Chapter 1: Introduction

The production of farmed foodfish and ornamental fish has become one of the fastest-growing segments of agriculture in the United States as well as around the world. The Food and Agricultural Organization of the United Nations estimates that aquaculture ventures produced 31 million tons of fish in 1998 (Food and Agriculture Organization of the United Nations, 2000). This high production mirrors a growing need for a readily-available source of protein by a swelling world population. However, intensive aquaculture production is severely limited by environmental, nutritional, and health factors. Among these problems, infectious disease is one of the most important, with an estimated 10% of all cultured aquatic species lost to infectious disease alone (Leong and Fryer, 1993; Heppell and Davis, 2000).

A common and serious bacterial disease of fish is mycobacteriosis, most often caused by one of the three following Gram-positive, acid-fast bacilli: *Mycobacterium mariunum*, *M. fortuitum*, and *M. chelonae*. Mycobacteriosis is a chronic progressive disease, causing systemic granulomas in vitually any tissue or internal organ and often leading to death (Austin and Austin, 1993; Smith, 1997). Given their ubiquitous nature in water and sediment, these organisms affect fish populations worldwide. Indeed, mycobacteriosis has been reported in over 160 species of fresh and saltwater fish (Chinabut, 1999), and all teleosts are considered potential hosts (Plumb, 1994). Perhaps most importantly, it has been reported to cause massive mortalities among fish grown in intensive aquaculture systems. Mycobacteriosis was first documented as a disease of cultured fish in the 1950's, when mortalities among hatchery-raised *Oncorhynchus* and *Salmo* spp. increased dramatically; morbidity even reached 100% in some populations (Wood and Ordal, 1958). Mycobacteriosis has been reported among other valuable cultured foodfish, such as striped bass, *Morone saxatilis* (Aronson, 1926), sea bass, *Dicentrarchus labrax* (Colorni, 1992), tilapia, *Oreochromis mossambicus* (Noga et al., 1990), summer flounder, *Paralichthys dentatus* (Hughes et al., 2002), plaice, *Pleuronectes platessa* (Timur et al., 1977), yellow perch, *Perca flavescens* (Daoust et al., 1989), snakehead, *Channa striatus* (Chinabut et al., 1990), and pejerrey, *Odonthestes*

bonariensis (Hatai et al., 1993). Fish cultured for the ornamental pet trade are also susceptible, with guppies, *Lebistes reticulatus* (Bragg et al., 1990), tetra, *Moenkhausia sanctaefilomenae* (Shamsudin et al., 1990), angelfish, *Pterophyllum* spp. (Smith, 1997), and goldfish, *Carassius auratus* (Talaat et al., 1999) having been reported with mycobacteriosis.

1. *Mycobacterium marinum*

Mycobacterium marinum is part of the order Actinomycetales and family Mycobacteriaceae. This pathogen is closely related to the *M. tuberculosis* complex, exhibiting 99.4% 16S rRNA sequence homology (Rogall et al., 1990), and was first isolated from tropical marine fish at the Philadelphia Aquarium (Aronson, 1926). Other *Mycobacterium* spp. can cause disease in fish, including: *M. fortuitum* (Cruz, 1938), *M. chelonei* (Arakawa and Fryer, 1984), *M. neoaurum* (Backman et al., 1990), and *M. simiae* and *M. scrofulaceum* (Lansdell et al., 1993). Furthermore, several other *Mycobacterium* spp. have been isolated from wild fish, including *M. piscium* (Bataillon et al., 1897), *M. chesapeaki* (Heckert et al., 2001), and *M. shottsii* (Rhodes et al., 2001), though these are closely related to *M. marinum* and may be reclassified as they are further studied.

Mycobacterium marinum is a pleomorphic, non-motile, non-sporulating, acid-fast, Gram-positive, and non-branching rod, usually approximately 1.5-3.0 µm in length and 0.2-0.6 µm in width. This bacterium is usually cultured with Middlebrook 7H10 or Lowenstein-Jensen media, and colonies are apparent in approximately 8-10 days at 25-28°C. Identification can be based on a variety of criteria, such as growth rate, acid-fastness, colonial morphology, specific biochemical test reactions, and DNA analysis (Talaat et al., 1997; Chinabut, 1999). Histologic stains commonly used to identify mycobacterial organisms include Brown-Hopps, Ziehl-Neelson, and modifications of the Fite's stain (Wolf and Smith, 1999; Hughes et al., 2002). A number of biochemical characteristics are also utilized to distinguish *M. marinum* from other *Mycobacterium* spp., and these properties include: pigment production (+), urease (+), thiopen-2-carboxylic acid hydrazide sensitivity (+), arylsulfatase (+), pyrazinamidase (+), tween

hydrolysis (+), catalase (-), and nitrate reduction (-) (Hedrick et al., 1987; Chinabut, 1999). Previous studies have also used 16S rRNA sequence analysis (Knibb et al., 1993; Heckert et al., 2001) and restriction enzyme assays (Talaat et al., 1997) to identify *M. marinum* infection.

Pathology

It is presumed that fish can be infected by ingestion of feed or water contaminated with feces, urine, exudate, or tissue from diseased animals (Chinabut et al., 1999). Mycobacteria may also infect fish through skin or gill lesions created by trauma or parasitic infection, and there is even evidence that transovarian passage of the bacteria can occur (Chinabut et al., 1994; Smith, 1997). After entry into the body, mycobacterial organisms spread throughout the body by the circulatory or lymphatic system (Chinabut, 1999).

If clinical disease develops, it may include signs of altered pigmentation, exophthalmia, ulceration and petechiation of the skin, emaciation, cachexia, and lethargy. Internally, a common observation is the enlargement of internal organs, including the anterior and posterior kidney, spleen, liver, and heart. Often, multiple white nodules can be observed grossly within these organs; these nodules are granulomas and have been reported in the brain, gills, gastrointestinal tract, and skeletal muscle. If extensive granulomatous inflammation develops, the normal organ architecture can be obliterated and organ function hindered.

In the acute form of the disease, mycobacteriosis is often characterized by necrosis and granulomatous inflammation in internal organs. Histologic examination exhibits a diffuse infiltration of macrophages and reticuloendothelial cells and the presence of mycobacterial organisms scattered throughout infected organs (Wolke and Stroud, 1978). Formation of multiple discrete granulomas is the most distinctive lesion of chronic *M. marinum* infection in finfish. Soft granulomas are composed of centrally-located caseous material in a sheath of epitheliod cells and macrophages, which are themselves surrounded by fibrous tissue (Talaat et al., 1999; Wolf and Smith, 1999). Hard granulomas are formed at an earlier stage of disease and lack a defined epithelioid

layer and caseous necrosis (Hedrick et al., 1987). In general, granulomas vary in size, often ranging from 80 to 500 µm (Sakanari et al., 1983), and usually contain necrotic cellular material and varying numbers of intracellular mycobacteria.

