JOANNE LAILA MAKI
Ichthyophthirius multifiliis Infection and Elements of Mucosal Immunity in the Channel Catfish, (*Ictalurus punctatus*)
(Under the direction of Dr. HARRY W. DICKERSON)

We are using the channel catfish (*Ictalurus punctatus*) infected with the ciliated protozoan parasite *Ichthyophthirius multifiliis* to investigate the mechanisms and cellular elements of mucosal immunity. Experiments were conducted to determine by ELISA levels of parasite-specific antibody in sera and cutaneous mucus of fish immunized against *I. multifiliis*. The systemic immune response of fish following surface exposure to parasites or intraperitoneal injection of purified i-antigen resulted in significant levels of *I. multifiliis*-specific sera antibody (Ab) at five weeks that increased over time until the end of sampling at 14 weeks. Cutaneous mucus of both treatment groups contained Ab levels that were much lower than those detected in sera. Parasite-exposed fish had mucus Ab levels beginning at three weeks post-treatment that corresponded with clearing of infection. In both treatment groups, cutaneous mucus Ab levels were not synchronous with sera levels over time suggesting that fish mucosal Ab arise by a mechanism other than passive diffusion from serum. In a second study, ultrastructural analysis of resin-embedded channel catfish head kidney and skin by transmission electron microscopy (TEM) identified a specific population of cells that were labeled by goat anti-catfish immunoglobulin (Ig)-Fab-2 fragments and a biotinylated rabbit-Fab made against goat-Fab followed by avidin-gold beads. Labeling was limited to the cytoplasmic vesicles while other lymphocytes, macrophages and epidermal mucus cells were negative. The labeled cells were characterized as antibody-secreting cells (ASC) based on their morphology and staining pattern. During the course of these studies, trophonts were detected within the peritoneal cavities of fish infected by surface exposure. This result

implies that *I. multifiliis* may invade tissues other than the exterior mucosal tissues of fish. Taken together, the TEM results are the first report of ASC in channel catfish skin while the ELISA results suggest that cutaneous antibodies may arise by local synthesis. These data support the hypothesis of a separate mucosal immune system in fish as well as the importance of cutaneous Ab in protective immunity against *I. multifiliis*.

INDEX WORDS: *Ichthyophthirius multifiliis,* channel catfish (*Ictalurus punctatus*), immobilization antigen, immunoglobulin, mucosal immunity, lymphocytes, antibody secreting cells (ASC), antibody, kinetics, light chains, B-cells.

ICHTHYOPHTHIRIUS MULTIFILIIS INFECTION AND ELEMENTS OF MUCOSAL IMMUNITY IN THE CHANNEL CATFISH, *ICTALURUS PUNCTATUS*

by

JOANNE LAILA MAKI

B. S., West Virginia University, 1979

D.V.M., Louisiana State University, 1983

M.S., The University of Tennessee, Knoxville, 1991

A Dissertation Submitted to the Graduate Faculty of The University of Georgia

in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GA

2002

ICHTHYOPHTHIRIUS MULTIFILIIS INFECTION AND ELEMENTS OF MUCOSAL IMMUNITY IN THE CHANNEL CATFISH, *ICTALURUS PUNCTATUS*

by

JOANNE LAILA MAKI

Approved:

Major Professor: Harry W. Dickerson

Committee: Corrie C. Brown
Donald L. Dawe
Duncan C. Krause
David S. Peterson

Electronic Version Approved:

Gordhan L. Patel
Dean of the Graduate School
The University of Georgia
August 2002

DEDICATION

I dedicate this dissertation to Mark King and my family, including Ashley, Christopher and Haley King, who know first-hand what it is like to live with a scientist. In every instance they have been supportive, caring and ever tolerant.

I also dedicate this work to my Aunt Laila Ferenz and my cat, Toby, who both passed away during its preparation. Since Laila loved cats I'm sure she would approve of the sentiment and not mind sharing.

ACKNOWLEDGEMENTS

I would like to acknowledge and thank the following individuals and agencies for their contribution to this scientific effort:

My major professor, Dr. Harry Dickerson, and committee members Drs. Corrie Brown, Ray Damian, Donald Dawe, Duncan Krause and David Peterson.

Collaborators at the University of Alabama at Birmingham, Drs. Jerry McGhee, Frits van Ginkel, Ray Jackson and Dr. Norman Miller at the University of Mississippi Medical Center in Jackson, Mississippi.

Individuals at the University of Georgia including Mary Ard, Vicki Burnley, Dr. Karin Everett, Dr. Penny Gibbs, Dr. Natalia Guseva, Tian Lin, Jane Noe, Dr. Buddy Steffens, Dr. Xuting Wang and members of the Medical Microbiology Department.

I would also like to thank The National Institutes of Health for financial support and Dr. Gary Madonna for his part in assisting in the submission of my grant application.

TABLE OF CONTENTS

Page

CHAPTER 1

LITERATURE REVIEW

1. Introduction:

Greater than eighty percent of the Earth's organisms inhabit aquatic ecosystems in which fishes comprise the largest, most diverse vertebrate class of more than 20,000 species (129, 145). Within the scientific community, researchers study fish for numerous reasons. General areas of interest include the husbandry and culture of fish as food, monitoring fish in their natural habitat as environmental sentinels and more recently the development of fish as non-mammalian biomedical models.

I have chosen the channel catfish, *Ictalurus punctatus*, infected with *Ichthyophthirius multifiliis* to investigate the mechanisms of mucosal immunity in fish. Channel catfish have been raised in the southern United States as a food fish since the 1950's (95). This species has also served as a comparative animal research model in humoral and cellular immunology (31, 49). *Ichthyophthirius multifiliis*, a ciliated, protozoan parasite of freshwater fishes, was noted in Chinese carp as early as the Sung dynasty (AD 960-1279)(39). It was known to be a transmissible, infectious disease of cultured fish as early as the 1800's (64, 166). Today, *I. multifiliis* is considered to be one of the most economically important pathogens in US aquaculture (176, 177). Our laboratory has studied *I. multifiliis* as it infects the channel catfish for more than twenty

years (42). Efforts have focused on the parasite's molecular biology and the host's immune response with the goal of developing an effective vaccine against the parasite.

Fish surviving *I. multifiliis* infection do not develop disease upon re-exposure and this resistant state has been reported in multiple fish species (10, 82, 86, 123, 136, 169, 186). After exposure to *I. multifiliis*, the protective immune response includes the presence of immunoglobulin within fish skin and cutaneous mucus and the production of antibodies that immobilize the infective form of the parasite in vitro (25, 36, 197). Immobilizing antibodies within mucosal tissues play an important role in protection against *I. multifiliis* but their origin remains unknown.

Channel catfish primarily produce one form of immunoglobulin, an IgM-like tetramer of 750 kDa, that is found both in the sera and external secretions (14, 50, 112, 113, 134, 135). Considerable experimental evidence supports the hypothesis that antibodies detected in the cutaneous mucus of fish arise independently from serum antibody but the actual sites of production and pathways of secretion have yet to be elucidated (38, 61, 62, 92, 106-109, 139, 150-152, 167). Possible mechanisms of secretion include passive or facilitated transport from sera or local production within epithelia by antibody-secreting cells. Determining how antibodies are transported to the surface of *I. multifiliis*-immune fish is relevant because passive transfer studies using parasite-specific antibodies have demonstrated a physiological barrier between the peripheral circulation and the skin (29, 48, 49, 104). Results of previous work, to be discussed in Section 4.4, strongly suggest that antibodies do not passively diffuse from sera to mucosal surfaces. Therefore, facilitated transport or local antibody production appears to be the probable mechanism of cutaneous antibody secretion in fish.

Given that fish do not have lymph nodes, immunological events such as antigen presentation, lymphocyte activation and cytokine expression could occur within skin and gills following *I. multifiliis* infection. If this is the case, then these mucosal tissues may serve as immunological inductive sites. If skin and gills also contain memory lymphocytes and/or antibody-secreting cells, then they have the capacity to be immunological effector sites. Further investigations are needed to determine what immunological events occur in the skin and gills of *I. multifiliis*-immune fish. If inductive and effector functions occur in these tissues then our experimental model will provide direct access to both arms of the mucosal immune response.

To further develop the channel catfish infected with *I. multifiliis* as a research model, experiments were conducted to investigate several different aspects of the host's mucosal immune system. Specifically, concentrations of *I. multifiliis*-specific antibody were measured in the sera and cutaneous mucus of channel catfish following immunization by surface exposure or intraperitoneal injection of purified i-antigen and Freund's incomplete adjuvant (Chapter 2). In a second experiment, putative antibody-secreting cells (ASC) were identified within normal catfish head kidney and skin based on the labeling of cytoplasmic Ig by Fab-2 fragments of a goat antibody raised against channel catfish sera Ig (See Chapter 3). During the course of these studies, *I. multifiliis* parasites were detected in the peritoneal cavities of channel catfish exposed to theronts by surface contact (Chapter 4). This unexpected finding requires a re-examination of the current concept that *I. multifiliis* infection is limited to the superficial mucosa of fish skin, gills and buccal cavity.

Methods used to generate the data presented in this body of work include an enzyme-linked immunosorbent assay (ELISA), immunohistochemistry and transmission electron microscopy. Taken together, the results of these experiments support a hypothesis that the skin and cutaneous mucus are components of the channel catfish immune system and emphasize the importance of cutaneous antibody in the specific immune response against *I. multifiliis*. These findings also support the idea that cutaneous antibodies arise in fish skin and mucus by mechanisms other than passive diffusion from serum. Such work provides a foundation for future experiments to investigate cellular trafficking and antigen-specific lymphocyte activation within the skin. Elucidation of such mechanisms will lead to a better understanding of mucosal immunity in fish and application of these concepts to the development of a vaccine against *I. multifiliis*.

2. The Parasite - *Ichthyophthirius multifiliis:*

2.1 Phylogeny: (Greek: *Ichthyo*-, fish; *phthir*-, a louse; *multi*-, many; *fil*-, a thread). In 1876, Fouquet described and named *Ichthyophthirius multifiliis* placing it in the subkingdom *Protozoa* and phylum *Ciliophora* (64). Since that time, DNA and RNA sequencing have radically affected how scientists classify organisms and changes are ongoing at all levels of phylogenic organization. A new clade, *Aveolata*, was recently proposed as a subkingdom based on protein and DNA sequencing (8). It is comprised of three groups of organisms: the ciliates, the dinoflagellates, and the apicomplexans based on sequencing and congruent morphological characteristics (158). Within this new grouping, the phylum *Ciliophora* remains intact and continues to contain a very diverse assortment of parasitic and free-living ciliates. *I. multifiliis* is a well-studied member of the order *Hymenostomatida* (class *Oligohymenophorea*) and is closely related to the

facultative fish parasite *Ophryoglena catenula* and the saprophytic, or histophageous, genus *Tetrahymena* (195, 196). At this time, the only other confirmed member of the family *Ichthyophthiriidae* is a ciliated, parasite of salt-water fishes, *Cryptocaryon irritans*. Recent rRNA sequencing, however, suggests the life cycle and morphological similarities between these two species are actually an example of convergent evolution (51). The literature contains references claiming that *Cryptocaryon* was previously described in 1938 and should be referred to as *Ichthyophthirius marinus* (130).

Members of the phylum *Ciliophora* share several morphological characteristics including cilia and basal bodies, microtubules and fibrils, two nuclei (i.e., macro- and micro-) and a defined mouth structure, called a cytostome. *Ichthyophthirius* and *Ophryoglena* share a unique structure called the organelle of Lieberkuhn (47). In addition to this morphologic similarity, ribosomal sequencing also supports the pairing of these two species (196). *Oligohymenophorea* contains other fish pathogens in addition to *I. multifiliis* and *C. irritans*. Obligate parasites within this class, *Chilodonella* and *Trichodina* spp., colonize gills and skin and are capable of killing heavily infected fish. *Epistylis* and *Tetrahymena* are considered to be facultative parasites that are associated with healthy and moribund fish, respectively (117). Within a related class, *Litostomatea*, there are two genera are of veterinary importance. The only zoonotic ciliate, *Balantidium* coli, causes diarrhea in both pigs and man. A second genus, *Entodinium*, contains symbionts that are critical to ruminant digestion.

2.2 Life Cycle and Biology:

Ichthyophthirius multifiliis has a simple life cycle consisting of three developmental stages each of which is ciliated: the infective form (theront), the host-

associated form (trophont) and the encysted environmental form (tomont)(82). The motile theront (~30 x 60 μm in size) has limited energy reserves and remains infectious for approximately three days (47). Theronts exhibit a characteristic directional and spiraling swimming pattern that differentiates them from *Tetrahymena*, a free-living aquatic ciliate that morphologically resembles *I. multifiliis*. Theronts are also attracted to light (i.e., phototactic) and find their host by chemotaxis. Fish mucus has been suggested as a source of molecular signals for the parasite (20).

The life cycle of *I. multifiliis* is influenced by water temperature. A single round of replication occurs in 4-5 days at water temperatures of 20-24^{o}C (59). The parasite cannot survive in water temperature greater than 30^{o}C. At colder temperatures (<10^{o}C) parasite development is slowed and this phenomenon has been used for long-term propagation under laboratory conditions (131).

I. multifiliis has been reported to cause mortalities in feral and cultured fish from the Equator to the Arctic Circle (147, 181). Epizootics usually occur during spring and summer months when warmer water temperatures increase parasite replication rate and reduced levels of dissolved oxygen cause stress in fish populations. Other factors influencing the severity of infection include stocking density, water quality, and susceptibility of various fish species (95). Outbreaks often occur in closed systems such as aquaria and ponds following the introduction of new fish carrying parasites.

Lesions associated with *I. multifiliis* infection have been well characterized. Infections are typically limited to the skin, gills and buccal cavity (58, 84, 184). Within these tissues, infections cause localized lymphocyte infiltration, focal necrosis and varying degrees of epithelial proliferation (37). In severe cases, sloughing of the

epidermis has been observed. In addition to these common sites, *I. multifiliis* has been reported within the cerebral cavity, circumorbital clefts and nasal pits of a naturally infected carp hybrid and the peritoneal cavities of channel catfish (121, 181, 184). Under laboratory conditions, *I. multifiliis* has been shown to survive and grow within the peritoneal cavities of channel catfish. This observation was made in experiments where fish were injected with live parasites via the intraperitoneal route (44).

During invasion, theronts are thought to secrete enzymes that facilitate their entry into the epidermal layers of the skin, gills and buccal cavity (57). Transformation to the trophont stage includes loss of the organelle of Lieberkuhn (47). Trophonts increase in size from 40-60 microns to greater than 200 microns in two to three days at temperatures of 20-25^{o}C. The parasite becomes grossly visible and by five days, each trophont may be 500 microns or more in size. At this point in their development they leave the host (activated by unknown stimuli) to encyst in the environment as tomonts. Within 18-24 hours, at 20-25^{o}C, the tomont has completed encystment and eight or nine rounds of division resulting in 200-500 daughter cells, or tomites. Approximately 24-30 hours after exiting the host, infectious theronts exit the cyst thus completing the life cycle (47).

2.3 Diagnosis, Treatment and Control:

Fish infected with *I. multifiliis* often demonstrate an aberrant behavior called "flashing" (i.e., darting or making quick movements against objects in their environment). This behavior may be seen early in infection before parasites are visible on the exterior surface of the fish. A definitive diagnosis of *I. multifiliis* can be made by light microscopic identification of the host-associated form (i.e., trophont) by either skin scrapings or gill clips (69, 95). Mild infections may resolve without treatment but in

closed systems multiple rounds of replication usually result in heavy parasite loads and high mortality. Infected fish often die due to impaired respiration and disrupted osmoregulation (85).

Chemical therapeutic agents reduce the number of parasites in the surrounding environment but do not kill host-associated forms of the parasite. Since parasites are shed from the host for an extended period, compounds must be added repeatedly to the water to ensure killing of all the parasites. Intervals between treatments as well as duration of treatment also vary since water temperature affects the rate at which the parasite develops. Raising the water temperature to 25-26^{o}C accelerates parasite replication and thus shortens overall treatment time. Not all fish can tolerate these higher temperatures, however, and raising water temperature may not always be practical (69). Frequent water changes also decrease the number of theronts re-infecting fish.

Effective chemical treatments for *I. multifiliis* include copper sulfate, potassium permanganate malachite green, salt and formalin (95). Commercial aquaculture operations are limited to using only those compounds approved for food fish and are hampered by the cost of treating large volumes of water for extended periods of time. The Federal Drug Administration (FDA) is currently reviewing the use of copper sulfate and potassium permanganate as parasiticides in food fish (63, 178). Malachite green, a teratogen, is restricted for use in ornamental fish in the United States.

Quarantine treatments that are effective against external parasites, including I. multifiliis, are non-iodized salt (1-2%) dips for 30 minutes or up to 0.3% in the water indefinitely. Solutions containing malachite green and formaldehyde (34-38% solution) are commercially available (69, 164). As with other infectious organisms, the best way to

control *I. multifiliis* is by prevention. Quarantine and preventative treatments can decrease the chance of introducing this parasite into a naïve fish population. Once *I. multifiliis* has become established in a closed system, however, it can be very difficult to eradicate.

2.4. Antigenic proteins:

As mentioned previously, fish recovering from mild *I. multifiliis* infections generally do not exhibit clinical disease upon subsequent exposure to the parasite. Immune fish produce antibodies in sera and mucus against abundant surface membrane proteins called immobilization antigens (i-antigens)(27, 45). *I. multifiliis* i-antigens range in size from 50-70 kDa and are glycosyl-phosphatidyl-inositol (GPI)-anchored proteins analogous to the i-antigens of *Tetrahymena* isolate (30, 101). In vitro immobilization assays, in which theronts are mixed with immune fish sera or i-antigen-specific monoclonal antibodies have identified five reactivity patterns, or serotypes of *I. multifiliis* (25, 27, 46, 48). Immobilization requires the cross-linking of i-antigens by bivalent antibody molecules and does not occur with monovalent Fab fragments (28). Polyclonal sera made in rabbits or mice, as well as *I. multifiliis* immune fish sera, immobilize only one parasite serotype (46, 197). Monoclonal antibodies raised against purified i-antigens immobilize only parasites that express that particular epitope on their surface and do not bind to reduced forms of the antigen on Western blot. This observation suggests that a certain percentage of antigenic determinants involved in immobilization are conformational (103).

3. The Host - *Ictalurus punctatus:*

3.1 Phylogeny:

Ictalurus punctatus belongs to a phylogenic group, or clade, of diverse fish species called *Teleostei* within a much larger class *Osteichthyes*. An internal bony skeleton, unique musculature of the jaw and gill region and a lung/swim bladder that connects to the gastro-intestinal tract define this class of fish. Within *Osteichthyes*, there is a sub-class of ray-finned fish (*Actinopterygii*) that are very diverse and consist of over 22,000 species in approximately 40 orders and 400 families. The majority of ray-finned fish are teleosts and thus bony fish comprise over one-half of the existing species of vertebrates (197). Although bony fishes are sometimes considered a homogenous group, they are much more heterogeneous than mammals (143).

A teleostean lineage called *Otophysi*, or *Ostariophysi* in older literature, contains over 25% of modern fishes of which the majority live in fresh water and is made up of *Sluriformes*, or catfishes; *Cyprinoformes* (carp, minnows, suckers and the distantly related zebrafish); *Characiformes* (tetras, pirhanas, hachetfish and pencilfish) and *Gymnotiformes* (electric eels and knifefish)(120). *Sluriformes* currently contains 30 families of which the channel catfish belongs to the family *Ictaluridae*. This one family contains over 2,000 species of catfishes that inhabit the inland and coastal waters of all continents (129).

3.2 Immune System:

Fishes are the earliest phylogenic animal group with an immune system showing clear similarities with mammals and birds (183). The teleost immune system has a strong innate component with acute and chronic inflammatory reactions, the ability to produce

antigen-specific antibodies and cellular immune functions as evidenced by graft rejection and anaphylaxis (143). Differences between fish and higher species include a limited number of immunoglobulin isotypes, poor affinity maturation of antibody after secondary antigen presentation and temperature dependence of both the humoral and cellular immune responses (146). In fish and other poikilotherms, the innate response is particularly important due to the influence of lower temperatures on the development of acquired immunity (195).

