammatory cell reaction (mixed populations), typical granulomas.
	Barbus poechii	Severe muscular necrosis, severe cellular infiltration, abundant granulomatous centers, well circumscribed.
Mambova	*Schilbe intermedius*	Severe muscular necrosis, islands of inflammatory cell aggregates in vacuoles, a non-granulomatous reaction.
	Hydrocinus vittatus	Epithelial desquamation, severe muscular necrosis, severe inflammation, abundant granulomatous lesions.
Machile	*Clarias gariepinus*	Muscular necrosis, vacuolation, severe inflammatory reaction, severe cellular reaction, a few granulomatous lesions.
	Sargochromis carlottae	Severe muscular necrosis with formation of vacuoles, hyaline degeneration, severe inflammatory reaction, typical mycotic granulomas affecting both muscle and skin.

4.3.0. Isolation of *A. invadans*

A. invadans was successfully isolated on Glucose-Peptone Media by culturing from spleen, kidney, liver and muscle (Fig. 4.17). Mycelial colonies were detected inside the media 5 days after inoculation. Furthermore, the fungus was washed in sterile pond water (Fig. 4.18) for zoospore sporulation (Fig. 4.19).

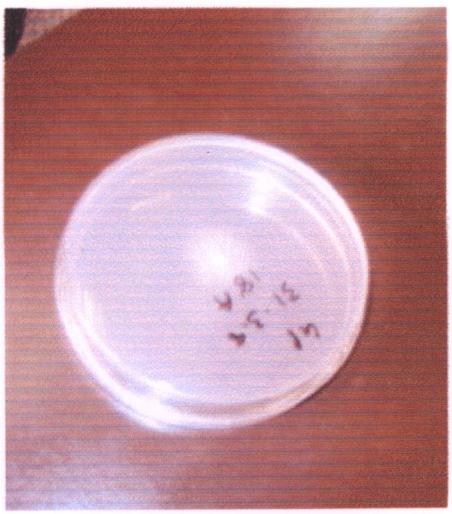


Fig. 4.17: A pure culture of *A. invadans* was isolated after repeated transfer of emerging hyphal tips through many plates of GP Media

Fig. 4.18: Small tubes containing fungus that was washed using sterile pond water.

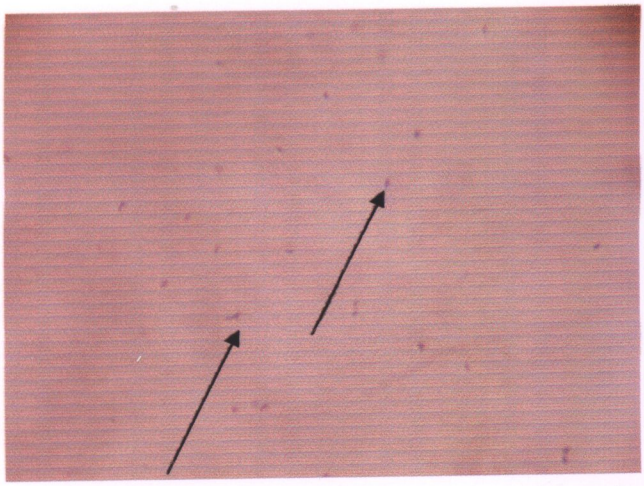


Fig. 4.19: *A. invadans*, rendered as motile zoospores (arrows) after incubation at temperatures between 18°C and 20°C for 24 hours. Ready for use in the Bio-toxicity assay.

4.4.0. Pathogenicity of *A. invadans*

a) Gross Pathology, Clinical Signs and Light Microscopy

Of the three species subjected to challenge experiments, only *Barbus Paludinosus* and *Oreochromis andersonii* were observed to be sensitive to *A. invadans*. *Oroechromis niloticus* proved to be resistant to the fungus.

A thorough examination of the fish that was inoculated without any compromise to the epithelium neither developed ulcers, even around the site of inoculation, nor displayed any significant pathological changes. The same was true for the EUS-resistant species, *Oreochromis niloticus*, which did not develop gross skin ulcers (Fig. 4.20); rather, their lesions appeared simply as reddened areas under the epidermis.

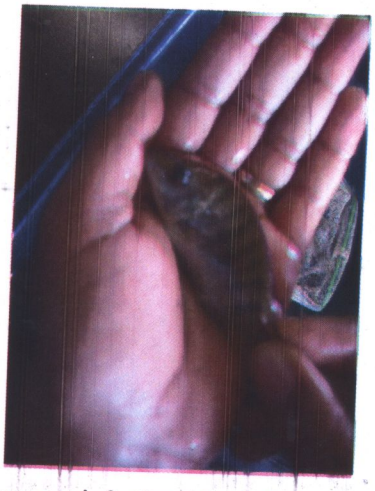


Fig. 4.20: *O. niloticus*, 32 days post-infection. No visible lesions on the dermis.

In the EUS-susceptible species (*O. andersonii* and *Barbus paludinosus*) gross pathological lesions were quite evident (Fig. 4.21 and 4.22).

Fig. 4.21; *O. andersoni*, showing a deep reddish-brown ulcer, which was seen to develop within seven days post-inoculation.

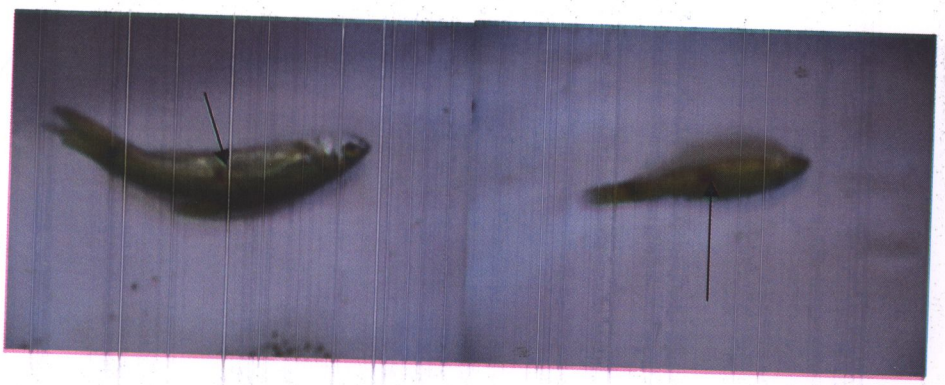


Fig. 4.22: *B. paludinosus* exhibiting typical EUS-like dermal ulceration

The control, inoculated with sterile pond water, did not show any clinical signs grossly (Fig. 4.23, white arrow).