Wolke and Stroud (1978) stated that granulomas found in finfish differ from those found in mammals in three ways: 1) giant cells are extremely rare in finfish, 2) calcification is rarely exhibited in finfish granulomas, and 3) finfish often exhibit high numbers of acid-fast organisms within granulomas. The validity of these findings appears dependant on the species of finfish affected, but in *Morone* spp., these findings are generally substantiated. Wolf and Smith (1999) experimentally infected *M. saxatilis* with *M. marinum* and studied the resulting granulomas; they found that multinucleated giant cells were rarely observed, mineralization was not present, and acid-fast bacteria were present in significant numbers in all areas of granulomatous inflammation. These findings are supported by studies of wild striped bass with mycobacteriosis (Hedrick et al., 1987; Heckert et al., 2001; Rhodes et al., 2001)

Treatment and Control

Treatment of diseased fish is difficult, partially because only two antibiotics, oxytetracycline (Terramycin®) and ormetoprim-sulfadimethoxine (Romet®), are approved by the Food and Drug Administration (FDA) and available for use in foodfish in the U.S.A. (Stoffregen et al., 1996). Neither antibiotic is licensed by the FDA for use against mycobacteriosis, and in fact, no chemotherapeutic is licensed for the treatment of *Mycobacterium* spp. in tropical, ornamental or foodfish. A number of therapeutic measures have been attempted with limited efficacy or feasibility. Dublin (1979) recommended use of the antimicrobials, rifampicin and isoniazid, which are commonly used against mammalian tuberculosis. Given the expense of these chemotherapeutics, this regimen was only suggested for treatment of valuable ornamental fish. Other chemotherapeutic cocktails have been attempted, with the use of rifampicin or cotrimoxazole plus ethambutol or rifampin plus doxycycline reported (Pattyn 1984). Van Duijn (1981) and Conroy and Conroy (1999) found that water baths of tetracycline or kanamycin sulfate, respectively, could help treat the acute stages of mycobacteriosis.

More recently, Lower and Poet (2001) treated external lesions related to *Mycobacterium* sp. infection with a series of enrofloxacin injections and found clinical resolution of the disease signs. However, none of these studies were complete clinical trials, and almost nothing is known about the pharmacokinetics of antituberculosis drugs in fish. Moreover, the question remains whether these treatments completely eliminate infection. A likely scenario is that treatments only eliminate overt clinical signs and these treated fish become asymptomatic carriers. In addition, even if treatment is attempted, it is further limited by the prospect of long-term, expensive antibiotic therapy and by the potential for developing resistant bacteria (Plumb, 1994). Therefore, many veterinarians and fish health professionals recommend destruction of infected fish, which often means destroying whole ponds or tanks of fish (Austin and Austin, 1993). This can result in significant economic loss for the producer or aquaculturist.

Reno (1998) reasoned that there are only a limited number of ways to control disease in cultured fish populations. These included reducing the spread of infectious agents within populations, chemotherapy, culling, and vaccination. The potential for disease among fish depends on the contact rate among infectious agents/fish and number of susceptible fish. Under intensive aquaculture conditions, the frequency of that contact is vastly increased. Beyond overcrowding and confinement, cultured fish are also subjected to other stressors such as handling, grading, fluctuating temperatures, poor water quality, and social stresses. Such factors exacerbate the susceptibility of fish to disease and thus further increase morbidity and mortality in the population (Noga et al., 1994).

Considering that chemotherapeutic treatement of piscine mycobacteriosis is not Federal Drug Administration-approved and usually unsuccessful, preventative measures are crucial. For example, fish should be obtained from specific pathogen-free sources and quarantined when received. Fish should be maintained under optimal conditions; factors such as poor nutrition, overcrowding, and poor water quality can result in stress, immunosuppression, and disease outbreaks. If fish carcasses are to be used as food, they should be heated to 76°C for 30 minutes to kill the mycobacteria (Thoen and Schliesser, 1984). Clinically infected fish or infected populations of fish should be humanely

euthanized and immediately destroyed by burying in quick lime or by burning (Chinabut, 1999). Phonolic compounds, alcohols, and formalin should be used as disinfectants when cleaning facilities and equipment. However, biofilm in the aquatic habitat can harbor the organism even after fish-holding structures and equipment are disinfected; indeed, biofilm bacteria appear to be more resistant to disinfection than free organisms (Schulze-Röbbecke and Fischeder, 1989).

Zoonosis

Mycobacterium marinum is also of human health concern because of its zoonotic potential. Disease caused by *M. marinum* infection has been well-documented since the 1970s, and human tuberculous skin lesions described between 1939 and 1954 were eventually attributed to *M. marinum* infection (Collins et al., 1985). Human *M. marinum* infection has increased in both immunocompetent and immunocompromised individuals in the last decade, even though the disease is likely under-reported and often misdiagnosed (Dobos et al., 1999).

Human *M. marinum* infections are often associated with direct handling of infected fish or with exposure to water, especially in swimming pools, saunas, and aquariums. The bacteria can gain entrance into the human body through traumatic lesions on the skin, and affected humans usually exhibit peripherally-located cutaneous granulomas. Infections are usually limited because the optimal growth temperature for this bacteria is 25-35°C, and growth appears to be inhibited at 37°C (El-Etr et al., 2001). However, in the laboratory, Clark and Shepard (1986) adapted a strain of *M. marinum* to grow at 37°C and demonstrated that *M. marinum* can disseminate through the mammalian body if the temperature restriction is removed.

Despite innocuous nicknames like “swimming pool granuloma” and “fish handler’s disease,” infections usually warrant long-term medical treatment and sometimes even surgery (Smith, 1997; Dobos et al., 1999). As with mycobacterial disease in poikilothermic organisms, *M. marinum* infections in mammals persist because the bacteria can survive in host cells. Specifically, *M. marinum* invades and proliferates in fibroblasts, epithelial cells, and macrophages (Mor, 1985; Ramakrishnan and Falkow,

1994). Aquatic mycobacteria can also occasionally spread to internal body systems of humans and have been isolated from pulmonary lesions (Chinabut, 1999), and from syonvial fluid and muscle (Blacklock and Dawson, 1979). Furthermore, there is heightened concern over pathogen exposure of immunocompromised individuals who own or work with fish. A number of cases of *M. marinum* infection have been documented among HIV-infected people, and these include disseminated infections and infections resistant to standard anti-tuberculous treatments (Glaser et al., 1994).