Bony and cartilaginous fish have organized lymphoid organs and the immunoglobulin molecule is a consistent characteristic within this vertebrate class (183). Fish larvae hatch at different stages of lymphocyte development, but progenitor lymphocytes are generally thought to originate in the embryonic kidney and migrate to the thymus. Following thymic proliferation, populations of lymphocytes migrate back to the head kidney, which in adult fish remains the equivalent of the mammalian bone marrow (143). Teleost lymphocytes mature and become antigen-specific by humoral (B-cell) or cellular (T-cell) pathways that have yet to be defined.

Since fish do not have lymph nodes or organized lymphoid tissue other than the thymus, spleen and kidneys, discrete secondary lymphoid sites are considered lacking. The capacity of fish intestine, skin and gills to respond to antigenic stimulation is especially of interest in the development of vaccines administered by immersion. The skin and gills are physical, physiological and immunological interfaces between fish and their aquatic environment. The apparent emphasis of innate immunity in fish and other aquatic species reinforces the importance of mucosal surface integrity and the vital role mucus plays in protecting them from physical damage, toxins or pathogenic organisms.

3.2.1 Innate Immunity:

3.2.1.1 Mucus and soluble factors:

In fish, the first line of immunological defense is a thick mucus layer that covers their skin, gills, buccal cavity and digestive tract. This physical barrier not only helps to reduce attachment of bacteria, parasites and fungi but is also critical for osmotic homeostasis and oxygen exchange (183). Excessively thick mucus or "shedding" of mucus is considered a general sign of disease and is commonly observed in parasitized or stressed fish. Cutaneous mucus is rich in glycosylated proteins and contains enzymes and lectin-like proteins that interact with specific carbohydrates common to bacterial cell walls (91). Agglutinins, factors distinct from immunoglobulins that adhere to foreign proteins, are also found in fish mucus. Lectins are thought to form the basis of self/non-self recognition in invertebrates, but their role in fish immunity remains speculative (5). Opsonins and histone-like proteins found in the surface mucus of fish are capable of bacterial lysis and parasite killing (21, 132, 195). Other nonspecific immune factors that occur in fish mucus include lysozyme, carbonic anhydrase, transferrin, and C-reactive protein (134, 156). The antibacterial nature of specific peptides in fish and amphibian skin are studied for possible application in human medicine (160).

Complement pathways occur in both fish sera and mucus. The alternative pathway and the lectin pathway are present in sera of the most primitive, jawless fish (*Agnatha*), and multiple lytic pathways has been proposed as possible mechanisms by which fish expand their innate capacity for immune recognition (170). The alternative pathway is evidenced by the lysis of bacteria incubated with normal fish sera and killing of the fish ectoparasite, *Gyrodactylis salaris* (76, 154). Complement has been implicated

in the lysis of *I. multifiliis* theronts in vitro but it requires an extended incubation time to result in parasite death (21). Complement may facilitate the killing of *I. multifiliis* within immune fish skin but this phenomenon has not been demonstrated experimentally.

Non-specific immune factors found in channel catfish by sequence analysis of cDNA libraries or in vitro culturing of cells include an interleukin 1-like protein (53, 159) and interferon β gene sequences (191). Cytokine-like proteins such as colony-stimulating factor (CSF), macrophage activating factor (MAF), interleukin-2, and tumor necrosis factor (TNF-α) have been described in other fish species (143).

3.2.1.2 Non-specific cells:

Neutrophils, granulocytes and macrophages are the predominant cell types present during the acute inflammatory phase of fish (3, 183). The inflammatory process and associated cellular elements also have been described in channel catfish skin (34). In general, granulomas are common sequelae to chronic inflammation and can effectively sequester pathogens within infected tissue (171).

The inflammatory responses associated with *I. multifiliis* infection include epithelial hyperplasia of the gills and epidermal layers of the skin and cellular infiltration. Cellular destruction and focal necrosis are also observed in areas adjacent to the parasite. Histological changes in the skin associated with infection include a predominant neutrophilic infiltration followed by increasing numbers of lymphocytes, eosinophils, and basophils. Such cells are often are seen near parasites before they leave the fish at 5-6 days post-infection (36). In previously exposed fish, neutrophils and macrophages were observed within the epidermis following subsequent infection with *I. multifiliis* theronts (37, 83). Thickened epidermal layers with disrupted cellular architecture and multiple

layers of alarm cells were observed in *I. multifiliis*-immune channel catfish three weeks after re-exposure to the parasite (Maki, unpublished). This general proliferative response, termed epithelial hyperplasia, appears subsequent to parasite re-infection and has been proposed to be the result of a non-specific immune stimulation (184).

Certain populations of fish leukocytes are capable of nonspecific cellular immune activities in a manner similar to mammalian natural killer cells (143). Channel catfish neutrophils become activated during bacterial infections (3, 60). Cells exhibiting natural killer cell characteristics, referred to as natural cytotoxic cells (NCC), kill transformed mammalian cells in vitro and have been proposed to be involved in the cytotoxic killing of *T. pyriformis* and *I. multifiliis* (55, 56, 70, 71). Although in the case of *I. multifiliis*, no direct experimental evidence supports this hypothesis. Agranular mononuclear leukocytes of rainbow trout (*Oncorhynchus mykiss*) and salmon (*Salmo salar*) are also capable of nonspecific killing of cells recognized as being foreign (i.e., virus infected fish cells or transformed mammalian cells) and are thought to fulfill similar functions in these species (72).

3.2.2 Acquired Immunity:

3.2.2.1. Lymphoid Organs:

In teleosts, there is a great degree of heterogeneity in the histological appearance and organization of lymphoid organs (146). As a general statement, the thymus, kidneys (i.e., head kidney and renal kidney) and spleen contain the majority of fish lymphocytes and are considered to be the components of the central lymphoid system (175). Fish do not have lymph nodes, a bursa of Fabricius, bone marrow or Peyer's patches (183).

The channel catfish thymus is located in the dorsal wall of the pharynx and does not change in size as the fish develops (73). It contains lymphocytes, which are termed thymocytes based strictly on their location, and seeds other organs with these cells early in development (173). The teleost thymus is located between the dermis and epithelium of the opercular cavity; it is relatively close to the external environment and thought to be directly involved in humoral and cellular immunity (24). In fish larvae, the thymus is separated from the external environment by one layer of cells and is thought to be the primary organ involved in early immune responses such as tolerance (146).

The head kidney produces erythroid, lymphoid and myeloid cells (174). Early in development the primordial kidney separates into the head kidney (i.e., pronephros) and the renal kidney (i.e., mesonephros). Channel catfish larger than four centimeters no longer have excretory tubules in the head kidney. In adult fish, the cell population of the head kidney is remarkably similar to the bone marrow of higher vertebrates (198, 199). In addition to hematopoiesis, this organ contains interrenal and chromaffin cells that are homologous to the mammalian adrenal cortex (73). The renal kidney contains distinct nephrons and renal tubules and islands of hematopoetic tissue similar to that found in the head kidney. The degree to which the channel catfish renal kidney contributes to the immune response is not known. It is of interest, however, that the posterior cardinal vein directly connects this organ to the head kidney.

The catfish spleen functions primarily as a site of red blood cell storage and destruction. As in mammals, there are areas of erythrocyte-rich red pulp and more basophilic white pulp in the spleen, but the degree of hematopoetic tissue is far less than in head kidney. Clusters of macrophages observed in the head kidney, renal kidney, and

spleen of normal channel catfish are known as melanomacrophage centers and are considered to be antigen-storage depots (73). Dendritic cells, commonly observed in the splenic germinal centers of higher vertebrate species, have not been demonstrated in fish (7).

3.2.2.2 Cellular Elements of Immunity:

Fish leukocytes are identified primarily on their histological staining patterns and morphological similarities to mammalian white blood cells (54, 201). Channel catfish peripheral blood leukocytes are identified as thrombocytes, lymphocytes, neutrophils, monocytes, eosinophils and basophils based on morphology and staining patterns at the light microscopic level (190). Cytochemical staining of thymus, head kidney, renal kidney and spleen identified cell populations with staining characteristics similar to mammalian lymphocytes (141).

Neutrophils, lymphocytes, monocytes and thrombocytes of the channel catfish and sea bass (*Dicentrarchus labrax*) have been characterized at the ultrastructural level (23, 60, 124, 125). The hematopoetic organs (kidneys and spleen) of the African catfish (*Clarias gariepinus*) contain erythroblasts, immature neutrophils, and plasma cells in addition to the above mentioned cells (11-13). Undifferentiated lymphoid-like cells lines capable of indefinite division and antibody secretion have been cultured from the channel catfish (127). Lymphocytes with cytoplasmic Ig have been identified within channel catfish head kidney and skin and characterized at the ultrastructural level (Maki, unpublished).

3.2.2.3 Humoral Immunity:

The immunoglobulin (Ig) molecules of both cartilaginous and bony fish occur as paired heavy and light chain structures (H_2L_2)(1, 143). Two forms of Ig (a 700-kDa hexamer and a 350-kDa tetramer) have been isolated from the sera of a marine species, the sheepshead (*Archosargus probatocephalus*)(110). Channel catfish predominantly produce an IgM-like tetrameric molecule that migrates as eight different molecular weight subpopulations when electrophoresed under denaturing conditions on polyacrylamide gels containing sodium dodecyl sulfate and cross-linked with 0.7% N,N'-diallyltartardiamide)(SDS-DPAGE)(113, 114). The presence of more than one heavy chain has been proposed based on the molecular weight of different sizes of Ig molecules purified from sera of immunized catfish by affinity chromatography (113, 141). Monoclonal antibodies made against channel catfish Ig heavy chains (3D11, 3E11 and 1H6) have been used to identify three different populations of the 700 kDa tetramer Ig in catfish sera (116). Monoclonal antibodies made against Ig light chains (3F12 and 1G7) recognize two populations in sera that electrophorese as three bands under reducing conditions by SDS-PAGE. The light chains (F and G) are found in sera at a ratio of 60:40 which is similar to human κ:λ light chains (111, 114). Changes in F and G light chain expression in the channel catfish over time have been correlated with maturation of the immune response but not with mucosal versus systemic Ig (113).

In carp (*Cyprinus carpio*), a separate subclass of mucosal Ig has been proposed based on reactivity of intestinal lymphocytes with Ig-specific monoclonal antibodies (149). Mucosal Ig in the channel catfish, however, has been determined to be identical to the form found in sera (115). Channel catfish immunoglobulin contains a J-chain

component that is thought to assist in the assembly of the molecule (126). The J-chain, a 15 kDa protein, exists in animals as primitive as sea cucumbers, oysters, earthworms and slugs. It is thought to play a role in immunoglobulin polymerization and transport across epithelial cells but its exact function is unknown (172). A second class of Ig, considered to be IgD-like, has recently been described in the channel catfish based on cDNA sequence analysis; however, its function remains unknown (192).

An interesting model has been proposed in which various "redox" forms of the same tetrameric Ig molecules of channel catfish (previously described in Lobb et al., 1983 and 1985) are produced rather than different Ig isotypes. It is postulated that a variation in the degree of disulfide cross-linking during synthesis results in Ig molecules with different affinities for opsonization, complement-mediated lysis and Fc binding on phagocytic cells (97).

The humoral antibody response of fish differs from the mammalian response in three ways. First, the rate at which antibody appears in sera is influenced by environmental temperature. Second, upon re-exposure to an antigen, there is a quantitative increase in sera antibody production but no isotype switch. Third, the change in antibody affinity over time is limited (7, 98). These differences fit a model in which the innate and primary immune responses are more important in fish with less emphasis on the secondary or anamnestic response (99, 195). Nevertheless, immunological memory occurs in fish indicating that memory B and T cells exist, even if their location is not yet known (146, 189).

3.2.2.4 Cell-mediated Immunity:

Other than the non-specific cytotoxic cells (NCC), channel catfish lymphoid cells associated with cellular immunity have not been identified as distinct populations (143). Negative panning of catfish peripheral blood mononuclear cells with immunoglobulin-specific mAbs produces a mixed population of surface Ig negative cells (sIg-) that have T cell-like activities in vitro (186). These cells are responsive to concanavalin A but not lipopolysaccharide (2, 162). A monoclonal antibody (CfT1) has been used to identify a population of lymphoid cells in channel catfish blood, spleen, and head kidney that may represent a thymocyte-derived subpopulation. These cells proliferate in vitro when exposed to concanavalin A. The antibody-binding epitope on the surface of these cells has been identified as a single chain protein of 35,000 MW (137). T cell receptor (TCR) and major histocompatibility protein gene sequences have been amplified from channel catfish cDNA libraries using polymerase chain reactions (PCR) (186, 193, 200).

4. Mucosal Immunity:

4.1 Phylogeny:

In mammals, the mucosal immune response is a highly orchestrated event involving multiple cell types requiring communication between the innate and specific immune systems. The end result is a humoral and cell-mediated response with immunological memory that is capable of protecting mucus-covered epithelial surfaces in an antigen-specific manner. A fascinating aspect of mucosal immunity is that antigen stimulation at one site may evoke a systemic immune response that protects at distant mucosal surfaces (165, 182). This finding is important because it suggests that mucosal

vaccination might yield a broader immune response with fewer adverse effects that those associated with systemic administration of the same antigen.

There seems to be at least two phylogenetic similarities between the external mucosal surfaces of aquatic and land-dwelling vertebrates, namely mucus-covered epithelial cells and the polymeric Ig molecule with a J-chain. It has been speculated that cooperation between the mucosal B-cell system and secretory epithelia is evolutionarily conserved in so many species because secretory antibodies are necessary for survival (18). Understanding the mechanisms of antigen-specific B-cell homing and synthesis of immunoglobulin at mucosal effector sites is of potential interest because such findings have direct application in vaccine development for both mammalian as well as non-mammalian species (i.e., birds and fish).

4.2 Mucosal immunity in mammals:

During the 1960's, the IgA dimer with its associated secretory protein was characterized (15, 33, 163). This event stimulated a strong interest in mucosal antibody and it role in intestinal health. Subsequent work on polymeric IgA and IgM transport across intestinal epithelia showed that the process was receptor mediated (16). In 1984, an article published in Nature provided direct evidence that the J-chain and secretory component were necessary to transport immunoglobulin through epithelial tissue (17). Since that time the secretory component has been renamed as the polymeric immunoglobulin receptor (pIgR) and the cellular events involved in IgA secretion have been confirmed (94, 179).

In humans, secretory immunoglobulin (IgA and a lesser amount of IgM) is synthesized in B cells located in lymphoid tissue associated with mucosal surfaces

(MALT). Both the dimeric IgA and pentameric IgM molecules contain a single J-chain molecule that is covalently bound to the Ig heavy chain (9). The J-chain binds to the pIgR on the epithelial cell and the complex is transported through the cell's cytoplasm. The Ig molecule is released on the apical surface of the epithelial cell following fusion of the pIgR-Ab complex with the cell membrane. Upon its release, a portion of the receptor remains with the Ig molecule (i.e., the secretory piece) which protects its from degradation within the enzymatic environment of the intestinal lumen or surface mucus (17-19, 75, 94).

In mammals, the MALT is divided into various components that are thought to communicate via a common mucosal B cell network. It is considered a secondary lymphoid organ along with the encapsulated spleen and lymph nodes (148). Lymphoid tissue associated with the gastrointestinal tract, including the Peyer's patches and M cells, are referred to as GALT. The GALT also includes the mesenteric lymph nodes and solitary lymphoid follicles in the appendix and distal large intestine (19). The nasal mucosa and tonsils located in mammalian nasal passages are referred to as NALT. Other systems include BALT (i.e., bronchi-associated), SALT (i.e., skin-associated), SGALT (i.e., salivary gland) and lymphoid tissues associated with the genitourinary tract (148, 165, 168).

Three characteristics that define the functions of the mammalian mucosal immune defense system are immune exclusion, immune regulation and immune elimination (18). These functions occur through the interplay of mucosal inductive and effector sites regardless of their location in the body. Inductive sites are those tissues from which antigen-primed B cells and up-regulated T cells are transported to regional lymph nodes

via lymph and blood. The manner in which antigen is presented (i.e., dendritic cells) and secondary signals (i.e., specific cytokines) within this microenvironment is thought to preferentially drive local proliferation and IgA secretion by differentiated and sensitized B-cells. Secretory antibody (IgA and IgM) is translocated through the epithelial layers of the effector sites and its passage is enhanced by the presence of mediators of the innate immune system. This cooperative, non-inflammatory defense system in which antigen-specific antibodies interact with non-specific defense molecules on mucosal surfaces is termed "immune exclusion" (18).

Regulation of the mucosal immune response occurs within the deeper layers of the epithelia or submucosa in concert with local and distant sites of lymphocyte priming and proliferation. Such interactions within the mucosal network are the sites of "immune regulation" and employ many yet to be discovered mechanisms including lymphocyte trafficking. When immune exclusion fails, antigens entering the epithelia are eliminated by local inflammatory reactions including the presence of antigen-specific antibody and activated T cells. This response culminates in the degradation and processing of the foreign proteins with the end result being "immune-elimination" (18).

The vast majority of research on the cellular components and mechanisms of mucosal immunity has been preferentially slanted towards mammalian systems. While the first steps were being taken in understanding mammalian mucosal immunity, parallel research was initiated in fish with the goal of developing immersion vaccines against bacterial pathogens. Investigations have focused on improving antigen uptake and generating humoral immune responses that were protective against challenge with a particular pathogen (7, 62, 128, 152). The common goal of both mammalian health and

aquaculture-based research is to understand how long-term, protective immunity can be induced at various mucosal surfaces. Studying phylogenic similarities and differences among the mucosal immune systems of various vertebrate species also may lead to information on mechanisms of mucosal immunity not attainable through the study of any one species alone.

4.3 Evidence of mucosal immunity in fish:

Scientists proposed more than thirty years ago that fish have a mucosal immune system (14, 50, 61). Mucosal tissues in teleosts that could serve as potential sites of lymphoid activity are the intestines, skin and gill. The majority of data supporting a separate mucosal immune system in fish is based on the fact that antigen-specific antibody occurs in mucus secretions. Immunoglobulin has been demonstrated in the bile, cutaneous mucus or gill mucus of plaice (*Pleuronectes platessa*), sheepshead (*Archosargus probatocephalus*), channel catfish (*Ictalurus punctatus*), ayu (*Plectoglossus altivelis*), carp (*Cyprinus carpio*), and brook trout (*Salvelinus fontinalis*)(61, 92, 107, 119, 149). Eggs of channel catfish and tilapia (*Oreochromis mossabicus*) are thought to contain immunoglobulin by maternal transfer (77, 169). Antigen-specific antibodies have also been demonstrated in the cutaneous mucus, bile and intestine after oral administration of antigens to plaice and carp (61, 149).

The presence of lymphocytes within mucus-covered tissues infers a capacity to generate a mucosal immune response. In channel catfish, lymphocytes were observed in surface mucus (134) and low numbers of lymphocytes have been identified in epidermal cell suspensions by flow cytometry (Maki, unpublished). Lymphocytes and immunoglobulin-containing cells have also been identified in the skin of rainbow trout

(138, 139). Lymphoid cells have been identified at the light microscopic level in the intestine of carp, goldfish, rainbow trout and tilapia (41, 52, 66, 151, 153). Intestinal B-lymphocytes have been labeled by monoclonal antibodies in carp, rainbow trout and channel catfish (40, 116, 151). Channel catfish intestinal lymphocytes have recently been characterized by flow cytometry, histochemistry and cytochemical staining (79). Approximately 5% of cells isolated from juvenile channel catfish intestine by enzyme degradation were designated as B cells, as labeled by an Ig-specific mAb, and >64% of cells were labeled by a mAb as neutrophils. The low number of B cells identified in normal intestine was proposed to be indicative of low antibody production. The authors hypothesize that if antibodies are present in fish intestinal secretions that they most likely arise from sera following antigen stimulation or inflammation. In every species studied to date, fish do not appear to have organized lymphoid tissue in their intestines and the degree to which this tissue contributes to the immune response under normal conditions is not known. Antigen delivery via anal intubation to carp, however, resulted in an immunological response in cutaneous mucus that was detected by ELISA (152).