Fig. 4.23; Two experimental fish (black arrows) showed gross lesions; where as the control (red arrow) did not develop any lesions at all.

One interesting observation in *B. paludinosus* was that the fish started showing signs of healing as atmospheric (and hence aquarium water) temperatures started rising, from early September onwards. Both specimens of fish had their wounds completely disappear by the middle of October (Fig. 4.24).

Fig.4.24: *Barbus paludinosus* recovered from EUS. The skin lesions disappeared completely.

Fig. 4.20: *O. niloticus*, 32 days post-infection. No visible lesions on the dermis.

In the EUS-susceptible species (*O. andersonii* and *Barbus paludinosus*) gross pathological lesions were quite evident (Fig. 4.21 and 4.22).

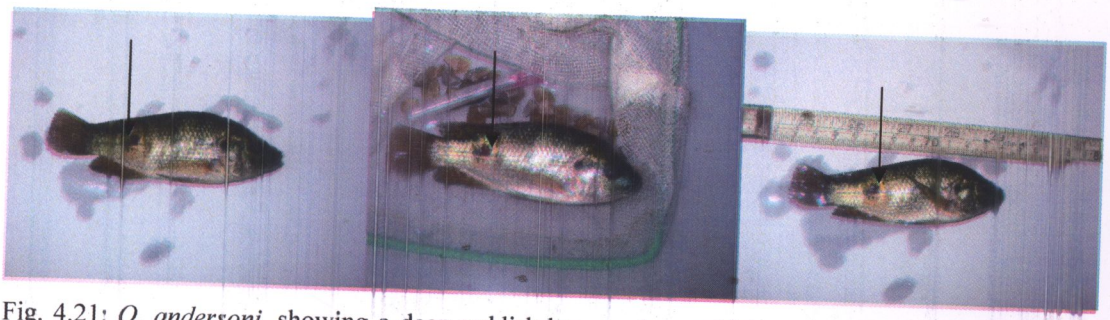


Fig. 4.21; *O. andersoni*, showing a deep reddish-brown ulcer, which was seen to develop within seven days post-inoculation.

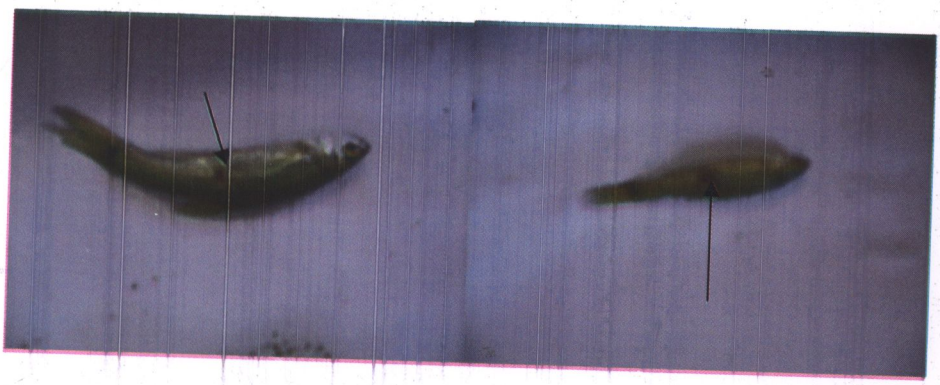


Fig. 4.22; *B. paludinosus* exhibiting typical EUS-like dermal ulceration

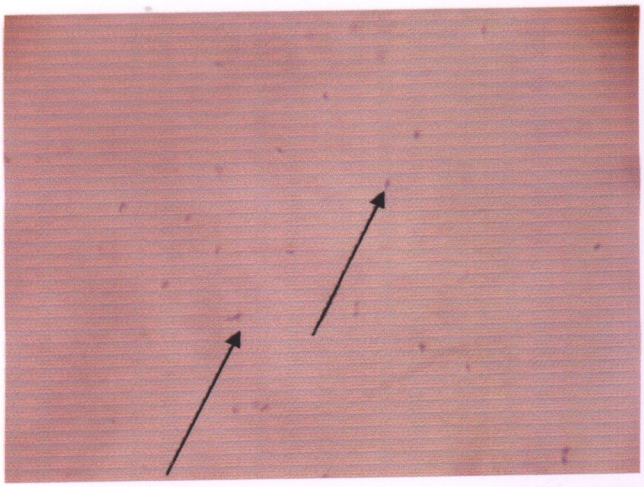


Fig. 4.19: *A. invadans*, rendered as motile zoospores (arrows) after incubation at temperatures between 18°C and 20°C for 24 hours. Ready for use in the Bio-toxicity assay.

4.4.0. Pathogenicity of *A. invadans*

a) Gross Pathology, Clinical Signs and Light Microscopy

Of the three species subjected to challenge experiments, only *Barbus Paludinosus* and *Oreochromis andersonii* were observed to be sensitive to *A. invadans*. *Oroechromis niloticus* proved to be resistant to the fungus.

A thorough examination of the fish that was inoculated without any compromise to the epithelium neither developed ulcers, even around the site of inoculation, nor displayed any significant pathological changes. The same was true for the EUS-resistant species, *Oreochromis niloticus*, which did not develop gross skin ulcers (Fig. 4.20); rather, their lesions appeared simply as reddened areas under the epidermis.

Fig. 4.20: *O. niloticus*, 32 days post-infection. No visible lesions on the dermis.

In the EUS-susceptible species (*O. andersonii* and *Barbus paludinosus*) gross pathological lesions were quite evident (Fig. 4.21 and 4.22).

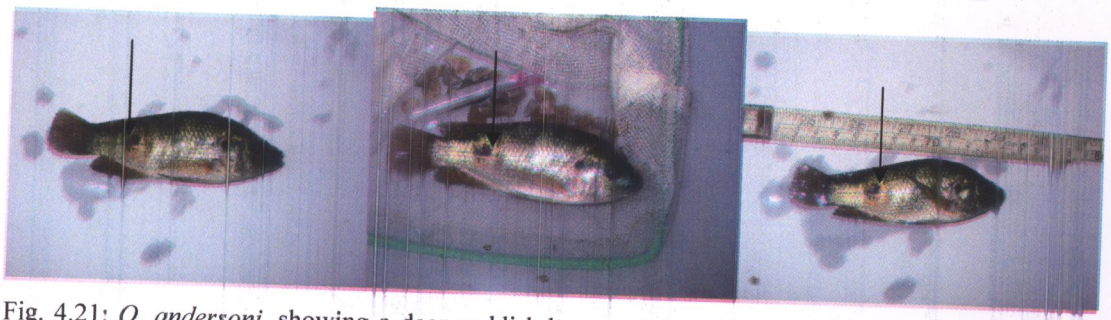


Fig. 4.21; *O. andersoni*, showing a deep reddish-brown ulcer, which was seen to develop within seven days post-inoculation.