2. Vaccination and Immunity

Because of the difficult management of fish mycobacteriosis and due to its zoonotic potential, development of an effective vaccine against fish pathogenic mycobacteria is vital. Today, there are multiple bacterins commercially available against bacterial fish pathogens, such as *Yersinia ruckeri*, *Aeromonas salmonicida,* and multiple *Vibrio* spp. (Evelyn, 1997), yet none exists for *Mycobacterium* spp. Thus development of an effective vaccine for *Mycobacterium* spp. for ornamental and cultured fish is vital.

Mycobacterium spp., including species pathogenic to mammals (i.e. *M. tuberculosis*, *M. leprae*, and *M. bovis*) and aquatic animals (i.e. *M. marinum*, *M. fortuitum*, and *M. chelonae*), are facultative intracellular pathogens. They cause severe, progressive, systemic disease, in part because they are able to evade the host immune system by residing and replicating inside of host's macrophages. For example, *M. marinum* and *M. tuberculosis* are able to avoid the endocytic pathway and persist in an intracytoplasmic vacuole because the vacuole does not fuse with lysosomes (Barker et al., 1997). Furthermore, *M. marinum* found in granulomas can express genes for proteins involved in metabolic and synthetic pathways, including transcription regulation, cell-wall synthesis, metabolite transport, and stress responses (Chan et al., 2002). This indicates that *M. marinum* bacteria are largely active in granulomas and can resist the host immune responses while ensuring their own long-term survival.

Because of their intracellular location, effective immune responses against *Mycobacterium* spp. require cell-mediated responses as well as humoral responses

(Ivanyi and Thole, 1994; Rook and Hernandez-Pando, 1996). Induced humoral antibodies have been shown to bind extracellular mycobacteria and hinder their movement to an intracellular position, but it is the cell-mediated responses that destroy mycobacteria-infected cells. Thus a vaccine for mycobacteriosis must stimulate both humoral and cell-mediated immunity.

The potential for a vaccine against piscine mycobacteria was suggested by Bartos and Sommer (1981). They were able to elicit cell-mediated immune responses in rainbow trout, *Salmo gairdneri*, from intraperitoneal immunization with *M. salmoniphilum* mixed with Freund's complete adjuvant. Later, Chen et al. (1996) vaccinated rainbow trout with intraperitoneal injections of extracellular products from various aquatic *Mycobacterium* spp. mixed with Freund's incomplete adjuvant. This resulted in enhanced levels of reactive oxygen species, phagocytes, lysozyme, and antibodies produced against the mycobacteria. Therefore, it has been shown that both the nonspecific and specific immune responses can be elicited by immunization with components or products of aquatic mycobacteria. However, it was not reported whether these immune responses were sufficient for protection against live bacterial challenge.

Recombinant Vaccines

Recombinant vaccines are relatively new to the field of fish medicine and have mostly been tested against viral and parasitic diseases, including viral hemorrhagic septicemia virus (VHSV; Lecocq-Xhonneux et al., 1994), infectious pancreatic necrosis virus (IPNV; Frost et al., 1998), and *Ichthyophthirius multifiliis* (He et al., 1997). Only one vaccine with a recombinant component has recently been available for commercial use in aquaculture, and this vaccine contained *Escherichia coli* expressing the IPNV VP2 protein (Leong et. al., 1997; Lorenzen, 1999).

Recombinant vaccines remain attractive constructs because they can be modified according to the required vaccine characteristics. For example, a protective antigen can be expressed on a bacterial host, thereby potentiating the immunostimulatory effects of the vaccine. Gilmore et al. (1988) expressed an IHNV glycoprotein on *E. coli* and produced protective immunity against IHNV challenge. Furthermore, antigens expressed

by the bacterial vector/hosts themselves can produce immune responses, thus making the vaccine multivalent. Noonan et al. (1995) expressed glycoprotein antigens from VHSV and IHNV on *Aeromonas salmonicida* and found that the vaccine induced protective immunity towards all three pathogens. Such an effect is important, because the immune responses towards one pathogen represented in the vaccine could potentially upregulate responses towards the other represented pathogens.

Despite the dearth of commercially-available recombinant vaccines, this strategy of vaccine construction is still being widely studied and has been largely successful inducing immune responses. Unfortunately, the stated success of recombinant vaccines has been based largely on the antibody responses in vaccinated fish (Lorenzen, 1999). The cell-mediated and non-specific immune responses, as well as the responses of these vaccinated fish to challenge, have not been well characterized.

DNA Vaccines

The development of DNA vaccines for fish has been increasingly studied in recent years. DNA immunization is based on the introduction of plasmid DNA encoding a protective antigen into animal tissue, which is then able to express the plasmid-encoded protein and induce subsequent immune responses (Heppell et al., 1998). Much effort has been invested into this technology, because DNA vaccines have multiple advantages over killed, attenuated, or subunit vaccines. DNA is very stable and is easily stored; plasmid constructs can be easily modified and can include more than one antigen-encoding gene; DNA vaccines eliminate the risk of residual virulence; and they can induce strong and long-lasting humoral and cell-mediated immunity (Heppell and Davis, 2000). Indeed, DNA vaccines are known to stimulate both non-specific and specific immune responses without the need for live organisms, replicating vectors, or adjuvants (Tanghe et al., 2000). The synthesis of antigen by DNA vaccination imitates natural infection by intracellular pathogens and leads to the subsequent cell-mediated responses and ultimately the generation of memory lymphocyte responses (Donnelly et al., 1997). Unfortunately, the cellular immune responses in fish are difficult to evaluate because of the lack of knowledge of the fish immune system and the unavailability of inbred strains

and appropriate assay reagents (Heppell and Davis, 2000). However, prior research has provided indirect evidence of cellular immune responses following DNA vaccination. For example, Kanellos et al. (1999) demonstrated antigen-specific proliferative responses among kidney-derived leukocytes after DNA immunization.