Immersion, dermal application, oral and anal intubation are methods that have been used experimentally to induce mucosal immune responses in fish (4, 100, 115, 119, 152, 167). Studies exposing fish to various antigens by immersion result in variable degrees of protection (as measured by challenge with virulent pathogens) and may not always result in the production of antigen-specific antibodies. The effectiveness of bath immunization is influenced by many factors including size and age of fish, physical size of antigen, water temperature, salinity/osmolarity, duration and replication of antigen

administration. Regardless of species, however, the skin and gill are considered to be the primary sites of antigen uptake (128).

The skin and gills have not been extensively studied as sites of antigen-specific immune activity. A polyclonal rabbit antibody was used to identify Ig-containing cells in the skin of rainbow trout after antigen exposure by immersion (138, 139). Cutaneous mucus antigen-specific antibody has been demonstrated in channel catfish and rainbow trout (115, 135, 149, 165, 188) and in the gill mucus of brook trout (119). Mucus antibody levels are typically several orders of magnitude lower than in sera. In channel catfish, for instance, total mucus Ig was found to be influenced by age, antigen exposure and was detected in nanogram quantities compared to milligrams in sera (202). It is apparent that immunoglobulin, whether it is antigen-specific or constitutive in nature, occurs in fish secretions. Although the mechanisms of mucosal antibody secretion in fish are not known at this time, its function can be considered to the same as it is in mammals (i.e., immune exclusion).

4.4. Systemic and mucosal immunity against *I. multifiliis*:

The channel catfish infected with *I. multifiliis* is an appropriate system to study the general characteristics of mucosal immunity but also the antigen-specific immune response against a virulent fish pathogen. We are interested in learning how fish develop immunity against *I. multifiliis* and specifically the role antibody plays in preventing infection. In addition to surface exposure, channel catfish have been immunized against *I. multifiliis* by intraperitoneal injection of live parasites, intraperitoneal injection of purified i-antigens with adjuvant, intramuscular injection of DNA encoding *I. multifiliis* i-antigen genes and injection of recombinant *Tetrahymena* expressing *I. multifiliis* i-

antigens (22, 27, 44, 105, 188). Regardless of route used, *I. multifiliis*-specific antibodies are produced in the sera and surface mucus of immunized fish (122). Serum antibodies levels are easily measured by immobilization assays, but detection of cutaneous mucus antibodies require more sensitive assays such as ELISA (187). *I. multifiliis*-specific antibody can be detected in surface mucus by immobilization but the mucus sample must be concentrated prior to analysis (197).

Based on experimental evidence using i-antigen-specific mAbs, a model of antibody-mediated protection against *I. multifiliis* has been proposed in which antibodies repel or immobilize parasites in the skin of immune fish (49). Passive transfer studies of *I. multifiliis*-specific antibodies have demonstrated that a physiological barrier exists between the peripheral circulation and the cutaneous mucosa (104). In these experiments, murine IgM-class monoclonal antibodies (mAbs) directed against the i-antigens of *I. multifiliis*, as well as the IgM tetramer in *I. multifiliis*-immune fish sera were administered via the intraperitoneal route to *I. multifiliis*-infected fish to effect passive protection. These large molecules failed to enter the cutaneous mucus but were detected in peripheral blood. Interestingly, IgG isotype mAbs, also directed against i-antigens, were transferred to the surface of the fish and caused the host-associated form of the parasite to exit the fish (28, 104). These results strongly suggest that *I. multifiliis* antibody is important in protection. The presence of *I. multifiliis* antibody in catfish sera and mucus also correlates positively with protection against infection (188). Additional laboratory evidence supporting this model includes the observation of theronts exiting the tail fin of immune fish within two hours of infection (35, 186).

Immunization studies have shown that intraperitoneal injection of live theronts induces a protective immune response in channel catfish against more than one *I. multifiliis* serotype. Fish immunized against G1.1 (serotype B) and G2 (serotype C) theronts by surface exposure developed immunity that prevented subsequent infection by each heterologous strain (102). In another study, fish immunized against G3 (serotype D) or G4 (serotype C) were also protected against virulent heterologous challenges that killed non-immunized control fish as well as *Tetrahymena*-injected fish within 12 days (93). Sera from fish in both experiments did not immobilize, or weakly immobilized, the heterologous strain of *I. multifiliis* in vitro. These results suggest that immunization by one parasite serotype induce a degree of cross-protection against other serotypes. Although immobilization assays using *I. multifiliis* immune fish sera are preferentially serotype dependent, these results indicate that cross-protective antigens do exist and to ensure protection against multiple serotypes, *I. multifiliis* vaccines must generate similar cross-protective immune responses.

The extent to which cell-mediated immunity is involved in protection against *I. multifiliis* has not been determined. Goldfish (*C. auratus*) immunized against *I. multifiliis* by intraperitoneal injection of live parasites developed a delayed hypersensitivity response (161). In carp, injection of corticosteroids to fish previously immunized against *I. multifiliis* abrogated protection. In other studies, the administration of steroids to *I. multifiliis*-immune channel catfish demonstrated a residual degree of protection compared to naïve fish (87, 88, 90). Channel catfish exposed multiple times to theronts followed by a single intraperitoneal injection of triamcinolone acetate were less resistant to infection

than those not receiving the steroid, but sera and mucus *I. multifiliis* antibodies were not significantly different between groups (197).

This model does not, however, exclude the interaction of other components of the immune system in generating the protective immune response against *I. multifiliis*. Innate factors, such as complement and local inflammatory reaction, may contribute to the cellular and humoral events occurring within fish skin. In immune fish, complement may serve to decrease the amount of antibody required to repel or immobilize the parasites. Binding of antibody to the invading organism may facilitate its killing by cytotoxic cells (i.e., NCC) within the skin. If these events occur in fish skin, however, *I. multifiliis*-specific antibody remains a central component in protective immune response against this parasite.

Studying the channel catfish infected with the protozoan parasite *I. multifiliis* provides us with an excellent opportunity to explore multiple facets of antigen presentation, processing and lymphocyte stimulation. By following the parasite-specific immune response over time, the location of memory lymphocytes and signals required for their activation may be identified. This model may also provide answers towards understanding how the innate immune system interacts with cells of the humoral and cell-mediated immune response to generate protection at mucus-covered surfaces. Such knowledge will be useful in the development of effective mucosal vaccines.

4.5 Vaccine Development:

I. multifiliis is one of six infectious agents currently responsible for the majority of aquaculture losses in the United States. Other diseases causing significant mortality in commercial fish operations are enteric septicemia (ESC), columnaris disease,

proliferative gill disease (PGD), winterkill and channel catfish virus (CCV)(176, 177). Enteric septicemia and columnaris are caused by bacteria, *Edwardsiella ictaluri* and *Cytophaga columnaris* (old name: *Flexibacter columnaris*), respectively (155). Recent efforts to develop vaccines against these pathogens include a modified, live vaccine consisting of a mutated strain of *E. ictaluri* (RE-33) that has a modified lipopolysaccharide membrane component (100).

Efforts are underway to develop vaccines against *C. columnaris*, however, other factors (i.e., immunosupression and concurrent disease) are thought to contribute to *C. columnaris* systemic infections and fish mortalities. The agent of PGD has been identified as a myxozoan parasite that requires an oligochete worm as an intermediate host. Therefore, secondary host eradication programs may precede or preclude PGD vaccine development. Winterkill is actually a complex syndrome that occurs during cold weather and may be related to poor water quality, co-existing parasite or bacterial infections. An invasive fungus, *Saprolegnia*, commonly colonizes affected fish but the role it plays in the disease is unknown at this time (95). Efforts are underway to develop vaccines against CCV, a herpesvirus, including a DNA vaccine encoding surface glycoprotein genes (133). In years past, conventional methods of vaccine production yielded effective formalin-killed bacterins against *Vibrio anguillarum* (i.e., vibriosis) and *Yersinia ruckeri* (i.e., enteric redmouth disease). However, biotechnological advances such as DNA vaccines or live-vectored vaccines may hold the key to controlling *I. multifiliis* and other pathogenic agents that are not amenable to traditional vaccine technology.

Early experiments evaluating *Tetrahymena pyriformis* as a heterologous vaccine against *I. multifiliis* met with limited success. Fish demonstrated partial protection to *I. multifiliis* after surface exposure, intraperitoneal injection of live organisms or injection with soluble *T. pyriformis* cilia preparations (43, 67, 68, 194). Intramuscular injection of soluble ciliary preparations resulted in *T. pyriformis*-agglutinating sera antibodies but protection against subsequent *I. multifiliis* infection was not determined (144). The concept of using *Tetrahymena sp*. alone as a heterologous *I. multifiliis* vaccine was seriously questioned after intraperitoneal injection of live *T. pyriformis* induced lymphoid stimulation in carp but did not protect fish from infection and sera from these fish failed to immobilize *I. multifiliis* theronts in vitro (89). In another experiment, heat-inactivated sera from *I. multifiliis*-exposed channel catfish did not react with *Tetrahymena* cilia in immunoblot assays (26). It has been suggested that exposure of fish to *Tetrahymena* may induce low levels of immunity by stimulation of non-specific innate immune factors (21).

In addition to surface exposure, fish have been immunized against *I. multifiliis* by intraperitoneal injection of live parasites, intraperitoneal injection of purified i-antigens with adjuvant, intramuscular injection of DNA encoding *I. multifiliis* i-antigen genes and injection of recombinant *Tetrahymena* expressing *I. multifiliis* i-antigens (22, 27, 44, 65, 105, 187). Two of these methods involving recombinant techniques (i.e., *I. multifiliis* DNA vaccines and the recombinant *Tetrahymena* vaccines) are described below.

2.5.1 DNA vaccines:

DNA vaccines, consisting of plasmids containing the genes of the desired expression product under control of suitable promoters are being evaluated in fish (81, 96). Reporter gene expression (i.e., luciferase and lacZ) were documented in rainbow

trout and zebrafish using an eukaryotic expression vector and a cytomegalovirus (CMV) promoter (80). Rainbow trout were protected against infection with viral hemorrhagic septicemia virus (VHSV) after immunization with DNA constructs containing the rhabdovirus envelope glycoprotein (G) gene sequences (118) and against the retrovirus hematopoetic necrosis virus (IHNV) when injected with the IHNV glycoprotein gene (6, 32). Seventy eight percent of channel catfish injected with DNA encoding two open reading frames of channel catfish herpes virus (IHV-1) also survived viral challenge (133). Thus far, the majority of DNA vaccine candidates being tested in fish have been against viral disease agents.

Partial protection against *I. multifiliis* has been demonstrated in channel catfish following immunization with DNA constructs encoding the 55-kDa i-antigen of the G5 serotype under the control of the CMV promoter (187). The vaccine construct consisted of the full-length i-antigen gene or a truncated form lacking coding sequence for either the N-terminal signal peptide or the C-terminal GPI anchor. The constructs were administered to fish intramuscularly as a single dose of 10, 1.0 or 0.1 μg of plasmid DNA or two doses of 1.0 μg/fish at a 2-week interval. Immunized fish produced low levels of i-antigen-specific antibodies in their sera two weeks after treatment and the relative survival rate between test groups and negative controls after parasite exposure were statistically significant (187).

DNA vaccines hold great promise for potential use in aquaculture in that they are inexpensive to manufacture, remove potential hazards associated with modified-live vaccines and are very stable (81). One potential problem is a general concern about administering genetically modified organisms (GMOs) to species raised for human

consumption. Cattle, swine and other livestock producers are addressing the same issue. Achievements or setbacks experienced during licensure of DNA vaccines in other species will impact their use in aquaculture.

2.5.2 Subunit and live-vectored vaccines:

Creating a subunit *I. multifiliis* vaccine is technologically challenging due to the anomalous codon usage that occurs in ciliates (142). Previous efforts to mutate and clone *I. multifiliis* i-antigen gene sequences into bacterial plasmids generated encouraging preliminary results (157). A cDNA fragment (1.2-kb) of the gene encoding the 48-kDa i-antigen of the G1 serotype (IAG48[G1]) was mutated by PCR primer mutagenesis. Ciliate gene sequences read as stop codon by *E. coli* (TAA and TAG) were changed to encode glutamine. The expressed protein ran as a 23-kDa product on SDS-PAGE and was detected by Western blot using rabbit antiserum against *I. multifiliis* serotype G1. Using a different approach, a 316-bp gene fragment of the 48-kDa i-antigen of the G1 serotype was generated from six synthetic oligonucleotides and cloned into an *E. coli* expression vector as a fusion protein. Sera collected from goldfish (*C. auratus*) immunized with the recombinant protein and Freund's complete adjuvant bound to *I. multifiliis* proteins on Western blot. Immunized fish were partially protected against a weakly virulent *I. multifiliis* challenge (78).

Recently, a full-length *I. multifiliis* gene product (IAG52A[G5/CC])(i.e., encoding the 52/55 kDa i-antigen of G5 (serotype D isolate) was generated by assembly polymerase chain reaction (PCR). Briefly, groups of oligonucleotides encoding pieces of the i-antigen template gene were combined in a set of nine reactions. The products of these reactions were combined in pairs to amplify intervening sequences. The PCR

product was synthesized by combining reactions in a step-wise fashion. An original set of nine reactions was reduced to one reaction in which the full-length product was amplified and purified. The cDNA encoding the entire G5 i-antigen gene was cloned into *E. coli* and mammalian cell expression vectors. It has been expressed in cell culture (COS-7 cells) and is being evaluated as a potential DNA vaccine in channel catfish (105).

A more novel approach to develop a vaccine against *I. multifiliis* involves expressing the i-antigen gene sequences in *Tetrahymena thermophila*, a free-living ciliate that is easily cultured under laboratory conditions. Expression of the *I. multifiliis* gene product is accomplished by biolistic bombardment of live, drug-sensitive *T. thermophila* mutants with gold beads coated with linearized plasmid DNA containing the i-antigen gene sequence. Homologous recombination and clonal selection, in the presence of paclitaxel, results in a population of *T. thermophila* transformants expressing the *I. multifiliis i-antigen* gene product (65).

Recombinant *T. thermophila*, expressing *I. multifiliis* i-antigen proteins, have been administered to channel catfish by intraperitoneal injection and immersion. Recent experiments have used organisms transformed with the *I. multifiliis* i-antigen gene and DNA encoding an inducible promoter from the metallothionein gene of *T. thermophila*. Adding cadmium to the *Tetrahymena* cultures during propagation induces expression of the i-antigen gene product. This induction system resulted in a >30-fold increase in the amount of *I. multifiliis* i-antigen expressed compared to previous constructs using the CMV promoter (187). Fish immunized with two doses of live recombinant *Tetrahymena*, encoding either (MTT.G1)-serotype A or (MTT.G5.1)-serotype D i-antigens by intraperitoneal injection two weeks apart produced serum antibodies that immobilized *I.*

multifiliis theronts in vitro and recognized unreduced forms of the protein on Western blots. Homologous *I. multifiliis* challenges resulted in 80-100% survival of immunized fish while 100% of control fish died (187).

Subunit or live-vectored *I. multifiliis* vaccines have an advantage over conventional injectable vaccines in their flexibility to be potential administered via the oral route or by immersion. The *T. thermophila* system provides a method to inexpensively produce large quantities of i-antigen and deliver the antigen as a live vaccine. These results not only demonstrate that *I. multifiliis* genes expressed in recombinant *T. thermophila* can induce a protective immune response in fish but they are also represent a significant advance in the development of a commercially viable recombinant vaccine against *I. multifiliis*.

4.5. Summary:

The majority of commercial fish vaccines are currently administered by injection into the peritoneal cavity or immersion (74, 185). However, the advent of new technology such as DNA vaccines, adjuvants other than aluminum hydroxide or oil emulsions, novel delivery mechanisms including live-vectored vaccines are being incorporated into the aquaculture industry (155). As the biotechnology revolution in human and veterinary medicine progresses, new technologies will be adapted to prevent diseases in fish.

As the world's population grows and further demands are placed on existing supplies of fish and aquatic organisms, financial interest in raising fish for food will also increase. A dramatic change is already occurring in that aquaculture is currently the largest and fastest growing sector of agriculture in the United States (177). *I. multifiliis* has been and continues to be an economically important pathogen in this industry.

Previous work in our laboratory has identified potential vaccine antigens and demonstrated the importance of i-antigens-specific antibodies in the protective immune response of fish against *I. multifiliis*. Recent experiments have shown potential success of DNA and live-vectored vaccines against this protozoan parasite. Results of studies presented in this body of work address the presence of a mucosal immune system in fish and measure *I. multifiliis* antibody in cutaneous mucus after specific routes of immunization. Taken together, an understanding of the molecular biology of the parasite and an understanding of the host's mucosal immune response will lead to the development of a successful vaccine against *I. multifiliis*.

REFERENCES

1. **Acton, R. T., P. Weinheimer, S. Hall, W. Niedermeier, E. Shelton, and J. Bennett.** 1971. Tetrameric immune macroglobulin in three orders of bony fishes. Proc Natl Acad Sci USA. **68(1):**107-111.
2. **Ainsworth, A. J., C. Dexiang, and T. Greenway.** 1990. Characterization of monoclonal antibodies to channel catfish, *Ictalurus punctatus*, leukocytes. Vet Immunol Immunopathol. **26:**81-92.
3. **Ainsworth, A. J.** 1992. Fish granulocytes: morphology, distribution and function. Ann Rev Fish Dis. **2:**123-148.
4. **Ainsworth, A. J., C. D. Rice, and L. Xue.** 1995. Immune responses of channel catfish, *Ictalurus punctatus* (Rafinesque) after oral or intraperitoneal vaccination with particulate or soluble *Edwardsiella ictaluri* antigen. J of Fish Dis. **18:**397-409.

5. **Alexander, J. B., and G. A. Ingram.** 1992. Non-cellular non-specific mechanisms in fish. Ann Rev Fish Dis. **2:**249-279.

6. **Anderson, E. D., D. V. Mourich, S. C. Fahrenkrug, S. LaPartra, J. Shepherd, and J. A. Leong.** 1996. Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Mol Mar Biol Biotechnol. **5:**114-122.

7. **Arkoosh, M., and S. Kaattari.** 1991. Development of immunological memory in rainbow trout (*Oncorhynchus mykiss*). I. An immunochemical and cellular analysis of the B cell response. Dev Comp Immunol. **15:**279-293.

8. **Baldauf, S. L., A. Roger, I. Wenk-Siefert, and W. Doolittle.** 2000. A kingdom-level phylogeny of eukaryotes based on combined protein data. Science. **290:**972-977.

9. **Bastian, A., H. Kratzin, K. Eckart, and N. Hilschmann.** 1992. Intra- and inter-chain disulfide bridges of the human J chain in secretory immunoglobulin A. Biol Chem Hoppe Seyler. **373:**1255-1263.

10. **Beckert, H., and R. Allison.** 1964. Some host responses of white catfish to *Ichthyophthirius multifiliis* Fouquet. Proceedings of the South State Assoc of Game and Fish Comm. **18:**438-441.

11. **Boomker, J.** 1979. The haemocytology and histology of the haemopoietic organs of South African fresh water fish. I. The haemopoietic organs of *Clarias gariepinus* and *Sarotherodon mossambicus*. Onderstepoort J Vet Res. **46:**217-222.

12. **Boomker, J.** 1981a. The haemocytology and histology of the haemopoietic organs of South African fresh water fish. III. The leucocytes, plasma cells and macrophages of *Clarias gariepinus* and *Sarotherodon mossambicus*. Onderstepoort J Vet Res. **48:**185-193.

13. **Boomker, J.** 1981b. The haemocytology and histology of the haemopoietic organs of South African fresh water fish. IV. Ultrastructure of some cells of *Clarias gariepinus* and *Sarotherodon mossambicus*. Onderstepoort J Vet Res. **48:**195-205.