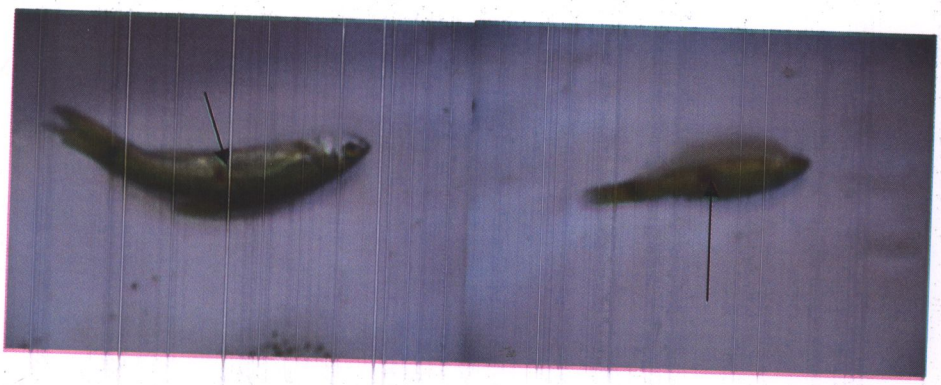


Fig. 4.22: *B. paludinosus* exhibiting typical EUS-like dermal ulceration

The control, inoculated with sterile pond water, did not show any clinical signs grossly (Fig. 4.23, white arrow).

Fig. 4.23; Two experimental fish (black arrows) showed gross lesions; where as the control (red arrow) did not develop any lesions at all.

One interesting observation in *B. paludinosus* was that the fish started showing signs of healing as atmospheric (and hence aquarium water) temperatures started rising, from early September onwards. Both specimens of fish had their wounds completely disappear by the middle of October (Fig. 4.24).

Fig.4.24: *Barbus paludinosus* recovered from EUS. The skin lesions disappeared completely.

b) Histopathology and Clinical Pathology

Microscopic examination of muscle, kidney, spleen, liver and gills from the fish that was challenged without any compromise to the epidermis showed only evidence of an inflammatory reaction, but definitely no fungal granulomas. A similar picture was observed in the muscle of the EUS-resistant *O. niloticus* (Fig. 4.25).

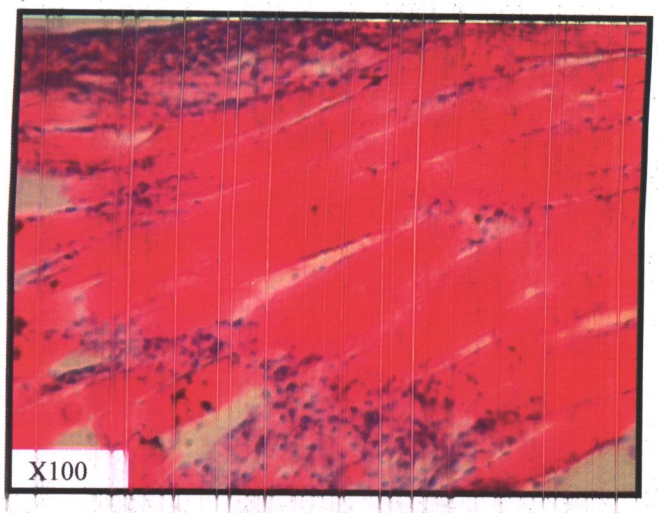


Fig. 4.25: Focus of bacterial infection at site of inoculation in muscle of EUS-resistant *O. niloticus* (H&E).

Microscopic examination of tissues from *O. andersonii* did show evidence of fungal hyphae and granulomas, typical of EUS (Fig. 4.26).

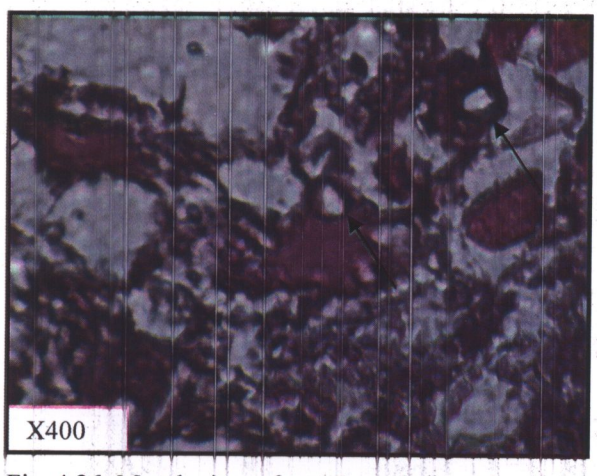


Fig. 4.26: Muscle tissue from *O. andersonii* found dead at day 15 post-infection (H & E).

Similarly, histopathology of a *B. paludinosus* fish that was sacrificed at day 17 post-infection, when the gross lesion was about 2 cm in diameter, revealed pathological features associated with the growth and invasion of fungal hyphae. Skeletal muscle was characterized by vacuolation, granulomatous inflammation, necrotic tissue debris, and the presence of inflammatory cells (Fig. 4.27).

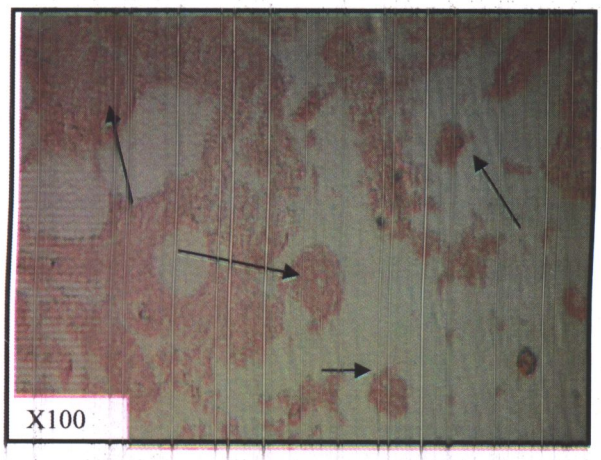


Fig. 4.27: Severe mycotic granulomas (arrows) in a muscle lesion from site of inoculation in *O. andersonii* found dead at day 15 post-infection (H & E). Note the fungal granulomas are quite evident.