Most importantly, DNA vaccines have already been shown to provide protection in fish to various intracellular pathogens, such as viral hemorrhagic septicemia, infectious hematopoietic necrosis virus, and bacterial kidney disease (Gomez-Chiarri et al., 1996; Boudinot et al., 1998; Lorenzen et al., 1998). Because these vaccines successfully induce protective immune responses against other intracellular pathogens, it strongly indicates that a DNA vaccine against *M. marinum* can be developed. However, before DNA vaccines can be utilized, researchers must also prove that these vaccines will not cause autoimmune disease, production of anti-DNA antibodies, or tolerance in fish (Heppell and Davis, 2000). Nonetheless, studies indicate that DNA vaccines have a promising future in fish medicine.

Mycobacterium spp. 85A Antigen

Numerous vaccines have been developed against mammalian mycobacteriosis using the 85A antigen (Ag85A). This protein is a member of the antigen 85 complex (Ag85), which is made up of the 30- to 32-kDa proteins, Ag85A, Ag85B, and Ag85C, and is encoded by the *fbpA*, *fbpB*, and *fbpC* genes, respectively. The Ag85A is conserved across *Mycobacterium* spp., including *M. tuberculosis*, *M. bovis* and *M. marinum* (Borremans et al., 1989; Ohara et al., 1997; Stinear et al., 2000).

The Ag85A is either secreted or retained in the cell wall and was originally found to have fibronectin-binding properties. Fibronectins are high molecular-weight glycoproteins found in plasma and tissues, and they are involved in cell motility and adhesion, inflammation, and phagocytic function (Bentley-Hibbert et al., 1999). The ability to bind extracellular matrix proteins such as fibronectin has been linked to increased virulence of pathogenic bacteria, because mycobacteria can thus bind host cells and disseminate throughout host tissues (Patti et al., 1994; Armitige et al., 2000).

The Ag85A has also recently been found to act as an essential mycolyltransferase during synthesis of the mycobacterial cell wall. More specifically, the Ag85A is partly responsible for transfer of mycolic acids to alpha-alpha'-trehalose to form alpha-alpha'-trehalose monomycolate and alpha-alpha'-trehalose dimycolate. These two products are essential components of the mycobacterial cell wall, which in turn contributes to disease persistence and protects the mycobacteria from environmental stress, immune responses, and antibiotics (Belisle et al., 1997; Armitige et al., 2000). Further evidence of its importance to cell wall function is provided by the expression of Ag85A after treatment with isoniazid (Garbe et al., 1996; Wallis et al., 1998). This therapeutic agent damages the mycobacterial cell wall, and Ag85A is subsequently highly upregulated as a means to help maintain the integrity of the cell wall. These findings indicate that the Ag85A is vital for the formation and maintenance of the mycobacterial cell wall and thus for the survival of the bacterial cell.

Previous studies have established that Ag85A, when delivered appropriately, induces immune responses and effective protection against infections of several mammalian mycobacterial species (Denis et al., 1998; Velaz-Faircloth et al., 1999; Naito et al., 2000). For example, studies have shown that a recombinant vaccinia virus expressing *M. tuberculosis* Ag85A and a recombinant *Brucella abortus* strain RB51 expressing *M. bovis* Ag85A are significantly immunostimulatory and protective against live *Mycobacterium* spp. challenge (Malin et al., 2000; Vemulapalli et al., 2002). Research has also shown that injection of plasmid DNA encoding the Ag85A results in specific antibody production and protective Th1-type immune responses against mammalian mycobacteriosis (Denis et al., 1998; Tanghe et al., 2000). These protective immune responses include both gamma interferon production and antigen-specific cytotoxic T-cell activity. Furthermore, because of the high homology between the 85A antigens of various mycobacterial species, immune responses to the Ag85A of one species can be cross-protective against infections by other mycobacterial species (Tanghe et al., 2000).

3. Piscine Animal Model

Mycobacteriosis is an important disease of ornamental fish, foodfish, and wild fish. Striped bass (*Morone saxatilis*) and its hybrids appear to be particularly susceptible to *M. marinum*, and infection generally leads to dramatic pathologic changes and often death (Wolf and Smith, 1999). Mycobacteriosis was first reported among striped bass at the Philadelphia Aquarium (Aronson, 1926) and later at the New York Aquarium (Nigrelli and Vogel, 1963). Sakanari et al. (1983) found subclinical infections in up to 68% of wild adult striped bass sampled from western US rivers and bays. Hedrick et al. (1987) described chronic mortalitity among juvenile striped bass raised in an intensive aquaculture system. Mortalities in this population reached upwards of 50%, while approximately 80% of the remaining fish were also infected. Lansdell et al. (1993) reported the frequent isolation of *M. marinum* from striped bass caught in the Pacific Ocean. More recently, investigators have looked into significant declines in striped bass health in the Chesapeake Bay and determined systemic mycobacteriosis as a major disease problem (Heckert et al., 2001; Rhodes et al., 2001). The majority of sampled fish exhibited external dermal ulcers, disseminated internal granulomas, low weight, decreased body fat, and a variety of opportunistic infections.

Hybrid Striped Bass

Striped bass and their hybrids belong to the family Percichthyidae of the order Perciformes. Four species of *Morone* can be found in the United States: the freshwater white bass (*M. chrysops*) and yellow bass (*M. mississippiensis*), the anadromous striped bass (*M. saxitilis*), and the brackish water white perch (*M. americana*). Hybrid striped bass (hybrids) are a cross between striped bass females (*Morone saxitilis*) and white bass males (*M. chrysops*) and are sometimes known as “Palmetto Bass.” All of the *Morone* spp. have been crossed with striped bass, but no other product has been as accepted by the commercial aquaculture community as the striped bass female by white bass male cross (Hodson, 1989).

Hybrid striped bass can survive in a variety of conditions. For example, they have an acceptable temperature range of 4 to 33°C, though optimum growth occurs within a temperature range of 25 to 27°C (Hodson, 1989). Furthermore, hybrid striped bass thrive in salinities of 0 to 25 ppt, while some even survive in seawater (32 ppt). Unlike some other hybrid fish, hybrid striped bass are fertile, with females reaching sexual maturity around 2-3 years of age and males becoming mature when they are 1-2 years old. Hybrids breed in the springtime, usually in March-May when temperatures are approximately 10-20°C. Eggs are released by the female, fertilized in the water column by males, and hatch in approximately 2 days. The newly hatched fry are not fully developed and cannot feed for the first few days, so they depend on a yolk sac for nutrients. After this period, young hybrids feed on zooplankton and insects and become pisciverous when suitably sized. Wild hybrids grow to approximately 1-1.5 kg in two years and usually live for approximately 5-6 years (Hodson, 1989). Hybrids harvested for food are generally sold at a weight of 0.65 to 1.2 kg and take 18-24 months to reach this market weight in an aquaculture facility. However, the growth rate depends on several factors, including water temperature, stocking density, feeding rate, and water quality.