14. **Bradshaw, C. M., A. S. Richard, and M. M. Sigel. 1971**. IgM antibodies in fish mucus. Proc Soc Exp Biol Med. **136:**1122-1124.

15. **Brandtzaeg, P., I. Fjellanger, and S. T. Gjeruldsen.** 1968. Immunoglobulin M: local synthesis and selective secretion in patients with immunoglobulin A deficiency. Science. **160:**789-791.

16. **Brandtzaeg, P.** 1974. Mucosal and glandular distribution of immunoglobulin components. Differential localization of free and bound SC in secretory epithelial cells. J Immunol. **112:**1553-1559.

17. **Brandtzaeg, P., and H. Prydz.** 1984. Direct evidence for an integrated function of the J chain and secretory component in epithelial transport of human polymeric IgA. Nature. **311:**71-73.

18. **Brandtzaeg, P., I. Nina Farstad, F.-E. Johansen, H. C. Morton, I. N. Norderhaug, and T. Yamanaka.** 1999a. The B-cell system of mucosa and exocrine glands. Immunol Rev. **171:**45-87.

19. **Brandtzaeg, P., E. Baekkevold, I. Farstad, F. Jahnsen, F. -E. Johansen, E. Nilsen, and T. Yamanaka.** 1999b. Regional specialization in the mucosal immune system: what happens in the microcompartments? Immunol Rev. **20(3):**141-151.

20. **Buchmann, K., and M. E. Nielsen.** 1999. Chemoattraction of *Ichthyophthirius multifiliis* (*Ciliophora*) theronts to host molecules. Int J Parasitol. **29:**1415-143.

21. **Buchmann, K., J. Sigh, C. Neilsen, and M. Dalgaard.** 2001. Host responses against the fish parasitizing ciliate *Ichthyophthirius multifiliis*. Vet Parasitol. **100:** 105-116.

22. **Burkart, M. A., T. G. Clark, and H. W. Dickerson.** 1990. Immunization of channel catfish, *Ictalurus punctatus* Rafinesque, against *Ichthyophthirius multifiliis* (Fouquet): killed versus live vaccines. J of Fish Dis. **13:**401-410.

23. **Cannon, M., H. Mollenhaur, T. Eurell, D. Lewis, A. Cannon, and C. Tompkins**. 1980. An ultrastructural study of the leucocytes of the channel catfish, (*Ictalurus punctatus*). J of Morphol. **164:**1-23.

24. **Chilmonczyk, S.** 1992. The thymus in fish: development and possible function in the immune response. Ann Rev Fish Dis. **2:**181-200.

25. **Clark, T. G., H. W. Dickerson, J. B. Gratzek, and R. C. Findley.** 1987. In vitro response of *Ichthyophthirius multifiliis* to sera from immune catfish. J Fish Biol. **31:**203-208.

26. **Clark, T. G., H.W. Dickerson, and R. C. Findley.** 1988. Immune response of channel catfish to the ciliary antigens of *Ichthyophthirius multifiliis*. Dev Comp Immunol. **12:**581-594.

27. **Clark, T. G., T. Lin, and H. W. Dickerson.** 1995. Surface immobilization antigens of *Ichthyophthirius multifiliis*: their role in protective immunity. Ann Rev Fish Dis. **5:**113-131.

28. **Clark, T. G., T. L. Lin, and H. W. Dickerson.** 1996. Surface antigen cross-linking triggers forced exit of a protozoan parasite from its host. Proc Natl Acad Sci USA. **93:**6825-6819.

29. **Clark, T. G., and H. W. Dickerson.** 1997. Antibody-mediated effects on parasite behavior: evidence of a novel mechanism of immunity against a parasitic protist. Parasitol Today. **13:**477-480.

30. **Clark, T. G., Y. Gao, J. Gaertig, X. Wang, and G. Cheng.** 2001. The I-antigens of *Ichthyophthirius multifiliis* are GPI-Anchored Proteins. J Eukaryol Microbiol. **48(3):**332-337.

31. **Clem, L.W., J.E. Bly, C. F. Ellsaesser, C. J. Lobb, and N. W. Miller.** 1990. Channel catfish as an unconventional model for immunological studies. J Exp Zool Suppl. **4:**123-125.

32. **Corbeil, S., S. E. Lapatra, E. D. Anderson, J. Jones, B. Vincent, Y. L. Hsu, and G. Kurath.** 1999. Evaluation of the protective immunogenicity of the N, P, M, NV and G proteins of infectious hematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*) using DNA vaccines. Dis Aquat Org. **39(1):**29-36.

33. **Crabbe, P. A., A. O. Carbonara, and J. F. Herman.** 1965. The normal human intestinal mucosa as a major source of plasma cells containing γA-immunoglobulin. Lab Invest. **14(3):**235-248.

34. **Croll, N., and J. Grizzle.** 1998. Ultrastructure of leukocytes in acutely inflamed dermis and muscle of channel catfish at different temperatures. J Aquat Animal Health. **10:**381-389.

35. **Cross, M. L., and R. A. Matthews.** 1992. Ichthyophthiriasis in carp, *Cyprinus carpio* L.: fate of parasites in immunized fish. J of Fish Dis. **15:**497-505.

36. **Cross, M. L.** 1993a. Antibody binding following exposure of live *Ichthyophthirius multifiliis* (*Ciliophora*) to serum from immune carp, *Cyprinus carpio*. Dis Aquat Org. **17:**159-164.

37. **Cross, M. L., and R. A. Matthews.** 1993b. Localized leukocyte response to *Ichthyophthirius multifiliis* establishment in immune carp, *Cyprinus carpio* L. Vet Immunol Immunopathol. **38:**341-158.

38. **Cross, M. L., and R. A Matthews.** 1993c. *Ichthyophthirius multifiliis* Fouquet (*Ciliophora*): the location of sites immunogenic to the host *Cyprinus carpio* (L.). Fish and Shellfish Immunol. **3:**13-24.

39. **Dashu, N., and L. Lien-Siang.** 1960. Studies on the morphology and life cycle of *Ichthyophthirius multifiliis* and its control, with a description of a new species. Acta Hydrobiology Sinica **2:**213-215.

40. **Davidson, G. A., A. E. Ellis, and C. J. Secombes.** 1993. Route of immunization influences the generation of antibody secreting cells in the gut of rainbow trout (*Oncorhynchus mykiss*). Dev Comp Immunol **17:**373-376.

41. **Davina, J., G. T. Rijkers, J. H. Rombout, and P. M. Lucy.** 1980. Lymphoid and non-lymphoid cells in the intestine of cyprinid fish. pp. 129-140. *In*

Development and Differentiation of Vertebrate Lymphocytes. J. D. Horton, ed., Elsevier/North-Holland Biomedical Press, New York, US.

42. **Dickerson, H. W., D. L. Dawe, J. B. Gratzek, J. Brown, and S. W. Pyle.** 1981. Induction of *Ichthyophthirius multifiliis* Fouquet infections in channel catfish *Ictalurus punctatus* Rafinesque: standardization of the procedure. Dev Biol Stand. **49:**331-336.

43. **Dickerson, H. W., J. Brown, D. L. Dawe, and J. B. Gratzek.** 1984. *Tetrahymena pyriformis* as a protective antigen against *Ichthyophthirius multifiliis* infection: comparison between isolates and ciliary preparations. J Fish Biol. **24:**523-528.

44. **Dickerson, H. W., A.L. Lohr, and J. B. Gratzek.** 1985. Experimental intraperitoneal infection of the channel catfish, *Ictalurus punctatus* (Rafinesque), with *Ichthyophthirius multifiliis* (Fouquet). J Fish Dis. **8:**139-142.

45. **Dickerson, H. W., T. G. Clark, and R. C. Findley.** 1989. *Ichthyophthirius multifiliis* has membrane-associated immobilization antigens. J Protozool. **36:**159-164.

46. **Dickerson, H. W., T. G. Clark, and A. A. Leff.** 1993. Serotypic variations among isolates of *Ichthyophthirius multifiliis* based on immobilization. J Euk Micro. **40:**816-820.

47. **Dickerson, H.W., and D.L. Dawe.** 1995. *Ichthyophthirius multifiliis* and *Cryptocaryon irritans* (Phylum *Ciliophora*), pp. 181-221. *In* P.T.K. Woo (ed.) Fish Diseases and Disorders. CAB International, Wallingford, UK.

48. **Dickerson, H. W., and T. G. Clark.** 1996. Immune response of fishes to ciliates. Ann Rev Fish Dis. **6:**107-120.

49. **Dickerson, H. W., and T. G. Clark.** 1998. *Ichthyophthirius multifiliis* a model of cutaneous infection and immunity in fishes. Immunol Rev. **166:**377-384.

50. **Diconza, J. J., and W. J. Halliday.** 1971. Relationship of catfish serum antibodies to immunoglobulin in mucus secretions. Aust J Exp Biol Med Sci. **49:**517-519.

51. **Diggles, B. K., and R. D. Adlard.** 1995. Taxonomic affinities of *Cryptocaryon irritans* and *Ichthyophthirius multifiliis* inferred from ribosomal RNA sequence data. Dis Aquat Org. **22:**39-43.

52. **Doggett, T. A., and J. E. Harris.** 1991. Morphology of the gut associated lymphoid tissue of *Oreochromis mossambicus* and its role in antigen absorption. Fish and Shellfish Immunol. **1:**213-227.

53. **Ellsaesser, C. F., and L. W. Clem.** 1994. Functionally distinct high and low molecular weight species of channel catfish and mouse IL-1. Cytokine. **6(1):**10-20.

54. **Ellis, A.** 1977. The leucocytes of fish: a review. J Fish Biol. **11:**453-491.

55. **Evans, D. L., S. S. Graves, D. Cobb, and D. L. Dawe.** 1984. Nonspecific cytotoxic cells in fish (*Ictalurus punctatus*) II. Parameters of target cell lysis and specificity. Dev Comp Immunol. **8:**303-312.

56. **Evans, D., and L. Jaso-Friedmann.** 1992. Nonspecific cytotoxic cells as effectors of immunity in fish. Ann Rev Fish Dis. **2:**109-121.

57. **Ewing, M. S., and K. M. Kocan.** 1985. *Ichthyophthirius multifiliis* (Ciliophora) invasion of gill epithelium. J Protozool. **32(2):**305-310.

58. **Ewing, M. S., and K. M. Kocan.** 1986. *Ichthyophthirius multifiliis* (Ciliophora) development in gill epithelium. J Protozool. **33(3):**369-374.

59. **Ewing, M. S., M. E. Lynn, and S. A. Ewing.** 1986. Critical periods in development of *Ichthyophthirius multifiliis* (*Ciliophora*) populations. J Protozool. **33(3):**388-391.

60. **Finco-Kent, D., and R. Thune.** 1987. Phagocytosis by catfish neutrophils. J Fish Biol. **31(Suppl A):**41-49.

61. **Fletcher, T. C., and P. T. Grant.** 1969. Immunoglobulin in the serum and mucus of the plaice (*Pleuronectes platessa*). Biochem J. **115:**65p.

62. **Fletcher, T. C., and A. White.** 1973. Antibody production in the plaice (*Pleuronectes platessa* L.) after oral and parenteral immunization with *Vibrio anguillarum* antigens. Aquaculture. **1:**417-428.

63. **Food and Drug Administration.** 1998. Aquaculture Drugs (A Chemical Hazard) Chapter 11. *In* Fish and Fishery Hazards Control Guide, Second Edition, pp. 115-131. FDA, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, DC.

64. **Fouquet, D.** 1876. Note sur une espece d'infusiores parasites des poisson d'eau douce. Arch Zool Exp Gen. **5:**159-165.

65. **Gaertig, J., Y. Gao, T. Tishgarten, T. G. Clark, and H. W. Dickerson.** 1999. Surface display of a parasite antigen in the ciliate *Tetrahymena thermophila*. Nature Biotech. **17:**462-465.

66. **Georgopoulou, U., and J. M. Vernier.** 1986. Local immunological response in the posterior intestinal segment of the rainbow trout after oral administration of macromolecules. **10(4):**529-537.

67. **Goven, B. A., D. L. Dawe, and J. B. Gratzek.** 1980. Protection of channel catfish, Ictalurus punctatus Rafinesque, against *Ichthyophthirius multifiliis* Fouquet by immunization. J of Fish Biol. **17:**311-316.

68. **Goven, B. A., D. L. Dawe, and J. B. Gratzek.** 1981. Protection of channel catfish (*Ictalurus punctatus*) against *Ichthyophthirius multifiliis* (Fouquet) by immunization with varying doses of *Tetrahymena pyriformis* (Lwoff) cilia. Aquaculture. **23:**269-273.

69. **Gratzek, J. B., E. B. Shotts, and D. L. Dawe.** 1992. Diseases and parasites of aquarium fish. pp. 227-268. *In* J.B. Gratzek and J. R. Matthews (eds.), Aquariology: the Science of Fish Health Management. Tetra Press, Blacksburg, VA.

70. **Graves, S., D. L. Evans, and D. L. Dawe.** 1985. Anti-protozoan activity of nonspecific cytotoxic cells (NCC) from the channel catfish *Ictalurus punctatus*. J Immunol. **134:**78-85.

71. **Graves, S., D. L. Evans, D. L. Dawe**. 1985. Mobilization and activation of nonspecific cytotoxic cells (NCC) in the channel catfish (*Ictalurus punctatus*) infected with *Ichthyophthirius multifiliis*. Comp Immunol Microbiol Infect Dis. **8:**43-51.

72. **Greenlee, A. R., R. A Brown, and S. S. Ristow.** 1991. Nonspecific cytotoxic cells of rainbow trout (*Oncorhynchus mykiss*) kill YAC-1 targets by both necrotic and apoptotic mechanisms. Dev Comp Immunol. **15(3):**153-164.

73. **Grizzle, J. M., and W. A Rogers.** 1976. Anatomy and Histology of the Channel Catfish. Auburn University, Agriculture Experiment Station. Auburn Printing, Inc., Auburn, AL.

74. **Gudding, R., A. Lillehaug, and O. Evansen.** 1999. Recent developments in fish vaccinology. Vet Immunol Immunopathol. **72(1-2):**203-212.

75. **Hanson, L. A., and P. Brandtzaeg.** 1993. The discovery of secretory IgA and the mucosal immune system. Immunol Today. **14:**416-417.

76. **Harris, P., A. Soleng, and T. Bakke.** 1998. Killing of *Gyrodactylus salaris* (*Platyhelminthes, Monogenea*) mediated by host complement. Parasitol. **117:**137-143.

77. **Hayman, J. R., and C. J. Lobb.** 1993. Immunoglobulin in the eggs of the channel catfish (*Ictalurus punctatus*). Dev Comp Immunol. **17:**241-248.

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

79. **Hebert, P., A. J. Ainsworth, and B. Boyd.** 2002. Histological enzyme and flow cytometric analysis of channel catfish intestinal tract immune cells. Dev Comp Immunol. **26:**53-62.

80. **Heppell, J., N. Lorenzen, N. Armstrong, T. Wu, E. Lorenzen, K. Einer-Jensen, and H. Davis.** 1998. Development of DNA vaccines for fish: vector

design, intramuscular injection and antigen expression using viral hemorrhagic septicemia virus genes as a model. Fish Shellfish Immunol. **8:**271-286.

81. **Heppell J., and H. Davis.** 2000. Applications of DNA vaccine technology to aquaculture. Adv Drug Dev. Rev. **43:**29-43.

82. **Hines, R. S., and D. T. Spira.** 1973. *Ichthyophthirius multifiliis* (Fouquet) in the mirror carp, *Cyprinus carpio* L. I. Course of infection. J. Fish Biol. **5:**385-392.

83. **Hines, R. S., and D. T. Spira.** 1973. *Ichthyophthirius multifiliis* (Fouquet) in the mirror carp, *Cyprinus carpio* L. II. Leukocyte response. J. Fish Biol. **5:**527-534.

84. **Hines, R. S., and D. T. Spira.** 1974a. *Ichthyophthirius multifiliis* (Fouquet) in the mirror carp *Cyprinus carpio* L., III. Pathology. J Fish Biol. **6:**189-196.

85. **Hines, R. S., and D. T. Spira.** 1974b. *Ichthyophthirius multifiliis* (Fouquet) in the mirror carp *Cyprinus carpio* L., IV. Physiological dysfunction. J Fish Biol. **6:**365-371.

86. **Hines, R. S., and D. T. Spira.** 1974c. Ichthyophthiriasis in the mirror carp *Cyprinus carpio* L. V. Acquired immunity. J of Fish Biol. **6:**373-378.

87. **Houghton, G., and R. A. Matthews.** 1986. Immunosuppression of carp (*Cyprinus carpio*) to ichthyophthiriasis using the corticosteroid triamcinolone acetonide. Vet Immunol Immunopathol. **12:**413-49.

88. **Houghton, G., and R. A. Matthews.** 1990. Immunosuppression in juvenile carp *Cyprinus carpio* L.: the effects of the corticosteroid triamcinolone acetonide and hydrocortisone 21-hemisuccinate (cortisol) on acquired immunity and the humoral antibody response to *Ichthyophthirius multifiliis* Fouquet. J of Fish Dis. **13:**269-280.

89. **Houghton, G., L. J. Healy and R. A. Matthews.** 1992. The cellular proliferative response, humoral antibody response, and cross reactivity studies of *Tetrahymena pyriformis* with *Ichthyophthirius multifiliis* in juvenile carp (*Cyprinus carpio* L.). Dev Comp Immunol. **16:**301-312.

90. **Houghton, G., and R. A. Matthews.** 1993. *Ichthyophthirius multifiliis* (Fouquet): survival within immune juvenile carp (*Cyprinus carpio* L.). Fish and Shellfish Immunol. **3:**157-166.

91. **Ingram, G. A.** 1980. Substances involved in the natural resistance of fish to infection – a review. J Fish Biol.**16:**23-60.

92. **Itami, T., Y. Takahashi, T. Oamoto, and K. Kubono.** 1988. Purification and characterization of immunoglobulin in skin mucus and serum of ayu. Nippon Suisan Gakkaishi. **54(9):**1611-1617.

93. **Jarrett, L. C.** 1997. Master's Thesis. The University of Georgia.

94. **Johansen, F.-E., I. N. Norderhaug, M. Roe, I. Sandlie, and P. Brandtzaeg.** 1999. Recombinant expression of polymeric IgA: incorporation of J chain and secretory component of human origin. Eur J Immunol. **29:**1701-1708.

95. **Johnson, M.** 1993. The veterinary approach to channel catfish. pp. 249-270. *In* L. Brown (ed.), Aquaculture for Veterinarians. Pergamon Press, Inc., Tarrytown, NY, USA.

96. **Jorgensen, J. B., A. Johansen, B. Stenersen, and A.-I. Sommer.** 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.

97. **Kaattari, S., D. Evans, and J. Klemer.** 1998. Varied re-dox forms of teleost IgM: an alternative to isotypic diversity? Immunol Rev. **166:**133-142.

98. **Kaattari, S., H. L. Zhang, I. Khor, I. Kaattari, and D. Shapiro.** 2002. Affinity maturation in trout: clonal dominance of high affinity antibody late in the immune response. Dev Comp Immunol. **26:**191-200.

99. **Klesius, P.** 1992. Immune system of channel catfish: and overture on immunity to *Edwardsiella ictaluri*. Ann Rev Fish Dis. **(2):**325-338.

100. **Klesius, P. H. and C. A. Shoemaker.** 1999. Development and use of modified live *Edwardsiella ictaluri* vaccine against enteric septicemia of catfish. Adv Vet Med. **41:**523-537.

101. **Ko, Y.-G., and G. A. Thompson.** 1992. Immobilization antigens from *Tetrahymena thermophila* are glycosyl-phosphatidyl-inositol-linked proteins. J Protozool. **39:**719-723.

102. **Leff, A. A., T. Yoshinaga, and H. W. Dickerson.** 1994. Cross immunity in channel catfish, *Ictalurus punctatus* (Rafinesque), against two immobilization serotypes of *Ichthyophthirius multifiliis* (Fouquet). J of Fish Dis. **17:**429-432.