Histopathology of the muscle at site of inoculation of *B. paludinosus* control fish showed a muscular necrosis, but no granulomatous inflammation (Fig. 4.28).

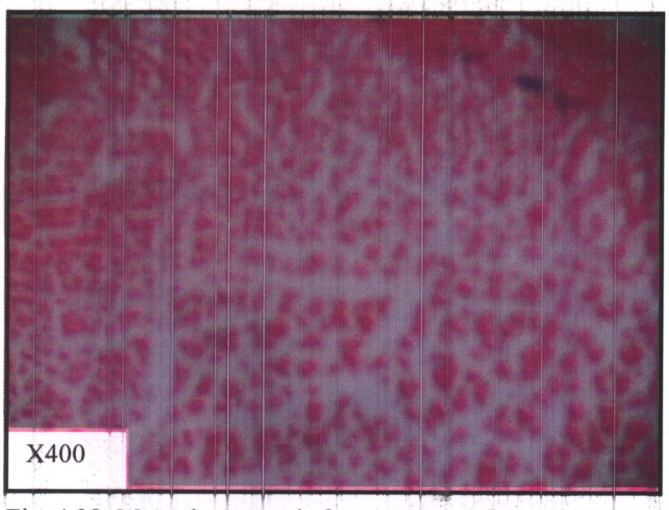


Fig. 4.28: Muscular necrosis from a control fish not exhibiting granulomatous inflammation. (H & E)

C) Isolation of *A. invadans* from the challenged fish

A. invadans was successfully re-isolated from the challenged fish that was susceptible after 15 days (Table 6). It was recovered from the muscles, as well as the kidneys and livers where the lesion had extended. The resistant fish species and controls did not have any fungal recoveries.

Table 6: Culture results from challenged fish species

Fish species and total number of fish (n=15)	**% of tissues yielding fungi on culture (n +ve)**			
	Muscle	**Liver**	**Spleen**	**Kidney**
Oreochromis niloticus	0	0	0	0
Oreochromis andersonii	93.3 (14)	66.7 (10)	0	73.3 (11)
Barbus paludinosus	80.0 (12)	0	0	60.0 (9)

CHAPTER FIVE

DISCUSSION

5.1.0. Fish Species Susceptibility

Some of the most advanced gross lesions in fish from the wild were observed in *Clarias gariepinus* and *C. ngamensis*. In this study variations among the *Clarias* species were clearly observed in spite of the study not really targeting species variations. In this case, the results revealed that *Clarias gariepinus* had more advanced lesions than *Clarias ngamensis*. Lio-Po *et al.,* (2000) mentioned *Clarias* species among those that get severely affected by EUS. One school of thought could be the fact that fish belonging to this genus survived long enough in the lagoons with very poor water quality, for lesions to have reached the advanced stages that they did. Although the water quality requirements of these fish are not fully known, it is noteworthy that the species can tolerate more unfavorable conditions than carps and other cultured species (Anonymous 1992). The other reason could be their skin anatomical arrangement. The fact that these species of fish have no scales probably makes it very easy for abrasion to occur whenever they are exposed to physical or chemical trauma. This may facilitate attraction of and infection by, zoospores of *A. invadans* (Kiryu and Shields unpublished data). The deep holes that were observed in catfish with worm-like parasites could be attributed to the fact that the parasites burrowed deeply into the muscle of the infected fish. Among the fish species collected from the field and closely scrutinized in the present study, *Tilapia sparmanii* was the only one that did not show any lesions. This may have been simply because none of the fish belonging to this species were infected in those particular sampling sites. However, the possibility that the *T. sparmanii* sampled from the Zambezi river basin in Sesheke district was resistant to infection with *A. invadans* can not be ruled out. That would be in line with studies by researchers in Japan (Hatai *et al.,* 1977), who have shown that some important culture species including tilapia, milk fish and Chinese carp are resistant to EUS.

One very important observation during the laboratory exposure was the complete failure of the experiment to reproduce EUS infection in *O. niloticus*. This is consistent with the findings in Bangladesh by Ahmed and Rab (1995), who, during their study, noted that where as ponds with some other species of fish (Thai silver barb, *Puntius gonionotus* (Bleeker) were severely affected (64.0 percent) by EUS, all *O. niloticus* monoculture ponds remained unaffected. In the Philippines, Yambot (1998) observed that although most freshwater fish species in the country have been affected by the Epizootic Ulcerative Syndrome (EUS), the *O. niloticus* remains resistant to the disease. This is an important finding for Zambia which is trying to promote and expand its aquaculture industry. Aquaculture would help to substantially alleviate the pressure on the agriculture sector. Thus, *O. niloticus* should be grown where EUS threat is apparent.

The other two fish species (*Barbus paludinosus* and *Oreochromis andersonii*) that were experimentally infected in the laboratory developed lesions within a few days post-inoculation, indicating that they are susceptible to infection with *A. invadans*. Infected *B. paludinosus* continued to swim actively, even when their gross lesions were quite advanced. On the other hand, *O. andersonii* even became moribund as the infection progressed. It may, therefore, be suggested that species susceptibility and response to infection with EUS may vary depending on various factors, among them species. In spite of the afore-mentioned findings of this study, however, note should be taken that researchers in other parts of the world noted that clinical signs of the affected fish species were the same (Chinabut 1998).

More than 100 fish species have been reported to be affected by EUS (Lilley *et al.*, 1992), but only relatively few reports have been confirmed by demonstrating the presence of mycotic granulomas in histological sections or by isolation of the pathogenic *Aphanomyces* fungus from tissues underlying ulcers. Some authors have commented that the most severely affected species in natural outbreaks are generally bottom dwellers (Llobrera and Gacutan 1987) or possess air-breathing organs (Roberts *et al.*, 1994). In the case of snakeheads, no particular size group appears to be more susceptible, with affected fish ranging from 40g to 900g (Cruz-Lacierda and Shariff

1995). However, there is a possibility that size or age may be significant in other species. For example, Indian major carp suffer high mortalities as fingerlings (Roberts *et al.,* 1989) but larger fish, although appearing ulcerated, have not been reported as dying in large numbers (Anonymous 1997). This could be attributed to the development of immunity against the disease. In India, the fish species documented to be most susceptible to EUS are *Channa* (20-100 percent), *Puntius* (5-100 percent), *Clarias* (10-30 percent), *Heteropneustes, Mystus* (5-75 percent), *Nandus,Cyprinus*, *Glossogobius* (10-60 percent), *Anabas* (10-55 percent) and *Mastacembelus* (10-35 percent) (Das 2002).