Hybrid striped bass are widely cultured for both the food and sportfishing markets. The popularity of these fish can be exemplified by their increase in production; 9.7 million pounds of hybrid striped bass were produced in 1999, an increase from the 10,000 pounds produced in 1986 (Kent SeaTech Corporation, 2000). In the United States Department of Agriculture 1998 Census of Aquaculture, production of hybrids in the United States was fourth in value ($28 million) and fifth in volume (9.4 million pounds) behind catfish (581 million pounds), salmon (104 million pounds), trout (72 million pounds), and tilapia (11.6 million pounds) (Carlberg et al., 2000). However, continued expansion of the hybrid striped bass culture industry is deterred by high production costs. These costs are associated with the need for feed, electrical energy, water, trained staff, and equipment. Loss of product due to disease also contributes significantly to production costs. Recent advances have been implemented to reduce costs in hybrid culture, and these advances include: 1) grading of fish after the first year to produce more

uniform fish sizes, 2) increasing stocking densities to increase yields, and 3) using extruded floating feeds with high protein levels to improve feed conversion ratios (Carlberg et al., 2000). In the future, further improvements must be made in increased feed conversion, year-round fingerling production, and disease and water quality management.

Because hybrids are commonly raised under intensive aquaculture situations (i.e. cages, ponds, tanks), they are often exposed to stressful conditions. A number of infectious and non-infectious problems are encountered in hybrid striped bass culture facilities. Non-infectious factors such as poor water quality, improper nutrition, and gas supersaturation can directly cause morbidity and mortality or predispose fish to infectious diseases. Healthy hybrids can generally resist many bacterial, fungal, parasitic and viral pathogens, but hybrids become susceptible to diseases when immunocompromised due to stress.

Bacteria are perhaps the most important pathogens in hybrid culture, causing severe mortalities and financial losses. Most bacterial pathogens of striped bass are ubiquitous in both the fish and water, and may not produce clinical disease. However, when fish become stressed or injured, these infectious agents may overcome host defense mechanisms and cause high losses. Outbreaks of disease appear to be closely linked to poor husbandry and management, and under intensive aquaculture conditions, disease can spread quickly through a production facility and result in high mortalities. Because of the potential and real impact of bacterial disease on *Morone* sp. aquaculture, eradication of one or more bacterial diseases from cultured populations may have a significant impact on the success of the bass farming industry.

Specific Research Objectives

Four objectives were established for this thesis, and the achievement of these objectives are described in the following chapters:

1) Develop a vaccine for piscine mycobacteriosis using the *M. marinum* 85A antigen (Ag85A).

2) Assess the degree and duration of the humoral and cellular immune responses generated by the vaccine.

3) Determine the effects of vaccine concentration on the immune responses generated.

4) Evaluate the degree of protection conferred by the vaccine against a live bacterial challenge with *M. marinum*.

Two hypotheses were tested by the vaccine work described in this thesis:

1) The vaccine will induce specific humoral and cellular immune responses and upregulate macrophage activity. Furthermore, higher vaccination doses will stimulate greater immune responses.

2) The vaccine will confer protection against live bacterial challenge with *M. marinum*. Higher vaccination doses will also produce more significant protection (i.e. lower splenic bacterial counts and longer survival post-challenge).

References

Arakawa, C.K., Fryer, J.L., 1984. Isolation and characterization of a new subspecies of *Mycobacterium chelonei* infections for salmonid fish. Helgolander Meeresuntersuchungen 37, 329-342.

Armitige, L.Y., Jagannath, C., Wanger, A.R., Norris, S.J., 2000. Disruption of the genes encoding antigen 85A and antigen 85B of *Mycobacterium tuberculosis* H37Rv: effect on growth in culture and in macrophages. Infect. Immun. 68, 767-778.

Aronson, J.D., 1926. Spontaneous tuberculosis in salt water fish. J. Infect. Dis. 39, 315-320.

Austin, B., Austin, D.A., 1993. *Mycobacterium* spp. In: Austin, B., Austin, D.A. (Eds.), Bacterial Fish Pathogens: Disease in Farmed and Wild Fish. Ellis Horwood, New York, pp. 45-50.

Backman, S., Ferguson, H.W., Prescott, J.F., Wilcock, B.P., 1990. Progressive panophthalmitis in chinook salmon, *Oncorhynchus tschawytscha* (Walbaum): a case report. J. Fish Dis. 13, 345-353.

Barker, L.P., George, K.M., Falkow, S., Small, P.L.C., 1997. Differential trafficking of live and dead *Mycobacterium marinum* organisms in macrophages. Infect. Immun. 65, 1497-1504.

Bartos, J.M., Sommer, C.V., 1981. *In vivo* cell mediated immune response to *M. tuberculosis* and *M. salmoniphilum* in rainbow trout (*Salmo gairdneri*). Dev. Comp. Immunol. 5, 75-83.

Bataillon, E., Dubard, L., Terre, L., 1897. Un nouveau type de tuberculose. Comptes Rendus Seances Societe Biologie 49, 446-449.

Belisle, J.T., Vissa, V.D., Sievert, T., Takayama, K., Brennan, P.J., Besra, G.S., 1997. Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. Science 276, 1420-1422.

Bentley-Hibbert, S.I., Quan, X., Newman, T., Huygen, K., Godfrey, H.P., 1999. Pathophysiology of antigen 85 in patients with active tuberculosis: Antigen 85 circulates as complexes with fibronectin and immunoglobulin G. Infect. Immun. 67, 581-588.

Beyer, T., Herrmann, M., Reiser, C., Bertling, W., Hess, J., 2001. Bacterial carriers and virus-like-particles as antigen delivery devices: role of dendritic cells in antigen presentation. Curr. Drug Targets Infect. Disord. 1, 287-302.

Blacklock, Z.M., Dawson, D.J., 1979. Atypical mycobacteria causing non-pulmonary disease in Queensland. Pathology 11, 283-287.

Borremans, M., De Wit, L., Volckaert, G., Ooms, J., De Bruyn, J., Huygen, K., Van Vooren, J-P., Stelandre, M., Verhofstadt, R., Content, J., 1989. Cloning, sequence determination, and expression of a 32-kilodalton-protein gene of *Mycobacterium tuberculosis*. Infect. Immun. 57, 3123-3130.