103. **Lin, T.-L., and H. W. Dickerson.** 1992. Purification and partial characterization of immobilization antigens from *Ichthyophthirius multifiliis*. J Protozool. **39(4):**457-463.

104. **Lin, T.-L., T. G. Clark, and H. W. Dickerson.** 1996. Passive immunization of channel catfish (*Ictalurus punctatus*) against the ciliated protozoan parasite *Ichthyophthirius multifiliis* by use of monoclonal antibodies. Infect Immunol. **64:**4085-4090.

105. **Lin, Y., G. Cheng, X. Wang, and T. G. Clark.** 2002. The use of synthetic genes for the expression of ciliate proteins in heterologous systems. Gene. **288(1-2):**85-94.

106. **Lobb, C. J., and L. W. Clem.** 1981. The metabolic relationships of the immunoglobulins in fish sera, cutaneous mucus and bile. J Immunol. **127:**1525-1529.

107. **Lobb, C. J., and L. W. Clem.** 1981a. Phylogeny of immunoglobulin structure and Function-X. Humoral immunoglobulins of the sheepshead, *Archosargus probatocephalus*. Dev Comp Immunol. **5:**271-282.

108. **Lobb, C. J., and L. W. Clem.** 1981b. Phylogeny of immunoglobulin structure and Function - XI. Secretory immunoglobulins in the cutaneous mucus of the sheepshead, *Archosargus probatocephalus*. Dev Comp Immunol. **5:**587-596.

109. **Lobb, C. J., and L. W. Clem.** 1981c. Phylogeny of immunoglobulin structure and Function - XII. Secretory immunoglobulins in the bile of the marine teleost *Archosargus probatocephalus.* Mol Immunol. **20(8):**615-619.

110. **Lobb, C. J., and L. W. Clem.** 1983. Distinctive subpopulations of catfish serum antibody and immunoglobulin. Mol Immunol. **20(8):**811-818.

111. **Lobb, C. J., M. Olson, and L. W. Clem.** 1984. Immunoglobulin light chains in a teleost fish. J of Immunol. **132(4):**1917-1923.

112. **Lobb, C. J.** 1985. Covalent structure and affinity of channel catfish anti-dinitrophenyl antibodies. Mol Immunol. **22(8):**993-999.

113. **Lobb, C. J.** 1986a. Structural diversity of channel catfish immunoglobulins. Vet Immunol Immunopathol. **12:**7-12.

114. **Lobb, C. J.** 1986b. Preferential expression of catfish light chain immunoglobulin isotypes in anti-dinitrophenyl antibodies. J Immunogenet. **13(1):**190-28.

115. **Lobb, C. J.** 1987. Secretory immunity induced in catfish, *Ictalurus punctatus*, following bath immunization. Dev Comp Immunol. **11(4):**727-738.

116. **Lobb, C. J., and M. Olsen.** 1988. Immunoglobulin heavy chain isotypes in a teleost fish. J of Immunol. **141:**1236-1245.

117. **Lom, J.** 1995. Trichodinidae and Other Ciliates (Phylum Ciliophora), pp. 229-262. *In* P.T.K. Woo (ed.) Fish Diseases and Disorders. CAB International, Wallingford, UK.

118. **Lorenzen, N., N. J. Olesen, and C. Koch.** 1999. Immunity to VHS virus in rainbow trout. Aquaculture. **172:**41-61.

119. **Lumsden, J. S., V. E. Ostland, P. J. Byrne, and H. W. Ferguson.** 1993. Detection of a distinct gill-surface antibody response following horizontal infection and bath challenge of brook trout *Salvelinus fontinalis* with *Flavobacterium branchiophilum*, the causative against of bacterial gill disease. Dis Aquat Org. **16:**21-27.

120. **Lundberg J. G.** March 1, 2001. Teleostei. The Tree of Life Web Project. http://tolweb.org/tree.

121. **Maki, J., C. Brown, and H. Dickerson.** 2001. Occurrence of *Ichthyophthirius multifiliis* within the peritoneal cavities of infected channel catfish *Ictalurus punctatus.* Dis Aquatic Org. **44:**41-45.

122. **Matthews, R. A.** 1994. *Ichthyophthirius multifiliis* Fouquet, 1876: infection and protective response within the fish host. pp. 17-42. *In* A. W. Pike and J. W. Lewis (eds.), Parasitic Diseases of Fish. Samara Publishing, Dyfed, U.K.

123. **McCallum, H. I.** 1986. Acquired resistance of black mollies, *Poecilia latipinna* to infection by *Ichthyophthirius multifiliis*. Parasitology **93:**251-261.

124. **Meseguer, J., M. Estaban, A. Garcia, R. Lopez, and B. Agulleiro.** 1990. Granulopoiesis in the head kidney of the sea bass (*Dicentrarchus labbrax* L.): an ultrastructural study. Arch of Histol and Cytol. **53:**287-296.

125. **Meseguer, J., M. Estaban, and B. Agulleiro.** 1991. Stromal cells, macropgages and lymphoid cells in the head-kidney of the sea bass (*Dicentrarchus labbrax* L.): An ultrastructural study. Arch Histol Cytol. **54(3):**299-309.

126. **Mestecky, J., R. Kulhavy, R. Schrohenloher, M. Tomana, and G. Wright.** 1975. Identification and properties of J chain isolated from catfish macroglobin. J of Immunol. **115(4):**993-997.

127. **Miller, N. W., M. A. Ryczyn, M. R. Wilson, G. W. Warr, J. P. Naftel, and L. W**. **Clem.** 1987. Development and characterization of channel catfish long term B cell lines. J of Immunol. **152:**2180-2189.

128. **Nakanishi, T., and M. Ototake.** 1997. Antigen uptake and immune responses after immersion vaccination. Dev Biol Stand. **90:**59-68.

129. **Nelson, J. S.** 1994. Fishes of the World. 3rd Edition. John Wiley & Sons, New York, NY, USA.

130. **Nigrelli, R. F., and G.D. Riggieri.** 1966. Enzootics in the New York Aquarium caused by *Cryptocaryon irritans* Brown, 1951 (*Ichthyophthirius marinum*

Sikama, 1961) a histophageous ciliate in the skin, eyes and gills of marine fish. Zoologica. **51:**97-102.

131. **Noe, J., and H. Dickerson.** 1995. Sustained growth of *Ichthyophthirius multifiliis* at low temperature in the laboratory. J Parasitol. **81(6):**1022-1024.

132. **Noga, E. J., Z. Fan, and U. Silphaduang.** 2001. Histone-like proteins in fish mucus are lethal to the parasite *Amyloodinium ocellatum*. Parasitol. **123(Pt 1):**57-65.

133. **Nusbaum, K. E., B. F. Smith, P. DeInnocentes, and R. C. Bird.** 2002. Protective immunity induced by DNA vaccination of channel catfish with early and late transcripts of the channel catfish herpesvirus (IHV-1). Vet Immunol Immunopathol. **84:**151-168.

134. **Ourth, D. D.** 1980. Secretory IgM, lysozyme and lymphocytes in the skin mucus of the channel catfish, *Ictalurus punctatus*. Dev Comp Immunol. **4:**65-74.

135. **Ourth, D. D.** 1986. Purification and quantification of channel catfish (*Ictalurus punctatus*) immunoglobulin. M J Ichthyol. **3:**140-143.

136. **Parker, J. C.** 1965. Studies on the natural history of *Ichthyophthirius multifiliis* Fouquet (1876), an ectoparasitic ciliate of fish. Ph.D. dissertation, University of Maryland, College Park, MD.

137. **Passer. B. J., C. H. Chen, N. W. Miller, and M. D. Cooper.** 1997. Identification of a T lineage antigen in the catfish. Dev Comp Immunol. **20(6):**441-450

138. **Peleterio, M. C. and R. H. Richards**. 1985. Identification of lymphocytes in the epidermis of the rainbow trout, *Salmo gairdneri* Richardson. J of Fish Dis. **8:**161-172.

139. **Peleterio, M. C. and R. H. Richards.** 1988. Immunocytochemical studies on immunoglobulin-containing cells in the epidermis of rainbow trout, *Salmo gairdneri* Richardson: influence of bath immunization. J Fish Biol. **32:**845-858.

140. **Petrie-Hanson, L. and A. Ainsworth.** 2000. Differential cytochemical staining characteristics of channel catfish leukocytes identify cell populations in lymphoid organs. Veterinary Immunol and Immunopathol. **73:**129-144.

141. **Phillips, J. O. and D. D. Ourth.** 1986. Isolation and molecular weight determination of two immunoglobulin heavy chains in the channel catfish, *Ictalurus punctatus*. Comp Biochem Physiol. **85B(1):**49-54.

142. **Prescott, D. M.** 1994. The DNA of ciliated protozoa. Microbiol Rev. **58(2):**233-267.

143. **Press, C. McL.,** 1998. Chapter 2. Immunology of Fishes. pp. 3-62. *In* Pastoret, P. P., P. Greibel, H. Bazin, A. Govaerts (eds.) Handbook of Vertebrate Immunology. Academic Press, San Diego, CA.

144. **Pyle, S. W., and D. L. Dawe.** 1985. Immune response of channel catfish, *Ictalurus punctatus* Rafinesque, to bacterial and protozoan antigens administered by three routes. Aquaculture. **46:**1-10.

145. **Rayl, A. J. S.** 2001. How to Create a Successful Fish Tale? In The Scientist. **15(16):**1.

146. **Rijkers, G. T., L. Frederix-Wolter, and W. B. vanMuiswinkel.** 1980. The immune system of cyprinid fish. *In* Phylogeny of Immunological Memory, M. J. Manning (ed.) Elsiever/North-Holland Biomedical Press, Amsterdam.

147. **Rinramaki-Kinnanen, P., and E.T. Valtonen.** 1997. Epizootiology of protozoans in farmed salmonids at northern latitudes. Intl J Parasitol. **27(10):**89-99.

148. **Roitt, I., and D. Male.** 1998. Introduction to the immune system. pp. 1-11. *In* Roitt, I., J. Brostoff, and D. Male. Immunology. 5th Edition. Mosby Int'l. Ltd., London, UK.

149. **Rombout, J. W., L. J. Blok, C. H. Lamers, and E. Egberts.** 1986. Immunization of carp (*Cyprinus carpio*) with a *Vibrio anguillarum* bacterin: indications for a common mucosal immune system. Dev Comp Immunol. **10(3):**341-351.

150. **Rombout, J. H. W. M., N. Taverne, M. van de Kamp, and A. J. Taverne-Theile**. 1993a. Differences in mucus and serum immunoglobulin of carp (*Cyprinus carpio* L.). Dev Comp Immunol. **17:**309-317.

151. **Rombout, J.W., A. J. Taverne-Thiele, and M. I. Villena.** 1993b. I. The gut-associated lymphoid tissue (GALT) of carp (*Cyprinus carpio* L.): An immunocytochemical analysis. Dev Comp Immunol. **17:**55-66.

152. **Rombout, J.W., A. A. van den Burg, C.T. van den Burg, P. Witte, and E. Egberts**. 1989. Immunological importance of the second gut segment of carp. III. Systemic and/or mucosal immune responses after immunization with soluble or particulate antigen. J Fish Biol. **35:**79-186.

153. **Rombout, J. W., P. H. Joosten, M. Engelsma, A. P. Vos, N. Taverne, and A.J. Taverne-Thiele.** 1998. Indications for a distinct putative T cell population in mucosal tissue of carp (*Cyprinus carpio* L.). Dev Comp Immunol. **22:**63-77.

154. **Sakai, D.** 1992. Repertoire of complement in immunological defense mechanisms of fish. Ann Rev Fish Dis. **2:**223-247.

155. **Shao, Z. J.** 2001. Aquaculture pharmaceuticals and biologicals: current perspectives and future possibilities. Adv Drug Dev Rev. **50:**229-243.

156. **Shepard, K. L.** 1994. Functions of fish mucus. Rev Fish Biol Fisheries. **4:**401-429.

157. **Sherrill, J.** 1995. Master's thesis. The University of Georgia.

158. **Siddell, M. E., K. S. Reece, B. S. Flores, N. A. Stokes, and E. M. Burreson.** 1997. Virginia Institute of Marine Science. The College of William and Mary.

159. **Sigel, M., B. Hamby, and E. Huggins.** 1986. Phylogenic studies on lymphokines. Fish lymphocytes respond to human IL-1 and epithelial cells produce an IL-1 like factor. Vet Immunol Immunopathol. **12:**47-58.

160. **Silphaduang, U., and E. J. Noga.** 2002. Peptide antibiotics in mast cells of fish. Nature. **414(6865):**710.

161. **Sin, Y., K. Ling, and T. Lam.** 1996. Cell-mediated immune response of goldfish, *Carassius auratus* (L.) to *Ichthyophthirius multifiliis*. J Fish Dis. **19:**1-7.

162. **Sizemore, R. C., N. W. Miller, M. Cuchens, C. Lobb, and L. W. Clem.** 1984. Phylogeny of lymphocyte heterogeneity: the cellular requirements for in vitro mitogenic response of channel catfish leukocytes. J of Immunol. **133(6):**2920-2924.

163. **South, M. A., M. D. Cooper, F. A. Wollheim, R. Hong, and R. A. Good.** 1966. The IgA system: I. Studies of the transport and immunochemistry of IgA in the saliva. J Exp Med. **123:**615-627.

164. **Southgate, P.** 1993. The veterinary approach to channel catfish. pp. 249-270. *In* L. Brown (ed.), Aquaculture for Veterinarians. Pergamon Press, Inc., Tarrytown, NY, USA.

165. **Staats, H. F., R. Jackson, M. Marinaro, I. Takahashi, H. Kiyono, and J. McGhee**. 1994. Mucosal immunity to infection with implications for vaccine development. Curr Opin Immunol. **6:**572-583.

166. **Stiles, C.W.** 1894. Report on a parasitic protozoan observed on fish in the aquarium. Bulletin of the U.S. Fisheries Commission **13:**173-190. Misc. Doc. 130. *In* House of Representatives Miscellaneous Documents of the 53rd Congress 2nd session. Government Printing Office, Washington, DC, USA.

167. **St. Louis-Cormier, E. A., C. K. Osterland, and P. D. Anderson.** 1984. Evidence for a cutaneous secretory immune system in rainbow trout (*Salmo gairdneri*). Dev Comp Immunol. **8:**71-80.

168. **Streilein, J. W.** 1983. Skin-associated lymphoid tissues (SALT): Origins and functions. J of Invest Dermatol. **80:**012s-016s.

169. **Subsasinghe, R. P., and C. Sommerville.** 1989. An experimental study into the possible protection of fry from *Ichthyophthirius multifiliis* Fouquet 1876 during mouthbrooding of *Oreochromis mossambicus* (Peters). Fish and Shellfish Immunol. **3:**97-105.

170. **Sunyer, J., and J. Lambris.** 198. Evolution and diversity of the complement system of poikilothermic vertebrates. Immunol Rev. **166:**39-57.

171. **Suzuki, Y., and T. Ida.** 1992. Fish granulocytes in the process of inflammation. Ann Rev Fish Dis. **2:**149-160.

172. **Takahashi, T., T. Wase, N. Takenouchi, M. Saito, K. Kobayashi, A. Moldoveane, J. Mestecky, and I. Moro.** 1966. The joining (J) chain is present in invertebrates that do not express immunoglobulins. Proc Natl Acad Sci USA. **93:**1886-1891.

173. **Tatner, M. F.** 1985. The migration of labeled thymocytes to the peripheral lymphoid organs in the rainbow trout *Salmo gairdneri* Richardson. Dev Comp Immunol. **9:**85-91.

174. **Temmink, J. H. M., and C. J. Bayne.** 1987. Ultrastructural characterization of leucocytes in the pronephros of carp (*Cyprinus carpio* L.). Dev Comp Immunol. **11:**125-137.

175. **Tomonaga, S., H. Zhang, K. Kobayashi, R. Fujii, and K. Teshima.** 1992. Plasma cells in the spleen of the aleutian skate, *Bathyraja aleutica*. Arch Histol Cytol. **55(3):**287-294.

176. **United States Department of Agriculture.** 1997. National Animal Health Monitoring System (NAHMS). Part I: Reference of 1996 U.S. Catfish Health and Production Practices.

177. **United States Department of Agriculture.** 1998. National Agriculture Statistics Service (NASS), Census of Agriculture: Aquaculture.

178. **U. S. Food and Drug Administration.** Center for Food Safety and Applied Nutrition. Fish and Fishery Product Hazards and Controls Guide. Appendix 5:FDA and EPA Guidance Levels.

179. **Vaerman, P., A. Langenries, D. Giffroy, P. Brandtzaeg, and K. Kobayashi.** 1998. Lack of SC/pIgR-mediated epithelial transport of a human polymeric IgA devoid of J chain: in vitro and in vivo studies. Immunol. **95:**90-96.

180. **Vallejo, A. N., N. W. Miller, and L. W. Clem.** 1992. Antigen processing and presentation in the teleost immune system. Ann Rev Fish Dis. **2:**73-89.

181. **Valtonen, E. T., and M. Koskivaara.** 1994. Relationship between the parasites of some wild and culture fishes in two lakes and a fish farm in central Finland. Intl J Parasitol. **24(1):**109-118.

182. **van Ginkel, F., H. Nguyen, and J. McGhee.** 2000. Vaccines for mucosal immunity to combat emerging infectious diseases. Emerg Infect Dis. **6(2):**123-132.

183. **van Muiswinkel, W.** 1995. The Piscine Immune System: Innate and Acquired Immunity. pp.729-750. *In* P.T.K. Woo (ed.) Fish Diseases and Disorders. Volume 1. CAB International, Wallingford, U.K.

184. **Ventura, M. T., and I. Paperna.** 1985. Histopathology of *Ichthyophthirius multifiliis* infections in fishes. J Fish Biol. **27:**185-203.

185. **Vinitnantharat, S., K. Gravningen, and E. Greger.** 1999. Fish Vaccines. Adv Vet Med. **41:**539-550.

186. **Wahli, T., and W. Meier.** 1985. Ichthyophthiriasis in trout: investigation of natural defense mechanisms. pp. 347-352. *In* A. E. Ellis (ed.) Fish and Shellfish Pathology. Academic Press, London.

187. **Wang, X.** 2001. Ph.D. Dissertation. The University of Georgia.

188. **Wang X., and H. W. Dickerson.** 2002. Surface immobilization antigens of the parasitic ciliate *Ichthyophthirius multifiliis* elicit protective immunity in channel catfish. Clin Diag Lab Immunol. **9(1):**176-181.

189. **Warr, G. W.** 1997. The adaptive immune system of fish. pp. 15-21. *In* Fish vaccinology. Gudding, R., Lillehaug, A., Midtlyng, P., Brown, F. (eds.) Dev Biol Stand. Basel, Karger. **90:**15-21.

190. **Williams, R., and M. Warner.** 1976. Some observations on the stained blood cellular elements of the channel catfish, *Ictalurus punctatus*. J Fish Biol. **9:**491-497.

191. **Wilson, M. R., H. Zhou, E. Bentgen, L. W. Clem, T. S. Stuge, G. W. Warr, N. W**. **Miller.** 1983. A comparison of vertebrate interferon gene families detected by hybridization with human interferon DNA. J Mol Biol. **166:**457-475.

192. **Wilson, M., E. Bengten, N. W. Miller, L. W. Clem, L. Dupasquier, and G. W Warr.** 1997. A novel chimeric heavy chain from a teleost fish shares similarities to IgD. Proc Natl Acad Sci USA. **94(9):**4593-4597.

193. **Wilson, M., H. Zhou, E. Bengten, L. Clem, T. Stuge, G. Warr, and N. Miller.** 1998. T cell receptors in channel catfish, structure and expression of TCR α and β genes. Mol Immunol. **9:**545-557.

194. **Wolf, K., and M. Markiw.** 1983. Ichthyophthiriasis: immersion immunization of rainbow trout (*Salmo gairdneri*) using *Tetrahymena thermophila* as a protective immunogen. Can J of Fish and Aquat Sci. **39:**1722-1755.