5.2.0. Diagnosis of EUS

Histopathology is currently the recommended method for confirmatory diagnosis of EUS (OIE, 2003; Lilley *et al.* 1998). Histopathological observations made from both naturally and artificially infected fish in the present study showed similar pathological pictures to those observed in other parts of the world. In the earliest stages of the disease, lesions showed some damage to the epidermal layer of the skin, resulting in an area of epithelial necrosis with some inflammatory cell infiltration. The pathology of *A. invadans* infection often involves considerable tissue damage some distance away from the hyphae (Wada *et al.,* 1994), implying that it secretes Extra-Cellular Products (ECPs) that are important to the process of infection. The nature of the ECPs from *A. invadans* remains obscure. This study has also confirmed this observation, where the *A. invadans* was isolated from kidney, liver and spleen of the fish from the wild. This is an indication that the fungus is very invasive. Zoospore injection studies have also confirmed this finding. In the artificially infected fish, *A. invadans* was re-isolated from kidney and liver of *Oreochromis andersonii*; and kidney of *Barbus paludinosus*, even though the zoospores were only inoculated into the muscle.

Histopathology of EUS mirrors the clinical findings and can be divided into five types of infectious processes (Chinabut and Roberts 1999). The histopathological picture seen in Fig. 4.13 could be typical *Type I,* based on the total lack of inflammatory response observed in the muscle. This could be attributed to the fact that the disease process was

so acute and severe that the fish succumbed before the inflammation took place (Chinabut and Roberts 1999). Hence, it may be considered that due to very poor inflammatory response, the fish might have offered a very insignificant or no resistance to *A. invadans* infection and that might be one of the major reasons for its high susceptibility to Epizootic Ulcerative Syndrome, and early death. These findings were also noted by Pradhan *et al.,* (2008). In their work, the group compared the inflammatory response in two different susceptible fish species, Indian major carp *Catla catla* and barb, *Puntius cauveriensis* to *Aphanomyces invadans*. They noted that in the case of barb where there was very weak or no inflammatory response, the severity of myonecrosis was so high that in the moribund fish, virtually no normal muscle fibers were observed in the lesion area. On the other hand, in the case of Indian major carp (*catla*), there was an active host inflammatory response and the severity of myonecrosis was less than that of the barb. In the present study, a very weak inflammatory response was observed, along with a secondary bacterial infection.

Fig. 4.14 is a typical example of *Type II pathology,* where an active inflammatory response is quite evident. Although in this type the fish do not survive long enough for extensive lesions to develop, the fungus does reach, and colonise the abdominal tissues, particularly the kidney (Fig. 4.15), and there may also be a secondary bacterial infection (Figs. 4.14 a and 4.14 b).

Sargochromis carlottae showed very well developed granulomas. This histopathological picture can be likened to the one described in snakeheads as *Type III pathology* by Chinabut and Roberts (1999). It is mostly observed in fish that have such a strong inherent resistance where they mount a profound inflammatory response which delays death so much that a wide range of pathological changes occur (Figs. 4.16a and 4.16b). In *Type III pathology*, extensive secondary infection may well be the cause of death (Chinabut and Roberts, 1999).

S. angusticeps showed *Type IV pathology*, which is similar to what Chinabut and Roberts (1999) observed in gray mullet (*Mugil cephalus*).

The present study was only able to demonstrate types *I, II, III and IV pathologies* in the fish samples from the wild. *Type V pathology* was demonstrated in the experimental infections of fish, where the *B. paludinosus* showed healing as water temperatures rose. Fungal hyphae were typically associated with a granulomatous response and extensive tissue necrosis. Marked inflammatory infiltration and formation of granulation tissue were also consistently observed in older lesions. Granulation tissue, an indication of tissue repair, developed to replace necrotic areas as observed by Kiryu *et al.* (2003).

Generally speaking, the fish that showed very well developed internal granulomas without frank ulcers, and those which showed clinical signs of tissue repair survived following challenge experiments. This is an indication that they mounted a very strong immune response against the fungus, and this saved them from succumbing. (Kiryu *et al.*, 2003). Hence, from the present study, it may be considered that infiltration of inflammatory cells plays an important role in resisting the *A. invadans* infection. It is well established that inflammation is the basic protective response of an organism and is the starting point which ultimately decides the overall resistance to any infection or injury (Pradhan *et al.*, 2008).

Isolation of the fungus *A. invadans* from tissues of infected fish in the microbiology laboratory, and subsequent reproduction of typical EUS lesions in susceptible fish species by inoculation with *A. invadans* zoospores, confirmed that microbiology can serve as a reliable diagnostic tool of EUS. Isolation of the fungus is one of the OIE recommended confirmatory tests (Chinabut and Roberts 1999).

This study has isolated and confirmed the presence of the oomycete *A. invadans* in susceptible fish species collected from the Zambezi River basin over a six-month period from October 2007 to April 2008. The fact that *A. invadans* was successfully isolated from tissues of only infected fish provides strong evidence that the fungus is the primary etiologic agent of EUS in susceptible fish species in the Zambezi River in Sesheke district, namely: *S. angusticeps, B. unitaeniatus, O. andersonii, B. paludinosus,*

C. gariepinus, *C. ngamensis*, *H. odoe*, *P. acuticeps*, *L. lunatus*, *S. intermedius*, *B. poechii*, *S. carlottae*, *B. lateralis*, *M. acutidens* and *H. vittatus*. Attempts to isolate the fungus from fish samples that did not show signs of EUS infection grossly and on histopathology proved futile. Fish used in the experimental infections also showed similar results. *A. invadans* can be consistently recovered from progressing, but not resolving, EUS lesions provided rigorous attention is given to obtaining uncontaminated innocula and suitable culture conditions are used (Roberts *et al.*, 1993). The present study successfully achieved this.