Boudinot, P., Blanco, M., de Kinkelin, P., Benmansour, A., 1998. Combined DNA immunization with the glycoprotein gene of viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus induces double-specific protective immunity and nonspecific response in rainbow trout. Virol. 249, 297-306.

Bragg, R.R., Huchzermeyer, H.F.A.K., Hanisch, M.A.M., 1990. *Mycobacterium fortuitum* isolated from three species of fish in South Africa. Onderstepoort J. Vet. Med. 57, 101-102.

Carlberg, J.M., Van Olst, J.C., Massingill, M.J., 2000. Hybrid striped bass: an important fish in US aquaculture. Aquacult. Mag. 26, 26-38.

Chan, K., Knaak, T., Satkamp, L., Humbert, O., Falkow, S., Ramakrishnan, L., 2002. Complex pattern of *Mycobacterium marinum* gene expression during long-term granulomatous infection. Proc. Nat. Acad. Sci. 99, 3920-3925.

Chen, S.C., Yoshida, T., Adams, A., Thompson, K.D., Richards, R.H., 1996. Immune response of rainbow trout to extracellular products of *Mycobacterium* spp. J. Aquat. Anim. Health 8, 216-222.

Chinabut, S., Limsuwan, C., Chanratchakool, P., 1990. Mycobacteriosis in the snakehead, *Channa striatus* (Fowler). J. Fish Dis. 13, 531-535.

Chinabut, S., Kanayati, Y., Pungkachonboon, T., 1994. Study of transovarian transmission of mycobacteria in *Betta splendens* Regan. In: Chou, L.M., Munro, A.D., Lam, T.J., Chen, T.W., Cheong, L.K.K., Ding, J.K., Hooi, K.K., Khoo, H.W., Phang, V.P.E., Shim, K.F., Tan, C.H. (Eds.), Proc. Third Asian Fisheries Forum. Asian Fisheries Society, Manila, Philippines, pp. 84-85.

Chinabut, S., 1999. Mycobacteriosis and nocardiosis. In: Woo, P.T.K., Bruno, D.W. (Eds.), Fish Diseases and Disorders: Viral, Bacterial and Fungal Infections. CAB International, New York, pp. 319-340.

Clark, H.F., Shepard, C.C., 1986. Effect of environmental temperatures on infection with *Mycobacterium marinum* (balnei) of mice and a number of poikilothermic species. J. Bacteriol. 86, 1057-69.

Colorni, A., 1992. A systemic mycobacteriosis in the European sea bass *Dicentrarchus labrax* cultured in Eilat (Red Sea) Bamidgeh. Israeli J. Aquacult. 44, 75-81.

Collins, C.H., Grange, J.M., Noble, W.C., Yates, M.D., 1985. *Mycobacterium marinum* infections in man. J. Hygiene 94, 135-149.

Conroy, G., Conroy, D.A., 1999. Acid-fast bacterial infection and its control in guppies (*Lebistes reticulatus*) reared on an ornamental fish farm in Venezuela. Vet. Rec. 144, 177-8.

Cruz, J.C., 1938. *Mycobacterium fortuitum* um novo bacilo acidoresistente patogenico para o homen. Acta Medical Rio de Janeiro 1, 297.

Daoust, P.Y., Larson, B.E., Johnson, G.R., 1989. Mycobacteriosis in yellow perch (*Perca flavescens*) from two lakes in Alberta. J. Wildl. Dis. 25, 31-37.

Denis, O., Tanghe, A., Palfliet, K., Jurion, F., van den Berg, T.P., Vanonckelen, A., Ooms, J., Saman, E., Ulmer, J.B., Content, J., Huygen, K., 1998. Vaccination with plasmid DNA encoding mycobacterial antigen 85A stimulates CD4+ and CD8+ T-cell epitopic repertoire broader than that stimulated by *Mycobacterium tuberculosis* H37Rv infection. Infect. Immun. 66, 1527-1533.

Dobos, K.M., Quinn, F.D., Ashford, D.A., Horsburgh, C.R., King, C.H., 1999. Emergence of a unique group of necrotizing mycobacterial diseases. Emerg. Infect. Dis. 5, 367-378.

Donnelly, J.J., Ulmer, J.B., Shiver, J.W., Liu, M.A. 1997. DNA vaccines. Ann. Rev. Immunol. 15, 617-648.

Donnelly, J.J., Liu, M.A., Ulmer, J.B., 2000. Antigen presentation and DNA vaccines. Am. J. Respir. Crit. Care Med. 162, S190-3.

Dulin, M.P., 1979. A review of tuberculosis (mycobacteriosis) in fish. Vet. Med. Small An. Clinician 74, 731-735.

El-Etr, S.H., Yan L., Cirillo, J.D., 2001. Fish monocytes as a model for mycobacterial host-pathogen interactions. Infect. Immun. 69, 7310-7317.

Evelyn, T.P.T., 1997. A historical review of fish vaccinology. In: Gudding, R., Lillehaug, A., Midtlyng, P.J., Brown, F. (Eds.), Fish Vaccinology. Developments in Biological Standardization. Karger, Basel, Switzerland, pp. 3-12.

Food and Agriculture Organization of the United Nations, 2000. Urban and periurban aquaculture. Regional Seminar on Feeding Asian Cities, Bangkok, Thailand, pp. 1-51.

Frost, P., Borsheim, K., Endresen, C., 1998. Analysis of the antibody response in Atlantic salmon against VP2 of infectious pancreatic necrosis virus (IPNV). Fish Shellfish Imm. 8, 447-456.

Garbe, T.R., Hibler, N.S., Deretic, V., 1996. Isoniazid induces expression of the antigen 85 complex in *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 40, 1754-1756.

Gilmore, R.D., Jr., Engelking, H.K., Manning, D.S., Leong, J.C., 1988. Expression in *Escherichia coli* of an epitope of the glycoprotein of infectious hematopoietic necrosis virus protects against viral challenge. Biotech. 6, 295-300.

Glaser, C.A., Angulo, F.J., Rooney, J.A., 1994. Animal-associated opportunistic infections among persons infected with the human immunodeficiency virus. Clin. Infect. Dis. 18, 14-24.

Gomez-Chiarri, M., Brown, L.L., Levine, R.P., 1996. Protection against *Renibacterium salmoninarum* infection by DNA-based immunization. In: Donaldson, E.M., MacKinlay, D.D. (Eds.), Aquaculture Biotechnology Symposium Proceedings, Physiology Section of the American Fisheries Society, pp. 155-157.