195. **Woo, P.T.K.** 1992. Immunological responses of fish to parasitic organisms. Annual Rev Fish Dis. **2:**339-366.

196. **Wright, A. D. G., and D. H. Lynn.** 1995. Phylogeny of the fish parasite *Ichthyophthirius multifiliis* and its relatives *Ophryoglena* and *Tetrahymena* (*Ciliophora, Hymenostomatia*) inferred from 18S ribosomal RNA sequences. Mol Biol Evol. **12(2):**285-290.

197. **Xu, C.** 1995. Ph.D. Dissertation. The University of Georgia.

198. **Zapata, A.** 1981. Lymphoid organs of teleost fish II. Ultrastructure of renal lymphoid tissue of *Rutilus rutilus* and *Gobio gobio*. Dev Comp Immunol. **5:**685-690.

199. **Zapata, A., and C.T. Amemiya.** 2000. Phylogeny of lower vertebrates and their immunological structures. Curr Top Microbiol Immunol. **248:**67-107.

200. **Zhou, H., E. Bengten, N. Miller, G. Warr, L. Clem, and M. Wilson.** 1997. T-cell receptor sequences in the channel catfish. Dev Comp Immunol. **21:**238.

201. **Zinkl, J., W. Cox, and C. Kono.** 1991. Morphological and cytochemistry of leucocytes and thrombocytes of six species of fish. Comp. Haematol Intl. **1:**187-195.

202. **Zillberg, D., and P. Klesius.** 1997. Quantification of immunoglobulin in the serum and mucus of channel catfish at different ages and following infection with *Edwardsiella ictaluri*. Vet Immunol Immunopathol. **58:**171-180.

"What we call the beginning is often the end. And to make an end is to make a beginning. The end is where we start from." T.S. Eliot

CHAPTER 2

THE SYSTEMIC AND CUTANEOUS MUCUS ANTIBODY RESPONSES OF CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) IMMUNIZED AGAINST *ICHTHYOPHTHIRIUS MULTIFILIIS*[1]

[1] Maki, Joanne L., and H. W. Dickerson. To be submitted to *Clinical and Diagnostic Laboratory Immunology*.

ABSTRACT

Channel catfish (*Ictalurus punctatus*) acquire protective immunity against the ciliated protozoan parasite *Ichthyophthirius multifiliis* following surface infection and treatment with formalin or inoculation with *I. multifiliis* surface immobilization antigens. In both cases, fish produce protective, immobilizing antibodies in their sera and cutaneous mucus. To further investigate the kinetics of antibody production, channel catfish were exposed to infective parasites or given a single injection of purified i-antigen (5.0 μg/fish) in Freund's incomplete adjuvant. Antibodies in blood and cutaneous mucus were measured subsequently over a 14-week period using an enzyme-linked immunosorbent assay incorporating a biotinylated goat antibody against channel catfish immunoglobulin. Infected and inoculated fish had mean serum antibody levels (A_{405}) at five weeks of 0.54 $\pm$ 0.17 and 0.35 $\pm$ 0.03, respectively, that were significantly higher (α = 0.05) than pre-treatment levels of 0.24$\pm$0.05 and continued to increase through the end of sampling at 14 weeks to 0.79 $\pm$ 0.30 and 0.71 $\pm$ 0.24, respectively. In contrast, cutaneous mucus antibody levels were much lower and varied significantly between replicates and treatment groups. Infected fish had mean mucus antibody levels that were significantly higher (α = 0.05) than pretreatment absorbance values of 0.21 $\pm$ 0.04 three to nine weeks after exposure to the parasite, reaching the highest level of 0.30 $\pm$ 0.07 at seven weeks. Individual injected fish had mucus antibody concentrations above pre-treatment values at 2, 3, and 11 weeks but the overall mean mucus antibody response of these fish was not significantly different from pretreatment absorbance values. Serum and mucus antibody responses of channel catfish against *I. multifiliis* following either infection or inoculation of i-antigen were not synchronous. *I. multifiliis* infection induced

a transient mucosal antibody response that coincided with resolution of infection and appeared to occur independently of serum antibody production.

Keywords: *Ichthyophthirius multifiliis*, mucosal immunity, channel catfish, *Ictalurus punctatus*, ELISA, antibody, i-antigen, mucus, kinetics

INTRODUCTION

The parasitic ciliate *Ichthyophthirius multifiliis* is one of the most virulent pathogens of freshwater fish. It has serious economic impact on aquaculture because of its widespread distribution and indiscriminate host specificity. Outbreaks occur throughout the world and usually result in high mortalities, primarily in intensively reared fish populations. Fish can become resistant to *I. multifiliis* following natural or experimentally induced infections. Acquired immunity has been well documented in many fish species including channel catfish, trout and carp (2, 20, 31, 42). A long-term goal of our research has been to understand the mechanisms of protective immunity against this parasite in order to develop an effective vaccine.

Sera from immune fish immobilize the organism in vitro, and it has been postulated that immobilizing antibodies serve to block parasite infection of the host (9, 17). Fish are protected against *I. multifiliis* following passive transfer of immobilizing murine mAbs that are transported to the skin (8, 9, 23). Immunoglobulin has also been demonstrated in the skin of fish immunized against *I. multifiliis* (44). These observations support the concept that antibodies are a key component of the immune barrier at the epithelial surface of fish. It has recently been shown that fish can be vaccinated against *I.*

multifiliis by intraperitoneal injection of surface proteins targeted by immobilizing antibodies (i.e., immobilization antigens) (15). Fish immunized by this procedure are immune to lethal challenge and produce antibodies against i-antigens in their blood and cutaneous mucus (4-6, 43, 44). Based on this and other work, an antibody-mediated mechanism of cutaneous immunity against *I. multifiliis* has been proposed (9, 11, 16, 20).

Channel catfish, like other teleosts, produce a single immunoglobulin class of tetrameric, IgM-like antibodies in their blood and mucus secretions that appear to be identical in structure (27, 28, 33). Although there is experimental evidence supporting the existence of a separate mucosal immune system in fish, it is currently unknown how mucus antibodies reach surface epithelia of the gut, gills and skin (1, 3, 10, 17-19, 24-26, 29, 30, 32, 34-38, 40). We would like to identify the sites where cutaneous mucus antibodies are induced and the mechanisms by which they are transported to the skin. Toward this end, we are using channel catfish infected with *I. multifiliis* as a model to investigate the cutaneous immune response of fishes to pathogens that invade through epithelial tissue (16).

In this report, we used an ELISA to compare over time the relative amounts of *I. multifiliis*-specific serum and cutaneous mucus antibodies elicited in naïve channel catfish following either surface infection or a single injection of purified i-antigen. We found that mucosal antibodies were produced following either *I. multifiliis* infection or the injection of purified antigen, and that in both cases their occurrence did not correlate with the production of serum antibodies. Our results suggest that parasite-specific antibodies detected in the cutaneous mucus of channel catfish exposed to *I. multifiliis*

(either by infection or injection) do not arise by passive transfer or simple exudation from the blood.

MATERIALS AND METHODS

Parasite propagation. The G5 *I. multifiliis* isolate used in this study has been previously characterized and its propagation methods described (13, 23).

Purification of *I. multifiliis* protein antigens*:* Immobilization antigens (i-antigens) of *I. multifiliis* (G5/serotype D) theronts were purified by Triton X-114 detergent extraction and murine monoclonal antibody (mAb) immunoaffinity chromatography (15, 22). Briefly, theront cell pellets re-suspended in 20 mM Tris-HCl were mixed with equal volumes of cold 2X extraction buffer containing 300 mM NaCl, 20 mM Tris-HCl (pH 7.5) and 2%(v/v) Triton X-114 (Sigma, St. Louis, MO) and 100 mM phenylmethyl-sulfonylfluoride (Sigma). Cells were lysed by repeated pipetting and gentle vortexing. Debris was removed by centrifugation 16,000g for 10 min at 4^{o}C. Supernatants were warmed to 30^{o}C and layered over cushions of 6% sucrose dissolved in 150 mM NaCl, 0.06% Triton X-114 and 10 mM Tris-HCl (pH=7.5) and incubated for 15 minutes at 30^{o}C to induce micelle formation. Micelles were separated by centrifugation (Beckman Model J2-21, Beckman Instruments, Fullerton, CA) at 300g in a swinging bucket rotor (Beckman: JS-13.1) for 10 min at RT. Supernatants were transferred to new tubes and the detergent phase re-extracted after the addition of equal volumes of 2X extraction buffer. I-antigens were removed from the detergent phase by precipitation with 10:1 (v/v) acetone (JB Baker, Phillipsburg, NJ) for 1 hr on ice and centrifugation at 10,000g for 15 min at 4^{o}C. The pellet was air-dried, resuspended in 10 mM Tris HCl

(pH= 7.5), flash frozen in liquid N_2 and stored at –80^{o}C (15). For the ELISA, aliquots of membrane protein were thawed to room temperature and diluted in 25 mM sodium acetate (pH 7.5) before use. The same preparation was further enriched for i-antigen using a column on which a monoclonal antibody specific for *I. multifiliis* G5 i-antigen (G3-61) was immobilized as described previously (22). The immunoaffinity chromatography purified form of i-antigen was used to inject fish by the intraperitoneal route.

Production of anti-catfish immunoglobulin antibody: Immunoglobulin (Ig) was purified from pooled channel catfish (*Ictalurus punctatus*) sera using an IgM purification column (ImmunoPure-IgM^{TM}, Pierce) following the manufacturer's recommended protocol. Fractions containing >0.5 μg protein, determined by absorbance (A_{280}), were pooled and de-salted using dextran columns (Pierce). A domestic mixed breed goat (*Capra hircus*) was injected three times over the course of three months with 1.0 μg of purified catfish Ig dissolved in 1.0 ml of PBS (136 mM NaCl; 2.7 mM KCl; 10.1 mM Na_2HPO_4; 1.5 mM KH_2PO_4)(pH 7.2) mixed 1:1 in Freund's incomplete adjuvant and administered via the intramuscular route (39). Blood was periodically collected from the jugular vein using a sterile needle and syringe. Serum was collected from clotted blood after overnight storage at 4^{o}C and aliquots stored at –20^{o}C. Goat Ig was purified from the sera using a Protein A/G column (Pierce). The goat antibody was biotinylated using a Sulfo-NHS-Biotinylation Kit (Pierce) and percent biotinylation calculated using a competitive binding assay incorporating 2-4'-hydroxy-azonbenzene benzoic acid (HABA) and avidin according to the manufacturer's instructions.

Experimental animals: Channel catfish fingerlings (mean weight = 18.5 $\pm$ 1.2 g) were obtained from The University of Georgia Agricultural Research Foundation's (UGARF) Aquaculture Facility, Athens, GA. These specific-pathogen-free fish were raised from formalin-treated eggs in containment and fed a commercial game fish ration once a day (Purina, St. Louis, MO). During the experiment, fish were observed daily and water quality monitored every other day for pH and NO_2 with standard test kits. Sodium bicarbonate (Fisher Scientific, Fair Lawn, NJ) was added as needed to maintain water alkalinity. Fish were randomly assigned to treatment groups and placed in twelve 76-liter glass aquaria on a self-contained, recirculating bio-filtered system at a density of 10 fish per aquarium and two aquaria per group. Water temperature ranged from 16 to 24^{o}C during the course of the experiment.

Immunization of fish with *I. multifiliis* protein: Fish were anesthetized with tricaine methane-sulfonate, MS-222 (Argent Chemicals, Redmond, Washington; 100-200 mg/L) dissolved in water that had been buffered with equal amounts of sodium bicarbonate (Fisher). Each fish received 5.0μg of affinity purified i-antigen diluted in 25 μl of PBS mixed 1:1 with Freund's incomplete adjuvant. Antigen was injected via the intraperitoneal route at the ventral surface midline using a 1-cc tuberculin syringe (Monoject^{R}, Sherwood Medical Company, St. Louis, MO) fitted with a 23-gauge x 1-inch needle (Becton Dickinson & Co., Franklin Lakes, NJ).

Exposure of fish to *I. multifiliis* parasites: Twenty catfish were exposed to *I. multifiliis* theronts (isolate G5; serotype D) maintained by passage on channel catfish (14). Unanesthetized fish were held in 2-liter plastic beakers containing charcoal-filtered water (200 ml/fish). They were exposed at a ratio of 5,000 theronts/fish at RT for 1 hour

and again two weeks later the procedure was repeated using 11,000 theronts/fish. Numerous parasites were observed on the exterior surface of all fish four days after the second exposure. Formalin (formaldehyde, 37% solution, J. T. Baker) was added to each tank at 0.26 ml/liter on the fifth day and every other day for three additional days. Complete water changes were performed twice a day during treatment. The fish appeared free of parasites three weeks after the infection was first observed and one fish died during treatment.

Collection of cutaneous mucus and sera: Cutaneous mucus and blood were collected from fish in both treatment groups two weeks before the experiment and at eight time points post-treatment over a fourteen week period. Four immunized fish and four *I. multifiliis*-exposed fish were sampled at weeks 1, 2, 3, and 5. Ten immunized and ten *I. multifiliis*-exposed fish were sampled at weeks 7, 9, 11 and 14. Fish were randomly selected from each tank, anesthetized and weighed. Mucus was collected prior to blood collection to prevent possible cross contamination of samples. After sampling, the tail fin of each fish was clipped for identification purposes to avoid sampling of the same fish on subsequent dates.

Mucus was processed as described below and stored at –80^{o}C. Blood (200–500 µl/fish) was collected from the caudal sinus using a 23-gauge x 1-inch needle and 1.0-cc syringe. Blood was transferred to 1.5 ml polypropylene tubes and refrigerated overnight (4-6^{o}C for 12-15 hr) to maximize clot retraction. The next day tubes were centrifuged at 12,000 rpm (10,000g) for 10 min in a tabletop microcentrifuge (Marathon 13K/M; Fisher) and sera transferred to 1.5ml tubes for storage (-20^{o}C).

Detection of *I. multifiliis*-specific antibodies by ELISA: Detergent-extracted *I. multifiliis* membrane protein (3.0-5.0 µg/ml) diluted in 25 mM sodium acetate (pH = 7.5) was applied to polyvinyl chloride 96 well plates (Falcon: 353912FB; Becton Dickinson) at 100 µl per well. Plates were incubated overnight at 4^{o}C, washed once with 200 µl of PBST/well and blocked with 1% normal goat sera (Sigma) diluted in PBST (1 hr at RT). Catfish sera diluted in PBS (1:80) and undiluted mucus samples were plated in triplicate wells at 100 µl/well. Control wells on each plate were replicates of diluted sera from *I. multifiliis*-exposed and non-exposed catfish. Mucus assays included control wells of pooled mucus from non-exposed fish. Plates were incubated 2-3 hr at RT or overnight at 4^{o}C and washed five times with PBST.

The biotinylated goat anti-catfish Ig antibody was applied at 0.25-0.50 µg/well. Plates were incubated for one hour at RT and washed five times with PBST. The primary antibody was followed by strepavidin-alkaline phosphatase conjugate (Sigma: E-2636 ExtraAvidin®) diluted in PBS (1:50,000). The enzyme substrate, p-nitrophenyl phosphate diethanol amine (BioRad, Hercules, CA) was added and the absorbance (A_{405}) values were determined on a kinetic microplate reader (Model: V-max, Molecular Devices, Corp., Sunnyvale, CA) at 30 minutes and 1 hour.

Standardization of cutaneous mucus collection: Previously published methods describing cutaneous mucus collection in fish used techniques such as rinsing, scraping and blotting the surface of fish with filter paper (1, 29, 45). In each instance additional water or PBS was added during processing that resulted in sample dilution. Prior to initiation of this experiment, a preliminary study was conducted to determine the standard error and dilution effect on total mucus protein. These variables were calculated based on

changes in absorbance at 280nm (A_{280}) of a pooled mucus sample before and after processing.

To prepare the pooled sample, cutaneous mucus was collected from five adult channel catfish (~1.4-2.3 kg each) obtained from the UGARF facility. Fish were anesthetized with tricaine methane-sulfonate, MS-222 (Argent Chemicals, Redmond, Washington; 100-200 mg/L) dissolved in water that had been buffered with equal amounts of sodium bicarbonate (Fisher). Each fish was placed on a flat surface covered with SaranWrap™ (S.C. Johnson and Son, Inc., Racine, WI). Mucus was collected from both sides of the fish by gently wiping with pieces of cotton (Johnson & Johnson, Skillman, NJ). Care was taken to not remove the entire mucus layer or abrade the skin during collection. Saturated cotton pieces were combined into 50 ml conical centrifuge tubes (Corning, Corning, NY) containing just enough PBS (approximately 10 ml/tube) to prevent drying. The cotton was pressed against the side of the tube with a spatula and discarded. The liquid remaining in each tube were pooled, absorbance (A_{280}) recorded and aliquots were stored frozen at –80^{o}C. This pooled mucus with an absorbance value (A_{280} = 0.6288) served as starting material for the following experiment.

Pieces of cotton (1.23 $\pm$ 0.33 g each) were placed in plastic weigh boats containing 1-2 ml of the pooled mucus sample. After 5-10 seconds, when the pieces were uniformly saturated, they were weighed again and individually inserted into 12 x 75mm-glass tubes (VWR Scientific Products, Buffalo Grove, IL) containing 500 µl of PBS/tube. The mucus samples were processed as described above and the contents transferred to individual 1.5 ml snap-lid polypropylene tube (Fisher) and centrifuged at 12,000 rpm (~10,000g) for 10 min in a tabletop microcentrifuge (Marathon 13K/M; Fisher).

Supernatants were collected and stored at –80°C. Absorbance values (A_{280}) of the processed mucus samples and original pooled mucus were compared and standard error calculated.

SDS-PAGE and Western blotting: One-dimensional sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of *I. multifiliis* antigens and Western blots were performed using previously published protocols (22). Purified i-antigen or detergent extracted membrane proteins (0.5 μg/lane) were electrophoresed on a 4% stacking/12% resolving polyacrylamide gels containing 1% sodium dodecyl sulfate in the stacking gel with or without β-mercaptoethanol (Sigma, St. Louis, MO) added to the loading buffer. Protein bands were visualized by staining with silver nitrate (BioRad, Hercules, CA) following the manufacturer's instructions.

Electrophoresed *I. multifiliis* membrane proteins were transferred to a polyvinylidene difluoride membrane (0.45μ)(Pierce), blocked with 3% bovine sera albumin (BSA)(Fraction V; Sigma) and incubated overnight at 4°C with *I. multifiliis*-exposed fish sera diluted 1:100 in PBS plus 0.05% Tween (PBST)(Sigma). After rinsing in PBST, the blots were incubated with 2.0 mg/ml of biotinylated goat anti-catfish Ig antibody diluted in PBST and 1%BSA at RT for 2 hr and washed. ExtraAvidin (1:50,000)(Sigma) in PBST was added and the blots incubated for 30 min at RT and washed again. The substrate (NBT/BCIP, 1-Step™; Pierce) was added and development stopped by rinsing the blots in water after 30 minute incubation at RT.

Statistical analyses*:* Tank and treatment group ELISA mean absorbance values and standard deviations were determined. Significance ($\alpha = 0.05$) was assigned by overlap of resulting confidence intervals. Normality was determined by the Shapiro-

Wilkes Test. Sera and mucus ELISA values, fish weights and mucus A_{280} absorbance values were analyzed for correlation by scatter plot matrix analysis and linear regression. Mucus collection standard error calculations used a 0.95 confidence interval. Software used to perform these analyses were GraphPad Prism® (Version 3.02)(GraphPad Software, San Diego, CA)(www.graphpad.com) and Jump® (JMP IN Version 3.2.6)(SAS Institute, Inc., Wadsworth Publishing, Belmont, CA).