5.3.0. Pathogenicity studies

A. invadans was highly pathogenic to *O. andersonii* in this study. *O. andersonii* experimentally inoculated with secondary zoospores of *A. invadans* endemic to Sesheke district developed skin ulcers similar to those observed in fish naturally infected in the wild. The fungus was re-isolated from experimentally challenged *O. andersonii* and *Barbus paludinosus*. Some of the fish were also inoculated with pond water obtained from one of the sampling sites. None of those fish showed the characteristic skin ulcers observed in wild fish, thus providing further evidence that *A. invadans* is responsible for the EUS-type ulcers observed in susceptible fish species throughout the Zambezi basin. This study was only able to reproduce EUS lesions in the laboratory by physical abrasion of the skin, followed by inoculation with the zoospores. Other researchers such as Callinan *et al.*, (1996a) had a similar experience. They reported outbreaks of EUS in estuarine fish in Australia associated with acid sulfate soil areas, and reproduced EUS by exposing susceptible fish to acid water and spores of *A. invadans*. In this case, a chemical abrasion had been induced by the acid sulfate. In Thailand, Kanchanakhan (1996) showed that EUS can be reproduced when susceptible snakeheads (*Chana* species) are injected with a particular strain of rhabdovirus and bathed in spores of *A. invadans*. In some cases, iridoviruses have been implicated.

The initial phase of the challenge experiments, where fish was inoculated with *A. invadans* without prior disruption to the epidermis, was unable to induce EUS lesions in the fish. This is because in some cases prior damage to skin is necessary before lesions

can be induced in fish (Jones and Hunt 1983). Through bio-toxicity assay, the experiment successfully reproduced Epizootic Ulcerative Syndrome, re-isolated *Aphanomyces invadans* and the histopathological lesions were indistinguishable from those seen in natural cases. These studies comply with Koch's postulates and have several important implications. They confirm *A. invadans* as a primary infectious agent of EUS in the Zambezi River Basin, and indicate that disruption of epidermal continuity may be a necessary precursor to fungal attachment and lesion induction (Callinan 1997). They also confirm the case definition of EUS, which is 'the presence of invasive *Aphanomyces* infection and necrotizing ulcerative lesions typically leading to a granulomatous response' (Roberts *et al.*, 1994).

Vishwanash *et al.* (1998), however, demonstrated the highly invasive abilities of *A. invadans* in tissues like intermuscular bones, gizzard and spinal cord. This provides an indication that under certain circumstances, the fungus may be able to invade the healthy skin of fish. The marked pathogenicity of *A. invadans* may result from its highly invasive nature. This invasiveness into the soft tissues has been described from both laboratory and field infections with ayu, *Plecoglossus altivelis* (Wada *et al.*, 1996), and Menhaden (Noga and Dykstra 1986; Noga *et al.*, 1988; Blazer *et al.*, 1999). The fungus, *A. invadans,* was subsequently re-isolated from tissues of the infected experimental fish. In this study, *A. invadans* was demonstrated in (by evidence of the typical granulomas on histopatholgy), as well as isolated from the spleens and kidneys of some of the infected fish from the wild. A similar situation was observed in the experimentally infected fish.

A number of organisms have been associated with EUS. The involvement of *Achlya* and *Saprolegnia* species in the pathogenicity of EUS has been previously examined. Limsuwan and Chinabut (1983) identified *Achlya* and *Saprolegnia* from ulcerated freshwater fish in Thailand; however, Tonguthai (1985) later dismissed these taxa as secondary or opportunistic invaders. Striped snakeheads *Channa striata* inoculated with several isolates of *Achlya* and *Saprolegnia* did not develop ulcers (Lilley and Roberts 1997). In Florida, Lim and Te Strake (1988) and McGarey *et al.* (1990) isolated several

oomycetes, including *Achlya bisexualis*, from ulcerated fish and water samples collected in the St. Johns River, but additional studies on pathogenicity were not undertaken. Bacteria such as *Eschericha coli, Aeromonas hydrophila, Pseudomonas aeruginosa, Staphylococcus epidermitis* and *Klebsiella* species have also been implicated (Kar 1999).

5.4.0. Factors predisposing fish to EUS infection

A number of factors have been hypothesized as either risk factors or determinants for EUS outbreaks. In this study the healing response observed in *B. paludinosus* could be attributed to the rise in temperatures that occurred as the weather conditions changed. *A. invadans* fails to thrive at 35°C and above. Kiryu *et al.* (2003) also reported a healing response in Atlantic Menhaden inoculated with *A. invadans*, as temperatures rose. Khan *et al.* (unpublished. data) indicated that susceptible species usually show lower rates of infection, and lesions may disappear after temperatures rise.

A. invadans is known to grow fastest in culture at temperatures between 26 to 30°C (Hatai and Egusa 1978; Fraser *et al.,* 1992; Lilley and Roberts 1997), and has been shown to grow in snakehead muscle tissue between 19 to 31°C (Chinabut *et al.,* 1995). However, further investigation has revealed that snakeheads are able to recover from *A. invadans* infection at higher temperatures (26-30°C), but are unable to prevent fungal invasion and eventually succumb to the disease at lower temperatures (19°C) (Chinabut *et al.,* 1995). The humoral and cellular immune responses of fish are known to be suppressed at low temperatures (Bly and Clem 1991), which may explain why higher mortalities from EUS occur when water temperatures are low. Naturally and artificially infected snakeheads have been shown to produce an antibody response against *A. invadans* (Thompson *et al.*, 1997), and the cellular macrophage response is also considered to be important in enabling fish to resist infection (Wada *et al.*, 1996).

Experimental injection challenges of native European and American fish species have shown that the pathogenic fungus, *A. invadans*, is capable of causing lesions in rainbow trout at 18°C (Thompson *et al.*, in press), but is less infective in stickleback

(*Gasterosteus aculeatus*) and roach (*Rutilus rutilus*) at 11°C to 16°C (Khan *et al.*, 1998). Observations on behavior of the hyphae at different temperatures were also made. At 20°C hyphae tended to invade deeper into the fish tissues, while at temperatures between 25°C and 30°C, hyphae actively grew outside the body surface around the lesions.