Hatai, K., Lawhavinit, O., Toda, K., Sugou, Y., 1993. Mycobacterium infection in pejerrey, *Odonthestes bonariensis* Cuvier & Valenciennes. J. Fish Dis. 16, 397-402.

He, J., Yin, Z., Xu, G., Gong, Z., Lam, T.J., Sin, Y.M., 1997. Protection of goldfish against *Ichthyophthirius multifiliis* by immunization with a recombinant vaccine. Aquaculture 158, 1-10.

Heckert, R.A., Elankumaran, S., Milani, A., Baya, A., 2001. Detection of a new *Mycobacterium* species in wild striped bass in the Chesapeake Bay. J. Clin. Microbiol. 39, 710-715.

Hedrick, R.P., McDowell, T., Groff, J., 1987. Mycobacteriosis in cultured striped bass from California. J. Wildl. Dis. 23, 391-395.

Heppell, J., Lorenzen, N., Armstrong, N.K., Wu, T., Lorenzen, E., Einer-Jensen, K., Schorr, J., Davis, H.L., 1998. Development of DNA vaccines for fish: vector design, intramuscular injection and antigen expression using viral hemorrhagic septicaemia virus genes as model. Fish Shellfish Immunol. 8, 271-286.

Heppell, J., Davis, H.L., 2000. Application of DNA vaccine technology to aquaculture. Adv. Drug Delivery Rev. 43, 29-43.

Hodson, R.G., 1989. Hybrid striped bass: Biology and life history. S. Regional Aquacult. Cent. Pub. 300, 1-4.

Hughes, K.P., Duncan, R.B., Smith, S.A., 2002. Renomegaly associated with a mycobacterial infection in summer flounder *Paralichthys dentatus*. Fish Pathol. 37, 83-86.

Ivanyi, J., Thole, J., 1994. Specificity and function of T- and B-cell recognition in tuberculosis. In: Bloom, B.R. (Ed.), Tuberculosis: Pathogenesis, Protection, and Control. ASM Press, Washington, D.C., pp. 437-458.

Jorgensen, J.B., Johansen, A., Stenersen, B., Sommer, A.I., 2001. CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (*Salmo salar* L.) leucocytes to produce supernatants with antiviral activity. Dev. Comp. Immunol. 25, 313-321.

Kanellos, T., Sylvester, I.D., Ambali, A.G., Howard, C.R., Russell, P.H., 1999. The safety and longevity of DNA vaccines for fish. Immunol. 96, 307-313.

Kent SeaTech Corporation, 2000. U.S. Production of hybrid striped bass during 1999. Kent SeaTech Corporation, San Diego, CA, pp. 1-15.

Knibb, W., Colorni, A., Ankaoua, M., Lindell, D., Diamant, A., Gordin, H., 1993. Detection and identification of a pathogenic marine mycobacterium from the European seabass *Dicentrarchus labrax* using polymerase chain reaction and direct sequencing of 16S rDNA sequences. Mol. Mar. Biol. Biotechnol. 2, 225-232.

Lansdell, W., Dixon, B., Smith, N., Benjamin, L., 1993. Isolation of several *Mycobacterium* species from fish. J. Aquat. An. Health 5, 73-76.

Leong, J.C., Fryer, J.L., 1993. Viral vaccines for aquaculture. Ann. Rev. Fish Dis. 3, 225-240.

Leong, J.C., Anderson, E., Bootland, L.M., Chiou, P.W., Johnson, M., Kim, C., Mourich, D., Trobridge, G., 1997. Fish vaccine antigens produced or delivered by recombinant DNA technologies. In: Gudding, R., Lillehaug, A. Midtlyng, P.J., Brown, F. (Eds.), Fish Vaccinology: Developments in Biological Standardization. Karger, Basel, Switzerland, pp. 267-277.

Lecocq-Xhonneux, F., Thiry, M., Dheur, I., Rossius, M., Vanderheijden, N., Martial, J., de Kinkelin, P., 1994. A recombinant viral haemorrhagic septicaemia virus glycoprotein expressed in insect cells induces protective immunity in rainbow trout. J. Gen. Virol. 75, 1579-1587.

Lorenzen, N., Lorenzen, E., Einer-Jensen, K., Heppell, J., Wu, T., Davis, H., 1998. Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss*, Walbaum) following DNA vaccination. Fish Shellfish Immunol. 8, 261-270.

Lorenzen, N., 1999. Recombinant vaccines: experimental and applied aspects. Fish Shellfish Immunol. 9, 361-365.

Lower, K., Poet, S., 2001. Use of enrofloxacin in the treatment of piscine mycobacteriosis. Compend. Contin. Ed. Practicing Vet. 692, 623-628.

Malin, A.S., Huygen, K., Content, J., Mackett, M., Brandt, L., Andersen, P., Smith, S.M., Dockrell, H.M., 2000. Vaccinia expression of *Mycobacterium tuberculosis*-secreted proteins: tissue plasminogen activator signal sequence enhances expression and immunogenicity of *M. tuberculosis* Ag85A. Microbes Infect. 2, 1677-1685.

Mor, N., 1985. Multiplication of *Mycobacterium marinum* within phagolysosomes of murine macrophages. Infect. Immun. 48, 850-852.

Naito, M., Matsuoka, M., Ohara, N., Nomaguchi, H., Yamada, T., 2000. The antigen 85 complex vaccine against experimental *Mycobacterium leprae* infection in mice. Vaccine 18, 795-798.

Nigrelli, R.F., Vogel, H., 1963. Spontaneous tuberculosis in fishes and in other cold-blooded vertebrates with special reference to *Mycobacterium fortuitum* Cruz from fish and human lesions. Zoologica 48, 131-143.

Noga, E.J., Wright, J.F., Pasarell, L., 1990. Some unusual features of Mycobacteriosis in the cichlid fish *Oreochromis mossambicus*. J. Comp. Pathol. 102, 335-344.

Noga, E.J., Kerby, J.H., King, W., Aucoin, D.P., Giesbrecht, F., 1994. Quantitative comparison of the stress response of striped bass (*Morone saxatilis*) and hybrid striped bass (*Morone saxitilis* x *Morone chrysops* and *Morone saxatilis* x *Morone americana*). Am. J. Vet. Res. 55, 405-409.