RESULTS

Immobilization antigens of the G5 *I. multifiliis* isolate. Detergent-extracted membrane proteins from *I. multifiliis* consisted primarily of a single group of proteins that when analyzed by SDS-PAGE had M_rs of approximately 55-kDa and 35 kDa under reducing and non-reducing conditions, respectively. These abundant membrane proteins were identified as immobilization antigens (i-antigens) by immunoaffinity chromatography using the immobilizing mouse monoclonal antibody G3-61 that was previously produced and characterized in our laboratory (24) (FIG. 2.1; Lanes 1-4). Sera from *I. multifiliis*-exposed channel catfish recognized only the unreduced i-antigen on Western blots (FIG. 2.1; Lanes 5-6). These results confirm previous studies that showed fish polyclonal antibodies bind to conformational epitopes of i-antigens and do not recognize reduced forms of the protein (7, 23, 43).

Detection of *I. multifiliis*-specific antibodies in blood and cutaneous mucus. We were interested in determining how different methods of parasite and antigen exposure affected the kinetics of antibody production in the channel catfish. Specifically, we sought to determine the primary systemic and mucosal immune responses of channel

catfish after infection and treated with formalin or receiving a single injection of purified i-antigen (5.0 μg/fish) in Freund's incomplete adjuvant. Antibodies against *I. multifiliis* were measured in blood and cutaneous mucus over a 14-week period by an enzyme-linked immunosorbent assay.

The parasite-specific ELISA used an affinity purified, mono-specific, polyclonal antibody against channel catfish immunoglobulin (Ig) that was produced in a goat. During assay development, sera from either previously immunized (4) or naïve channel catfish were incubated at various dilutions (1:10-1:256) with *I. multifiliis* membrane proteins coated onto 96-well plastic plates. Channel catfish antibody that bound specifically to antigen was detected by the addition to each well of 0.25-0.5 μg of the biotinylated goat antibody. From these initial tests (data not shown), a catfish serum dilution of 1:80 provided a consistent signal to compare the serum antibody response over time. The standard error among ELISA assays was <0.05. Similar preliminary tests on cutaneous mucus determined that parasite-specific antibody activity was reduced to background levels at dilutions as low as 1:4. Thus, in subsequent assays only undiluted mucus samples were analyzed.

Systemic antibody responses. Naïve channel catfish that survived infection with *I. multifiliis* had mean serum antibody absorbance values of 0.54 $\pm$ 0.17 at five weeks that were significantly higher compared to pre-exposure sera antibody levels that continued to increase to a maximum mean value of 0.79 $\pm$ 0.30 at 14 weeks (FIG 2.2A). The mean serum antibody levels between the two replicates in this group (10 fish per replicate) were statistically similar (except at Week 9); indicating that surface infection produced a relatively uniform serological response in the twenty, randomly selected, out-bred

channel catfish used in this experiment. The mean serum absorbance values between the two replicates of infected fish remained within 0.29 OD_{405} units throughout the sampling period.

A single intraperitoneal injection of purified i-antigen in incomplete Freund's also produced a relatively uniform serological response in twenty outbred channel catfish (FIG. 2.3A) comparable to that seen in fish infected with the live parasite. Both replicates of inoculated animals (10 per replicate) produced i-antigen-specific serum antibodies at five weeks post-injection with a mean absorbance value of 0.35 $\pm$ 0.03 that was significantly higher than the pre-treatment serum antibody absorbance mean of 0.24 $\pm$ 0.05. The serum antibody response continued to increase to a maximum mean value of 0.71 $\pm$ 0.24 at 14 weeks. The mean serum absorbance values of the two replicates remained within 0.22 OD_{405} units throughout the sampling period.

Cutaneous antibody responses. Compared to the systemic response, cutaneous mucus antibody levels were very low in both the infected and inoculated groups of fish (FIG 2.2B & 2.3B). This result was expected as relatively low mucus antibody responses have been reported previously in fishes immunized against *I. multifiliis* (and other antigens) (1, 29, 43, 45). Our collection method was reproducible with a relatively low standard error (S. E. = 0.175). As a result of a dilution factor (~1.7) introduced by mucus collection and processing, mucus absorbance values obtained in the ELISA were estimated to be approximately 60% less than the actual amount of parasite-specific antibody that was present on the surface of the fish. Due to low concentrations of antibody in mucus and apparent variations in immune response among individual fish, the significance of changes in mean mucus absorbance values was difficult to determine.

Nevertheless, 17 parasite-exposed fish and 12 antigen-injected fish had individual mucus antibody absorbance values that were significantly higher than the overall pretreatment mean of 0.21 $\pm$ 0.04. ELISA absorbance values of 0.396 and .325 OD_{405} were detected in two injected fish at 14 weeks, and one infected fish had a value of 0.395 OD_{405} at 7 weeks. Both tanks of infected fish had mean mucus antibody levels above 0.21 $\pm$ 0.04 from three to nine weeks with a peak value of 0.30 $\pm$ 0.07 at seven weeks. Wide standard deviations at each time point, however, essentially rendered these changes statistically insignificant (FIG. 2.2B). One replicate group of injected fish had individual mucus absorbance values above the overall pretreatment mucus mean of 0.21 $\pm$ 0.04 at 2 and 3 weeks after injection, but the mean values between replicates for this same period were not statistically significantly from one another or background values (FIG 2.3B). The same replicate group of injected fish had two individuals with relatively high antibody levels of 0.325 and 0.396 at 14 weeks, but the replicate group mean value for that time point was low (0.22 $\pm$ 0.08). Mucus absorbance values of the other replicate in this treatment group were not significantly different from the pretreatment values throughout the sampling period.

Comparison of systemic and mucosal antibody responses. Correlation analyses were performed on 148 matched samples consisting of serum antibody ELISA values, mucus antibody ELISA values, fish weight and mucus total protein (FIG. 2.4). Fish weight was used as a relative measure of fish size. Total mucus protein was estimated by absorbance (A_{280}). A low positive correlation (0.337) was observed between sera titer and fish weight. This result was expected as the fish continued to grow over time as they responded to antigenic stimulation. A low positive correlation value

(0.034) also was observed between mucus titer and mucus OD_{280}. All other analyses had slightly negative values (-0.076 to –0.24). Correlation analyses performed on a subset of samples (n = 20) determined that fish having the highest serum antibody levels did not have correspondingly high mucus antibody levels (–0.116).

In summary, the primary systemic antibody response of both treatment groups (*I. multifiliis*-infected or i-antigen-injected fish) resulted in increasing antibody levels in the blood that were at their highest at the end of sampling at 14 weeks. In contrast, antibodies in cutaneous mucus were detected at much lower levels in both treatment groups and had returned to, or were below, pretreatment levels by 14 weeks. These results suggest that comparable primary systemic antibody responses were elicited in channel catfish by either treatment while the cutaneous mucus antibody responses differed significantly from the systemic antibody responses.

DISCUSSION

Channel catfish that received a single injection of *I. multifiliis* i-antigen emulsified in incomplete Freund's adjuvant developed a primary serum antibody response by five weeks that continued to increase through the end of sampling at 14 weeks. Fish that were infected with *I. multifiliis* (and subsequently treated to cure the infection) produced serum antibodies at levels and times comparable to those observed following injection of i-antigen. Mean mucus antibody levels in both treatment groups, however, were at much lower concentrations at all sampling time points, peaked at seven weeks, and returned to pretreatment levels by eleven weeks. Over the same time period following either antigen injection or parasite infection, mucus antibodies appeared transiently and disappeared

while the serum specific antibody concentration continued to rise. Fish inoculated with i-antigen produced lower levels of specific antibody in the mucus than fish infected with the parasite. In either case, the kinetics of the cutaneous mucosal antibody response appears to differ from that of the systemic immune response. Thus, although the precise mechanism of cutaneous mucus antibody secretion remains unknown, our results suggest that it is unlikely that mucus antibodies arise simply by passive diffusion from the blood.

Understanding how antibodies are targeted to fish skin is central to elucidating the mechanisms of mucosal immunity in fishes, and has practical application to the development of vaccines against *I. multifiliis* (7, 16). Although the systemic and mucosal antibody responses of fishes can occur separately (29, 30, 34, 35, 38), the actual mechanisms and sites of cutaneous antibody induction, production and secretion have yet to be elucidated (17, 42). Immunoglobulins in the mucus of the plaice (*Pleuronectes platessa* L.), ayu (*Plectoglossus altivelis*), sheepshead (*Archosargus probatocephalus*) and channel catfish are physically and immunologically identical to immunoglobulin molecules isolated from the blood (17-19, 21, 25-29, 33). Nevertheless, antibodies located in surface mucus do not appear to arise by diffusion or active transport. Experimental evidence in the sheepshead (a common marine fish) showed purified immunoglobulin molecules isolated from the blood could not be detected in the cutaneous mucus or bile when radio-labeled and subsequently injected intravenously back into the same fish (24). Passive transfer studies in channel catfish using immobilizing mouse monoclonal antibodies or sera from *I. multifiliis*-immune fish demonstrated that a physiological barrier seems to exist between the peripheral circulation and the skin that blocks passive transfer of antibody molecules greater in size

than 750 kDa (23). Other experimental evidence supports the existence of a separate mucosal immune system in fishes as well (1, 34-38, 40, 41, 45).

Histological changes associated with *I. multifiliis* infection include infiltration of neutrophils followed by the appearance of other granulocytes and macrophages. Inflammatory processes associated with tissue destruction and repair also occur (12, 29). What remains unknown is whether parasite antigens are processed in the skin or carried to distant sites such as the head kidney or spleen for presentation and lymphocyte stimulation or if these events occur locally. Differences in the cutaneous mucosal antibody response between treatment groups indicate that additional immunological events, potentially within the skin, resulted in parasite-specific antibody in surface mucus of infected and cured fish beginning three weeks after exposure; a point in time that also corresponded to the resolution of clinical infection. Demonstrating B lymphocytes and/or antibody-secreting cells within fish skin will add to the idea that channel catfish skin is immunologically active and a component of a mucosal immune system.

In conclusion, we wished to determine if parasite infection preferentially elicited the production of cutaneous antibody and if injection of antigen elicited primarily a systemic antibody response. Interestingly, the primary systemic antibody response of the injected and infected fish appeared similar in duration and magnitude. This result demonstrated that one dose of i-antigen without the addition of the immune stimulatory components found in Freund's complete adjuvant is capable of stimulating a sustained serological response similar to that seen in surface infected fish. In this experiment we did not test if fish were protected against parasite challenge. We know from previous studies, however, that fish surviving a single round of infection by surface exposure to *I.*

multifiliis are typically immune, as are fish injected via the intraperitoneal route with live theronts (4). While, in preliminary vaccination experiments i-antigen injected without adjuvant did not elicit protection against lethal challenge (Wang and Dickerson, unpublished). Because we did not use an active adjuvant in this study, differences in cutaneous and serum antibody levels observed between infected fish and inoculated fish could be due to an insufficient inflammatory response induced by incomplete Freund's adjuvant leading to a weak mucosal immune response in the injected fish.

The mechanisms by which fish exposed to *Ichthyophthirius multifiliis* antigens (with or without adjuvant) develop protective immunity against subsequent infection are currently under investigation. Demonstration of parasite-specific antibodies in the skin and surface mucus is central to the current hypothesis that parasite-specific antibodies within the skin and gills are responsible for protective immunity. As we learn more about the processes of antibody induction and secretion in the surface mucus of immune fish, we hope to better understand the mechanisms of mucosal immunity.

ACKNOWLEDGEMENTS

This work was supported by a National Institutes of Health, NIAID, Mentored Clinical Scientist Award-AI01429. We would like to thank Dr. Natalia Guseva for providing the purified i- antigen used to inject the fish and Jane Noe for excellent technical support in the care and maintenance of fish and parasite cultures. Members of the Statistic Department, The University of Georgia, Athens, GA participated in experimental design and analysis.

REFERENCES

1. **Ainsworth, A. J., C. D. Rice, and L. Xue.** 1995. Immune responses of channel catfish, *Ictalurus punctatus* (Rafinesque), after oral or intraperitoneal vaccination with particulate or soluble *Edwardsiella ictaluri* antigen. J Fish Dis. **18:**397-409.
2. **Beckert, H., and R. Allison.** 1964. Some host responses of the white catfish to *Ichthyophthirius multifiliis* (Fouquet). Proc S E Assoc Game Fish Comm. **18**:438-441.
3. **Bradshaw, C. M., A. S. Richard, and M. M. Sigel.** 1971. IgM antibodies in fish mucus. Proc Soc Exp Biol Med. **136:**1122-1124.
4. **Burkart, M. A., T. G. Clark, and H. W. Dickerson.** 1990. Immunization of channel catfish, *Ictalurus punctatus* Rafinesque, against *Ichthyophthirius multifiliis* (Fouquet): killed versus live vaccines. J Fish Dis. **13**:401-410.
5. **Clark, T. G., H. W. Dickerson, J. B. Gratzek, and R. C. Findley.** 1987. In vitro response of *Ichthyophthirius multifiliis* to sera from immune catfish. J Fish Biol. **31**:203-208.
6. **Clark, T. G., H. W. Dickerson, and R. C. Findley.** 1988. Immune response of channel catfish to ciliary antigens of *Ichthyophthirius multifiliis*. Dev Comp Immunol. **12:**581-594.
7. **Clark, T. G., T. L. Lin, and H. W. Dickerson.** 1995. Surface immobilization antigens of *Ichthyophthirius multifiliis*: Their role in protective immunity. Ann Rev Fish Dis. **5:**113-131.

8. **Clark, T. G., T. L. Lin, and H. W. Dickerson.** 1996. Surface antigen cross-linking triggers forced exit of a protozoan parasite from its host. Proc Natl Acad Sci USA. **93:**6825-6829.

9. **Clark, T. G., and H. W. Dickerson.** 1997. Antibody-mediated effects on parasite behavior: Evidence of a novel mechanism of immunity against a parasitic protist. Parasit Today **13(12):**477-480.

10. **Cobb, C. S., M. G. Levy, and E. J. Noga.** 1998. Acquired immunity to amyloodiniosis is associated with an antibody response. Dis Aquat Org. **34**:125-133.

11. **Cross, M. L., and R. A. Matthews.** 1992. Ichthyophthiriasis in carp, *Cyprinus carpio* L.: fate of parasites in immune fish. J Fish Dis. **15:**497-505.

12. **Cross, M. L., and R. A. Matthews.** 1993. Localized leukocyte response of to *Ichthyophthirius multifiliis* establishment in immune carp, *Cyprinus carpio* L. Vet. Immunol Immunopathol. **38**;341-358.

13. **Dickerson, H. W., D. L. Dawe, J. B. Gratzek, J. Brown, and S. W. Pyle.** 1981. Induction of *Ichthyophthirius multifiliis* Fouquet infections in channel catfish *Ictalurus punctatus* Rafinesque: standardization of the procedure. Dev Biol Stand. **49:**331-336.

14. **Dickerson, H. W., A. L. Lohr, and J. B. Gratzek**. 1985. Experimental intraperitoneal infection of the channel catfish, *Ictalurus punctatus* (Rafinesque) with *Ichthyophthirius multifiliis* (Fouquet). J Fish Dis. **8**:139-142.

15. **Dickerson, H. W., T. G. Clark, and R. C. Findley.** 1989. *Ichthyophthirius multifiliis* has membrane-associated immobilization antigens. J Protozool. **36:**159-164.

16. **Dickerson, H. W., and T. G. Clark.** 1998. *Ichthyophthirius multifiliis*: a model of cutaneous infection and immunity in fishes. Immunol Rev. **166:**377-384.

17. **Diconza, J. J., and W. J. Halliday.** 1971. Relationship of catfish serum antibodies to immunoglobulin in mucus secretions. Aust J Exp Biol Med Sci. **49:**517-519.

18. **Fletcher, T. C., and P. T. Grant.** 1969. Immunoglobulin in the serum and mucus of the plaice (*Pleuronectes platessa*). Biochem J. **115:**65p.

19. **Fletcher, T.C., and A. White.** 1973. Antibody production in the plaice (*Pleuronectes platessa* L.) after oral and parenteral immunization with *Vibrio anguillarum* antigens. Aquaculture. **1:**417-428.

20. **Hines, R. S., and D. T. Spira.** 1974. Ichthyophthiriasis in the mirror carp *Cyprinus carpio* (L.). V. Acquired immunity. J Fish Biol. **6**:373-378.

21. **Itami, T., Y. Takahashi, T. Oamoto, and K. Kubono.** 1988. Purification and characterization of immunoglobulin in skin mucus and serum of ayu. Nippon Suisan Gakkaishi. **54(9):**1611-1617.

22. **Lin, T. L., and H. W. Dickerson,** 1992. Purification and partial characterization of immobilization antigens from *Ichthyophthirius multifiliis*. J Protozool. **39**:457-463.

23. **Lin, T. L., T. G. Clark, and H. W. Dickerson.** 1996. Passive immunization of channel catfish (*Ictalurus punctatus*) against the ciliated protozoan parasite *Ichthyophthirius multifiliis* by the use of murine monoclonal antibodies. Infect Immun. **64:**4085-4090.

24. **Lobb, C. J., and L. W. Clem.** 1981. The metabolic relationships of the immunoglobulins in fish serum, cutaneous mucus and bile. J Immunol. **127:**1525-1529.

25. **Lobb, C. J., and L. W. Clem.** 1981. Phylogeny of immunoglobulin structure and function. XI. Secretory immunoglobulins in the cutaneous mucus of the sheepshead, *Archosargus probatocephalus*. Dev Comp Immunol. **5:**587-596.

26. **Lobb, C. J., and L. W. Clem.** 1981. Phylogeny of immunoglobulin structure and function-XII: Secretory immunoglobulins in the bile of the marine teleost *Archosargus probatocephalus*. Mol Immunol. **18(7):**615-619.

27. **Lobb, C. J., and L. W. Clem.** 1983. Distinctive subpopulations of catfish serum antibodies and immunoglobulin. Mol Immunol. **20(8):**811-818.

28. **Lobb, C. J.** 1985. Covalent structure and affinity of channel catfish anti-dinitrophenyl antibodies. Mol Immunol. **22(8):**993-999.

29. **Lobb, C. J.** 1987. Secretory immunity induced in catfish, *Ictalurus punctatus,* following bath immunization. Dev Comp Immunol. **11:**727-738.

30. **Lumsden, J. S., V. E. Osterland, P. J. Byrne, and H. W. Ferguson.** 1993. Detection of a distinct gill-surface antibody response following horizontal infection and bath challenge of brook trout *Salvelinus fontinalis* with *Flavobacterium branchiophilum*, the causative agent of bacterial gill disease. Dis Aquat Org. **16:**21-27.

31. **McCallum, H. I.** 1986. Acquired resistance of black mollies *Poecilia latipinna* to infection by *Ichthyophthirius multifiliis*. Parasitology **93:**251-261.

32. **Ourth, D. D.** 1980. Secretory IgM, lysozyme and lymphocytes in the skin mucus of the channel catfish, *Ictalurus punctatus*. Dev Comp Immunol. **4:**65-74.

33. **Ourth, D. D.** 1986. Purification and quantification of channel catfish (*Ictalurus punctatus*) immunoglobulin. M J Ichthyol. **3:**140-143.

34. **Peleterio, M. C., and R. H. Richards.** 1988. Immunocytochemical studies on immunoglobulin-containing cells in the epidermis of rainbow trout, *Salmo gairdneri*. Richardson: influence of bath immunization. J Fish Biol. **32:**845-858.

35. **Rombout, J. W., L. J. Blok, C. H. Lamers, and E. Egberts.** 1986. Immunization of carp (*Cyprinus carpio*) with a *Vibrio anguillarum* bacterin: indications for a common mucosal immune system. Dev Comp Immunol. **10(3):**341-351.

36. **Rombout, J. H. W. M., N. Taverne, M. van de Kamp, and A. J. Taverne-Theile.** 1993a. Differences in mucus and serum immunoglobulin of carp (*Cyprinus carpio* L.). Dev Comp Immunol. **17:**309-317.

37. **Rombout, J.W., A.J. Taverne-Thiele, and M. I. Villena.** 1993b. I. The gut-associated lymphoid tissue (GALT) of carp (*Cyprinus carpio* L.): An immunocytochemical analysis. Dev Comp Immunol. **17:**55-66.