The outbreaks of EUS are stress related. It is unlikely that the mere presence of the pathological agents would lead to the disease if all other factors are optimal. The turbidity of water in areas where fish samples from the wild were obtained was quite high, signifying poor quality. That may have contributed to the stressful conditions for the fish, causing them to easily succumb to infection. In the challenge experiments of the present study, when no water changes took place in the infection tanks and the quantity of feed was increased, excessive algae growth and deteriorating water quality resulted, creating very stressful conditions for the fish. Whenever one or more stress factors increase in magnitude such that fish immune systems cannot guard against the pathological agents, an outbreak of EUS may be observed (Pathiratne and Jayasinghe 2001). Lilley *et al* (1998) noted that an EUS outbreak can occur only when susceptible fish, infective forms of the fungus and suitable environmental conditions are present at a particular site. EUS outbreaks in Bangladesh were associated with farming of susceptible fish species in ponds which had previously been derelict, or ponds which had been treated with pesticides to remove predators and other undesirable fish prior to stocking (Ahmed and Rab 1995). It is also possible that soil and/sediment characteristics influence outbreak occurrence in freshwater settings although no definite associations have yet been identified. Macintosh (1986) found that sediments at many outbreak sites were slightly acidic and had low calcium content. The author suggested that such soils would account for the poorly buffered acidic water and high levels of aluminum and iron in water samples from such sites. Ahmed and Rab (1995) noted an association between EUS outbreaks and ponds having reddish sandy soils, and suggested the associated relatively high turbidities in these ponds may have been stressful to fish.

Changes in water quality and agricultural run-off due to floods may cause stress for fish, and maybe a component to predispose to EUS. The exclusive use of rainwater and underground water in fish ponds would reduce the risk of infection with EUS, due to the absence of parasites and microbial flora (Munro and Roberts 1989). This would probably explain the fact that when affected fish are transferred at an early stage to very clean water conditions, bathed in antibiotic or limed water, losses are reduced and a significant number of fish may recover (Chinabut and Roberts 1999). During this study, some of the fish that were caught in areas with fast-flowing water were actually showing signs of wound healing. Lagoons showed a very high relative risk of EUS infection with more widespread and enlarged gross lesions. These findings support those by Khan and Lilley (2002), who showed that haors harbored the highest levels of EUS infection. A haor is a depression in flood plains located between two or more rivers, which functions as an internal drainage basin. The fact that infected fish showed signs of improvement when they found their way to areas of fast flowing better quality water may also support the assumption that the quality of water is significant from an aetiological point of view. In some fish species, age may play a role in susceptibility to EUS infection. In menhaden, a larger proportion of fish below the age of 1 were shown to be affected than fish above the age of 1 (Levine *et al.*, 1990).

Low pH (below 6.4) is one of the risk factors associated with outbreaks of Epizootic Ulcerative Syndrome. Sammut *et al.* (1996) showed that low pH causes stress and induces significant lesions in fish. In susceptible fish species, this would invariably enhance infection with *A. invadans* upon exposure to the pathogen. The average pH of water at the sampling sites in the present study was 6.2. This may explain the infection that occurred in the fish collected at those locations. Some other researchers in Australia demonstrated the importance of pH in the pathogenesis of EUS. Using aquarium trials, they subsequently induced Red Spot Disease in fish exposed sublethally to artificially acidified water (at both pH 3 and pH 5) and pathogenic *Aphanomyces* spores, even at low concentrations of monomeric aluminium (Callinan *et al.*, 1996). They observed that infectivity of *A. invadans* reduces at higher pH.

High prevalence of infection with *Aphanomyces invadans* reportedly occurs in years with high rainfall, or in regions with low salinities (Callinan *et al.*, 1989; Levine *et al.*, 1990; Noga 1993; Lilley *et al.*, 1998). *A. invadans* has a low salinity tolerance and will not grow well in salinities above 15 practical salinity units (psu) (Dykstra *et al.*, 1986, Levine *et al.*, 1990, Blazer *et al.*, 2002). The secondary zoospores of *A. invadans* lose their infectivity above 4 to 6 psu (Fraser *et al.*, 1992; Lilley *et al.*, 1998; Kiryu *et al.*, 2002), indicating that infections must occur below 4 psu (Kiryu *et al.*, unpubl. data). Rainfall is thought to contribute to epizootics of EUS by reducing salinity that allows the oomycete to sporulate. Rainfall also induces sub-lethal exposure to acidified runoff; resulting in epidermal necrosis and exposure of the dermis (Sammut *et al.*, 1996), while dissolved oxygen is reduced to less than 1 ppm followed by the concomitant damage to the epidermis (Plumb *et al.*, 1976).

Heavy rainfall in an area can bring the risks of water quality deterioration, directly through agricultural runoff, and indirectly by introducing infectious agents into the waterways. In the Tar-Pamlico rivers estuary, North Carolina, Levine et al (1990) found higher prevalence of menhaden infected with ulcerative mycosis at lower salinities (2.5–9.6 psu) compared to higher salinities (13.5–15.7 psu). Other researchers (Noga *et al.*, 1993) showed that the most damaging outbreaks in the Pamlico River coincided with unusually high rainfall and reduced salinity.

In this study, the main critical factors proposed to be involved in the occurrence of this disease in this part of the Zambezi basin are:

1) Flooding: During the 2006/2007 rainy season, serious floods were experienced in the Zambezi River basin. Compared to the subsequent rainy season, when water levels did not rise too high, the prevalence of EUS was relatively higher during the former period. Kabata (1985) explained that floodwater is one of the risk factors of EUS, probably because it is a route of entry for pathogens. Roberts *et al.* (1989) also described floodwater as a powerful means for spreading EUS throughout Bangladesh. After the

water levels have subsided, acidity in the rivers increases and this may cause abrasion of the fish skin and exposure of the dermis.

2) Turbidity: The water was extremely turbid in some sampling sites. This is a sign of poor water quality. As Ahmed and Rab (1995) observed, the turbidity may have contributed to stress in the fish, rendering them very susceptible to EUS. The organic debris and algae blooms observed must have resulted in high levels of ammonia upon decomposition, causing low concentrations of dissolved oxygen.

3) Crowding: Not only were the lagoons too shallow but they also had large numbers of fish and very poor water quality of low pH, i.e. below 6.2. This made the fish very vulnerable.

4) Water temperature: At that time of the year water temperatures were conducive for *Aphanomyces invadans* sporulation.

5.5.0. Economic impact of EUS

Published estimates of direct economic losses due to EUS mortalities in several affected countries are listed by Lilley *et al.* (1998). Projecting future losses, the most conservative estimate of the cost of fish losses due to EUS in Australia, the Philippines and Indonesia until the year 2027 has been calculated at US$63 million (Anonymous 1998b).