Noonan, B., Enzmann, P.J., Trust, T.J., 1995. Recombinant infectious hematopoietic necrosis virus and viral hemorrhagic septicemia virus glycoprotein epitopes expressed in *Aeromonas salmonicida* induce protective immunity in rainbow trout. Appl. Environ. Microbiol. 61, 3586-3591.

Ohara, N., Ohara-Wada, N., Kitaura, H., Nishiyama, T., Matsumoto, S., Yamada, T., 1997. Analysis of the genes encoding the antigen 85 complex and MPT51 from *Mycobacterium avium*. Infect. Immun. 65, 3680-3685.

Patti, J.M., Allen, B.L., McGavin, M.J., Hook, M., 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. Annu. Rev. Microbiol. 48, 585-617.

Pattyn, S.R., 1984. *Mycobacterium marinum*. In: Kubica, G.P., Wayne, L.G. (Eds.), The Mycobacteria: A Sourcebook Part B. Marcel Dekker, New York, pp. 1137-1139.

Plumb, J.A., 1994. Miscellaneous bacterial diseases. In: Plumb, J.A. (Ed.), Health Maintenance of Cultured Fishes: Principal Microbial Diseases. CRC Press, Inc., Boca Raton, FL.

Ramakrishnan, L., Falkow, S., 1994. *Mycobacterium marinum* persists in cultured mammalian cells in a temperature-restricted fashion. Infect. Immun. 62, 3222-3229.

Reno, P.W., 1998. Factors involved in dissemination of disease in fish populations. J. Aquat. An. Health 10, 160-171.

Rhodes, M.W., Kator, H., Kotob, S., van Berkum, P., Kaattari, I., Vogelbein, W., Floyd, M.M., Butler, W.R., Quinn, F.D., Ottinger, C., Shotts, E., 2001. A unique *Mycobacterium* species isolated from an epizootic of striped bass (*Morone saxatilis*). Emerg. Infect. Dis. 7, 896-899.

Rogall, T., Flohr, T., Böttger, E.C., 1990. Differentiation of *Mycobacterium* species by direct sequencing of amplified DNA. J. Gen. Microbiol. 136, 1915-20.

Rook, G.A.W., Hernandez-Pando, R., 1996. The pathogenesis of tuberculosis. An. Rev. Microbiol. 50, 259-284.

Sakanari, J.A., Reilly, C.A., Moser, M., 1983. Tubercular lesions in Pacific coast populations of striped bass. Trans. Am. Fish. Soc. 112, 565-566.

Schulze-Röbbecke, R., Fischeder, R., 1989. Mycobacteria in biofilms. Zentralblatt für Hygiene und Umweltmedizin 188, 385-390.

Shamsudin, M.N., Tajima, K., Kimura, T., Shariff, M., Anderson, I.G., 1990. Characterization of the causitive organism of ornamental fish mycobacteriosis in Malaysia. Fish Pathol. 25, 1-6.

Smith, S.A., 1997. Mycobacterial infections in pet fish. Semin. Avian Exot. Pet. 6, 40-45.

Stinear, T.P., Jenkin, G.A., Johnson, P.D.R., Davies, J.K., 2000. Comparative genetic analysis of *Mycobacterium ulcerans* and *Mycobacterium marinum* reveals evidence of recent divergence. J. Bacteriol. 182, 6322-6330.

Stoffregen, D.A., Bowser, P.R., Babish, J.G., 1996. Antibacterial chemotherapeutants for finfish aquaculture: a synopsis of laboratory and field efficacy and safety studies. J. Aquat. An. Health 8, 181-207.

Talaat, A.M., Reimschuessel, R., Trucksis, M., 1997. Identification of mycobacteria infecting fish to the species level using polymerase chain reaction and restriction enzyme analysis. Vet. Microbiol. 58, 229-237.

Talaat, A.M., Trucksis, M., Kane, A.S., Reimschuessel, R., 1999. Pathogenicity of *Mycobacterium fortuitum* and *Mycobacterium smegmatis* to goldfish, *Carassius auratus*. Vet. Microbiol. 66, 151-164.

Tanghe, A., Denis, O., Lambrecht, B., Motte, V., van den Berg, T., Huygen, K., 2000. Tuberculosis DNA vaccine encoding Ag85A is immunogenic and protective when administered by intramuscular needle injection but not by epidermal gene gun bombardment. Infect. Immun. 68, 3854-3860.

Thoen, C.O., Schliesser, T.A., 1984. Mycobacterial infections in cold-blooded animals. In: Kubica, G.P., Wayne, L.G. (Eds.), The Mycobacteria: A Sourcebook Part B. Marcel Dekker, New York, pp. 1297-1311.

Timur, G., Roberts, R.J., 1977. The experimental pathogenesis of focal tuberculosis in the plaice (*Pleuronectes platessa* L.). J. Comp. Path. 87, 83-87.

Van Duijn, C., 1981. Tuberculosis in fishes. Journal Small An. Practice 22, 391-411.

Velaz-Faircloth, M., Cobb, A.J., Horstman, A.L., Henry, S.C., Frothingham, R., 1999. Protection against *Mycobacterium avium* by DNA vaccines expressing mycobacterial antigens as fusion proteins with green fluorescent protein. Infect. Immun. 67, 4243-4250.

Vemulapalli, R., He, Y., Sriranganathan, N., Boyle, S.M., Schurig, G.G., 2002. *Brucella abortus* RB51: enhancing vaccine efficacy and developing multivalent vaccines. Vet. Microbiol. 90, 521-532.

Wallis, R.S., Perkins, M., Phillips, M., Joloba, M., Demchuk, B., Namale, A., Johnson, J.L., Williams, D., Wolski, K., Teixeira, L., Dietze, R., Mugerwa, R.D., Eisenach, K., Ellner, J.J., 1998. Induction of the antigen 85 complex of *Mycobacterium tuberculosis* in sputum: a determinant of outcome in pulmonary tuberculosis treatment. J. Infect. Dis. 178, 1115-1121.

Wolf, J.C., Smith, S.A., 1999. Comparative severity of experimentally induced mycobacteriosis in striped bass *Morone saxatilis* and hybrid tilapia *Oreochromis* spp. Dis. Aquat. Organ. 38, 191-200.

Wolke, R.E., Stroud, R.K., 1978. Piscine mycobacteriosis. In: Montali, R.J. (Ed.), Mycobacterial infections of zoo animals. Smithsonian Institution Press, New York, pp. 86-104.

Wood, J.W., Ordal, E.J., 1958. Tuberculosis in Pacific salmon and steelhead trout. Fish Commission Oregon 25, 1-38.