In Kerala, India, despite official announcements that unaffected fish could safely be eaten, panicky consumers were so alarmed that they even shunned safe mussels and ducks during the time of the outbreak (Sanjeevaghosh 1992). In Alappuzha, water for domestic use had to be supplied in tankers since people were afraid to use supposedly contaminated lake water even for washing (Sanjeevaghosh 1992). According to Tonguthai (1986), a similar loss of confidence in freshwater fish occurred in Thailand too, in 1982 to 1983, during the initial outbreak of EUS, and this led to financial losses of over US$8.7 million.

In Zambia, fish farming is currently not very developed, but might become more common in the near future. EUS could pose a very serious threat to small-scale fish farmers, causing them serious financial difficulties from sudden disease losses, or from reduced production levels due to disease. Losses in wild fisheries could deprive the poorer sections of the community from access to cheap sources of animal protein. EUS could also threaten the future of aquaculture in Zambia and the Southern African region.

CHAPTER SIX

RECOMMENDATIONS

For the control, management and prevention of future outbreaks of EUS the following measures that could help are being proposed and recommended:

1) It may be beneficial for fish breeders to use EUS-resistant species like the *O. nilotucus*, instead of very susceptible species, as demonstrated by this study. This information could benefit fish farmers in Zambia where aquaculture is still in its infancy.

2) Increasing Calcium and magnesium levels in the ponds would benefit fish skin and induce encystment in fungal zoospores, thereby making them fall out of suspension (Khan and Lilley 2002).

3) Hygiene/other disease: Allowing cattle to wash and drink in the pond after ploughing or grazing in other areas would give a high relative risk, possibly due to the transport of pathogens with the cattle. Netting with dried or disinfected nets, and requiring buyers to do the same, would contribute to much lower relative risk values. The use of equipment that has been transported between farms (e.g. by buyers) is likely to provide a source of infective material, and drying or disinfection is recommended (Lilley and Inglis 1997).

4) Treatments for therapeutic purposes include antibiotics and chemicals. Though these have brought some satisfactory results, they have many undesired side-effects too. These include residues, cost increases, development of bacterial resistance, and negative impact on the environment (Areechon, 1992).

5) Fish should be protected from parasites. A high relative risk has been demonstrated in ponds where farmers believe fish were affected by parasites. A number of parasites have been isolated from EUS-affected fish (Tonguthai 1986) and may either be possible vectors for the pathogen, or a stress-inducing factor in EUS outbreaks. Subasinghe (1993)

demonstrated such an association between the level of infection by *Trichodina spp.* and the susceptibility of *Channa striata* to EUS infection. The mechanism of attachment of these parasites can cause skin rupture, and might facilitate infection by the EUS fungus.

6) To save the worst affected fish species from extinction, they could be bred under captivity for ranching operations in addition to conserving them in their natural habitats.

7) It is important to take public educational measures and allay the natural fears of farmers, fishers and consumers about any wider effects of EUS.

Further Investigations Proposed

a) Environmental factors like temperature, alkalinity, hardness and pH, are important in initiating EUS. But only further, more rigorous, experimental work can determine which ones are really critical in relation to the development of EUS in susceptible fish of the Zambezi river basin. The absence of adequate data on the relationship between a fish and its environment is an obstacle to unraveling the complex relationship between EUS and the environment. Further studies under laboratory and field conditions, would create a clearer picture, which would help scientists come up with better control measures and hence reduce, or even eliminate the devastating effects of EUS.

b) Researchers in the United States of America were able to show that one zoospore could give rise to multiple lesions, confirming the observation that hyphae travel along the myosepta. (Kiryu *et al.*, 2002). The present study, however, did not investigate the minimum number of zoospores necessary to give rise to lesions in susceptible fish species. Therefore there is need to embark on this work.

c) Researchers in different parts of the world like the USA, South East Asia and Australia (Callinan *et al.*, 1995, Lilley *et al.*, 1998 and Kiryu *et al.*, 2003) have clashing views on whether or not *A. invadans* can invade an intact epidermis of fish. This clearly indicates that the relationships between integument damage, exposure, development of lesions and fish mortality require further study.

d) Khan and Lilley (2002) found out during an interview-based questionnaire survey that some farmers and fishermen opined that aquatic birds, fish-eating birds, reptiles and mammals might transmit EUS from one place to another by preying on easy to catch EUS-affected fish and dropping uneaten portions in unaffected water bodies. Further work to investigate this possibility is needed especially in the Zambezi river basin where a lot of crocodiles are found.

e) Very few studies have been undertaken to confirm resistance among imported species of fish, and the mechanism of resistance. A better perspective of this phenomenon would ensure the use of more EUS-resistant fish species on fish farms.

f) The Extra-Cellular Products (ECPs) that Wada *et al.* (1994) purported to be secreted by *A. invadans* and important to the process of infection need to be closely analysed so that their nature can be better understood.

g) Molecular epidemiological studies may help establish the source of the pathogen of EUS, *A. invadans,* to the Southern African Zambezi river basin.

CHAPTER SEVEN

CONCLUSIONS

1. The study has isolated, confirmed and documented the presence of *A. invadans* as a highly infectious, pathogenic and invasive primary pathogen.

2. In this study, the following species of fishes were confirmed to be susceptible to EUS: *Clarias ngamensis*, *C. gariepinus*, *Barbus poechii*, *Seranochromis angusticeps*, *Brycinus lateralis*, *Micralestes acutidens*, *Sargochromis carlottae*, *Hydrocinus vittatus*, *Phryngochromis acuticeps*, *Schilbe intermedius*, *Hepsetus odoe*, *Labeo lunatus*, *Oreochromis andersonii*, *Barbus unitaeniatus* and *Barbus paludinosus*.

3. *Tilapia sparmanii* was the only species that did not exhibit any lesions among those sampled from the field.

4. The fish that was experimentally infected in the laboratory (*Barbus paludinosus* and *Oreochromis niloticus*) developed skin ulcers similar to those observed in fish infected in the wild.

5. Histopathology and microbiology have been successfully used in this study to diagnose EUS in fish samples collected from the Zambezi River Basin in Sesheke district of Zambia, as well as to confirm the presence of the fungus *A. invadans* in experimentally challenged fish in the laboratory.

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