38. **Rombout, J.W., A. A. van den Burg, C.T. van den Burg, P. Witte, and E. Egberts.** 1989. Immunological importance of the second gut segment of carp. III. Systemic and/or mucosal immune responses after immunization with soluble or particulate antigen. J Fish Biol. **35:**79-186.

39. **Sambrook, J., E. F. Fritsch, and T. Manniatis.** 1989. *In* Molecular Cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, MA, US.

40. **St. Louis-Cormier, E. A., C. K. Osterland, and P. D. Anderson.** 1984. Evidence for a cutaneous secretory immune system in rainbow trout (*Salmo gairdneri*). Dev Comp Immunol. **8:**71-80.

41. **Van Muiswinkel, W.** 1995. The Piscine Immune System: Innate and Acquired Immunity. *In* P. T. K. Woo (ed.) Fish Diseases and Disorders. Vol. 1. CAB International, Wallingford, UK.

42. **Wahli, T., and W. Meier.** 1985. Ichthyophthiriasis in trout: an investigation of natural defense mechanisms. *In* A. E. Ellis (ed.), Fish and Shellfish Pathology. Academic Press, London, United Kingdom.

43. **Wang, X., and H.W. Dickerson.** 2002. Surface immobilization antigen of the parasitic ciliate *Ichthyophthirius multifiliis* elicits protective immunity in channel catfish (*Ictalurus punctatus*). Clin Diag Lab Immunol. **9(1):**176-181.

44. **Xu, C.** 1995. Ph.D. thesis. The University of Georgia.

45. **Zillberg, D., and P. H. Klesius.** 1997. Quantification of immunoglobulin in the serum and mucus of channel catfish at different ages and following infection with *Edwardsiella ictaluri.* Vet Immunol Immunopathol. **58:**171-180.

Figure 2.1. Reduced and unreduced forms of *I. multifiliis* G5 i-antigen and Western Blot demonstrating binding of *I. multifiliis*-exposed channel catfish sera to the unreduced form of i-antigen. The G5 i-antigen was purified by Triton X-114 detergent solubilization, phase separation and immunoaffinity column chromatography using mAb G3-61. Proteins were resolved by SDS-PAGE on a 12% acrylamide gel and stained with silver nitrate (BioRad). Lane 1, pre-stained protein molecular weight markers (Broad Range, BioRad); Lane 2, reduced Triton X-144 G5 membrane protein (0.5 μg) with a predominant band of ~50 kilodaltons (k-Da); Lane 3, reduced immunoaffinity purified i-antigen (0.5 μg); Lane 4, unreduced immunoaffinity purified i-antigen (0.5 μg)(~40 kDa); Lane 5, reduced i-antigen (0.5 mg) transferred to PVDF membrane and incubated with sera from a channel catfish previously exposed to *I. multifiliis*. Catfish Ig was detected by incubating the blot with 2.0 μg/ml of a Protein A-purified goat anti-channel catfish Ig antibody followed by rabbit anti-goat antibody conjugated to alkaline phosphatase and NBT-BCIP (BioRad). Lane 6, unreduced i-antigen (0.5 μg) transferred to PVDF membrane and treated in a manner similar as described for Lane 5.

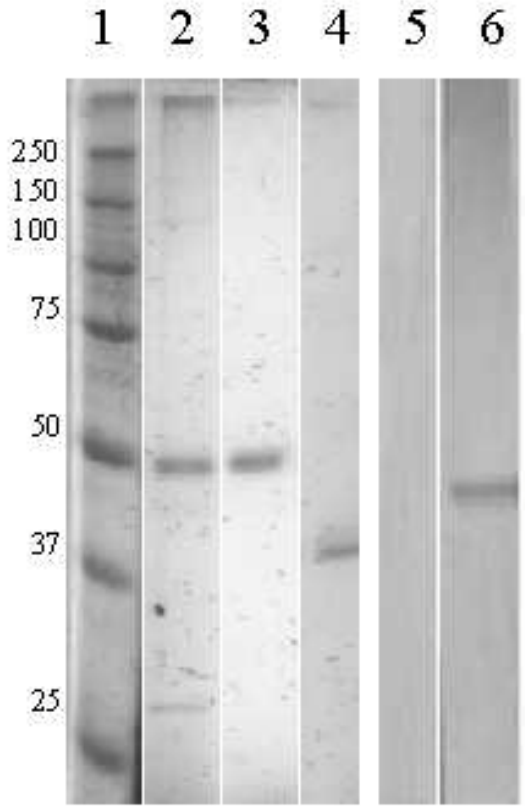
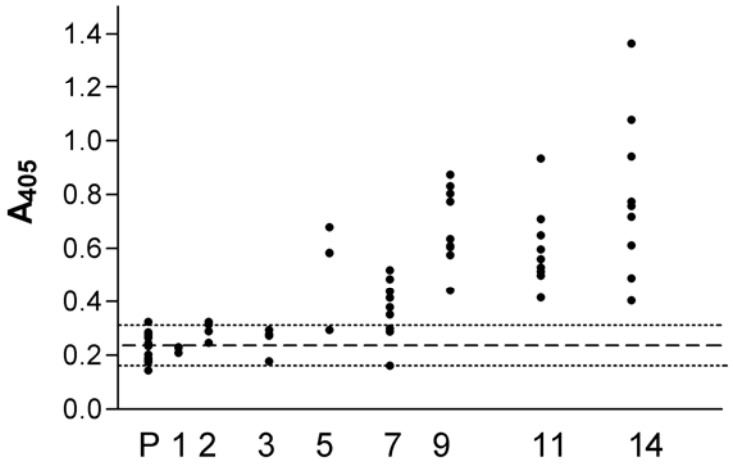

1
2
3
4
5
6
250
150
100
75
50
37
25
40 kDa

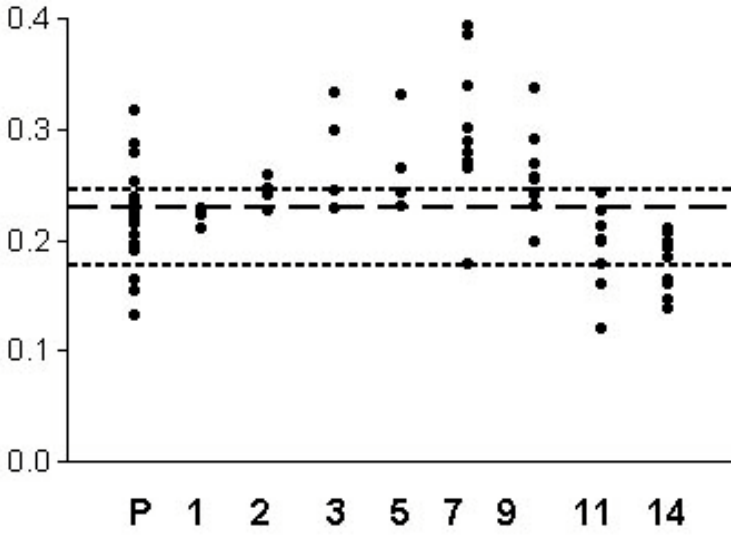
Figure 2.2. *I. multifiliis*-specific ELISA absorbance values of channel catfish serum (A) and mucus (B) following immunization by surface exposure to parasites and formalin treatments. Twenty channel catfish were exposed to 11,000 parasites per fish and treated with formalin. Two fish were sampled from each tank during weeks 1, 2, 3 and 5. Five fish were sampled from each tank during weeks 7, 9, 11 and 14. Sera (diluted 1:80 in phosphate buffered saline) and undiluted mucus were applied to ELISA plates containing Triton X-114-purified *I. multifiliis* membrane proteins. Catfish immunoglobulin was detected using a biotinylated goat anti-catfish immunoglobulin antibody as described in Material and Methods. Dashed lines represent mean absorbance values of Replicates 1+2 pretreatment (P) mucus (0.19 ± 0.03) and serum (0.24 ± 0.02). Dotted lines represent upper and lower limits of all pre-treatment mucus (0.21 ± 0.04) and serum ($.24 \pm 0.05$) samples.

Figure 2.2A. ELISA absorbance values of diluted channel catfish sera (1:80) following immunization by surface exposure to I. *multifiliis* theronts (11,000 per fish) and formalin treatments.



Week	Replicate 1 Mean ± SD	Replicate 2 Mean ± SD	1+2 Mean ± SD
Pre-treat	0.239 ± 0.06	0.244 ± 0.03	0.241 ± 0.05
1	0.220 ± 0.01	0.228 ± 0.001	0.224 ± 0.01
2	0.306 ± 0.02	0.321 ± 0.01	0.289 ± 0.05
3	0.276 ± 0.003	0.236 ± 0.08	0.256 ± 0.05
5	0.631 ± 0.07	0.440 ± 0.21	0.536 ± 0.17
7	0.326 ± 0.10	0.427 ± 0.10	0.370 ± 0.11
9	0.574 ± 0.08	0.829 ± 0.11	0.679 ± 0.14
11	0.517 ± 0.07	0.708 ± 0.17	0.601 ± 0.15
14	0.662 ± 0.22	0.957 ± 0.33	0.793 ± 0.30

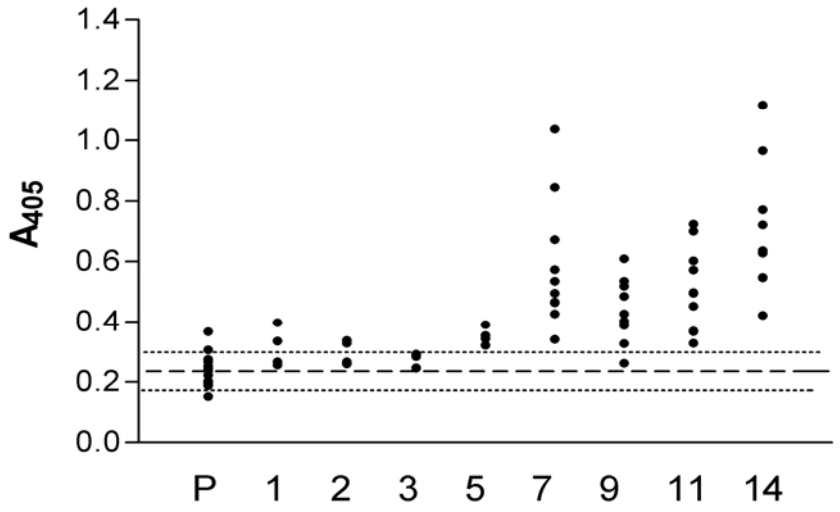
Figure 2.2B. ELISA absorbance values of undiluted channel catfish mucus following immunization by surface exposure to I. *multifiliis* theronts (11,000 per fish) and formalin treatments.



Week	Replicate 1 Mean ± SD	Replicate 2 Mean ± SD	1+2 Mean ± SD
Pre-treat	0.235 ± 0.03	0.217 ± 0.06	0.226 ± 0.05
1	0.219 ± 0.01	0.226 ± 0.004	0.222 ± 0.01
2	0.250 ± 0.01	0.238 ± 0.01	0.244 ± 0.01
3	0.238 ± 0.01	0.317 ± 0.02	0.278 ± 0.05
5	0.237 ± 0.01	0.299 ± 0.05	0.268 ± 0.05
7	0.269 ± 0.06	0.340 ± 0.06	0.301 ± 0.07
9	0.260 ± 0.02	0.256 ± 0.06	0.259 ± 0.04
11	0.211 ± 0.02	0.174 ± 0.04	0.194 ± 0.04
14	0.184 ± 0.03	0.173 ± 0.02	0.179 ± 0.03

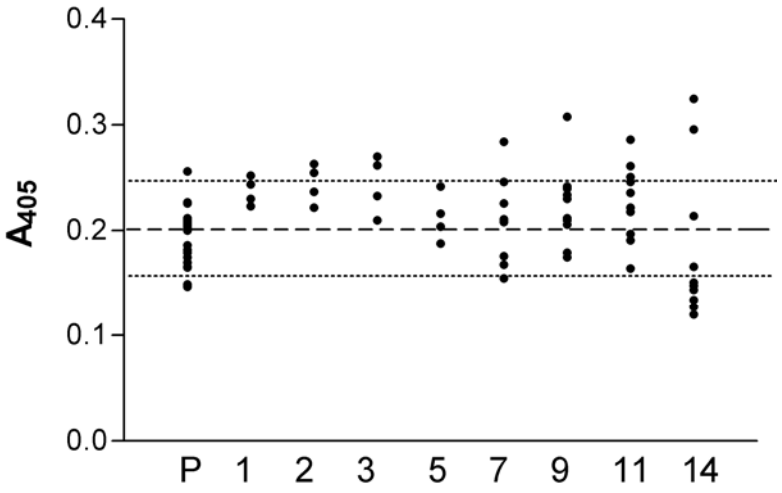
Figure 2.3. *I. multifiliis*-specific ELISA absorbance values of channel catfish sera (A) and mucus (B) following immunization by intraperitoneal injection of purified *I. multifiliis* i-antigen. Twenty channel catfish received 5.0 μg of immunoaffinity purified *I. multifiliis* i-antigen in Freund's incomplete adjuvant. Two fish were sampled from each tank during weeks 1, 2, 3 and 5. Five fish were sampled from each tank during weeks 7, 9, 11 and 14. Sera (diluted 1:80 in phosphate buffered saline) and undiluted mucus were applied to ELISA plates containing Triton X-114-purified *I. multifiliis* membrane proteins. Bound catfish antibody was detected using a biotinylated goat anti-catfish immunoglobulin antibody as described in Material and Methods. Dashed lines represent mean absorbance values of Replicate 1+2 pretreatment (P) mucus (0.19 ± 0.03) and sera (0.24 ± 0.02). Dotted lines represent upper and lower limits of all pre-treatment mucus (0.21 ± 0.04) and sera ($.24\pm0.05$).

Figure 2.3A. ELISA absorbance values of channel cat fish sera (diluted 1:80) following immunization by intraperitoneal injection of 5.0 μg of purified *I. multifiliis* i-antigen in Freund's incomplete adjuvant.



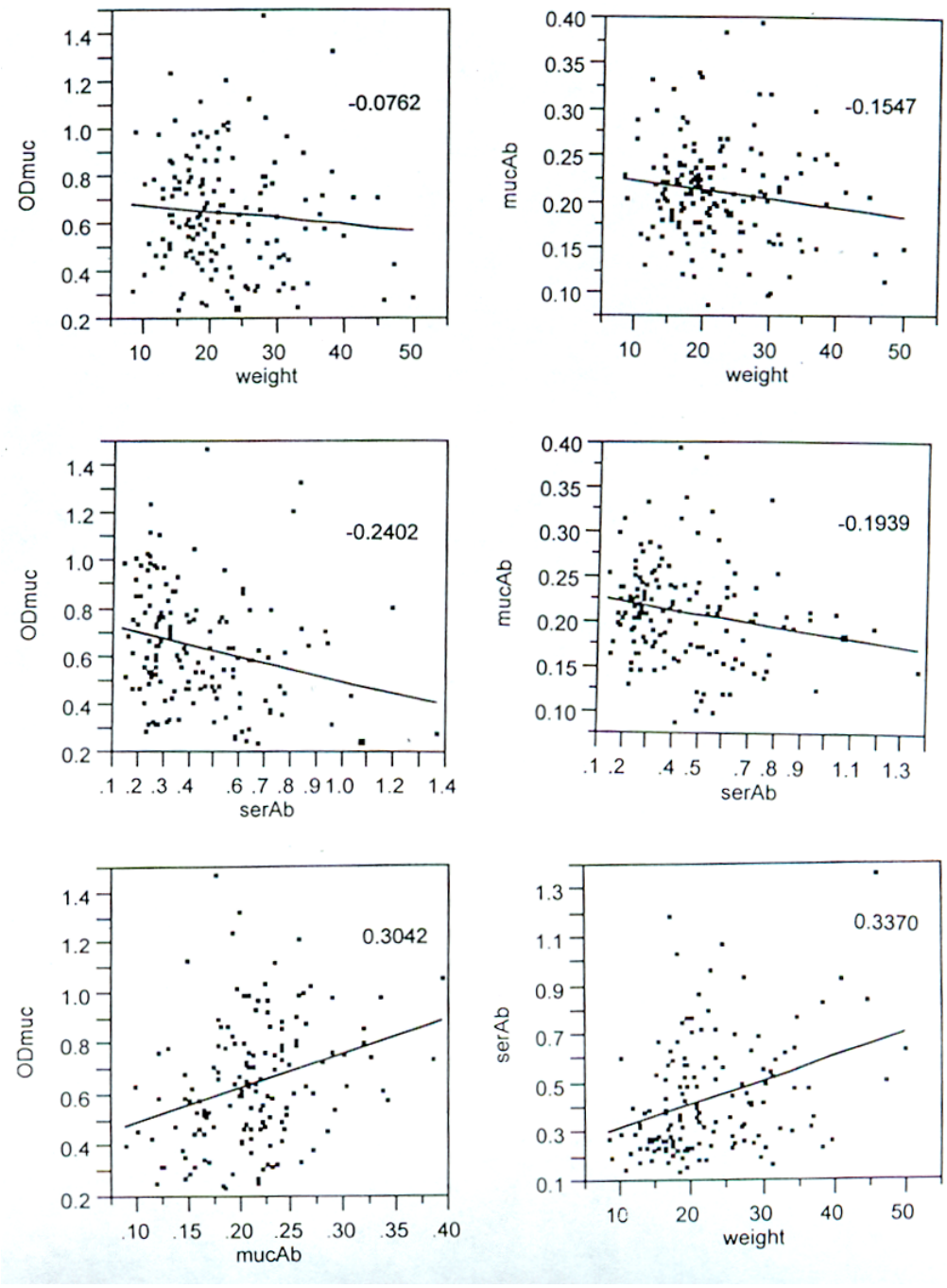
Week	Replicate 1 Mean ± SD	Replicate 2 Mean ± SD	1+2 Mean ± SD
Pre-treat	0.264 ± 0.07	0.224 ± 0.03	0.240 ± 0.05
1	0.366 ± 0.04	0.261 ± 0.01	0.314 ± 0.07
2	0.262 ± 0.005	0.324 ± 0.02	0.293 ± 0.04
3	0.288 ± 0.01	0.246 ± 0.001	0.267 ± 0.02
5	0.349 ± 0.01	0.356 ± 0.05	0.352 ± 0.03
7	0.513 ± 0.10	0.658 ± 0.28	0.586 ± 0.21
9	0.417 ± 0.14	0.452 ± 0.07	0.434 ± 0.10
11	0.402 ± 0.07	0.620 ± 0.09	0.511 ± 0.14
14	0.727 ± 0.16	0.698 ± 0.03	0.714 ± 0.24

Figure 2.3B. ELISA absorbance values of channel cat fish mucus following immunization by intraperitoneal injection of 5.0 µg of purified *I. multifiliis* i-antigen in Freund's incomplete adjuvant.



Week	Replicate 1 Mean ± SD	Replicate 2 Mean ± SD	1+2 Mean ± SD
Pre-treat	0.177 ± 0.02	0.210 ± 0.02	0.193 ± 0.03
1	0.234 ± 0.02	0.241 ± 0.02	0.237 ± 0.01
2	0.230 ± 0.01	0.259 ± 0.01	0.244 ± 0.02
3	0.222 ± 0.02	0.266 ± 0.01	0.243 ± 0.03
5	0.202 ± 0.02	0.223 ± 0.03	0.212 ± 0.02
7	0.189 ± 0.03	0.232 ± 0.03	0.210 ± 0.04
9	0.219 ± 0.02	0.233 ± 0.05	0.226 ± 0.04
11	0.198 ± 0.02	0.256 ± 0.02	0.227 ± 0.04
14	0.141 ± 0.02	0.224 ± 0.08	0.192 ± 0.09

Figure 2.4. Correlation analysis of fish weight, individual serum (1:80 dilution) ELISA absorbance values, cutaneous mucus ELISA absorbance values and cutaneous mucus total protein concentration as estimated by absorbance at A_{280}. Individual values were analyzed by scatterplot matrix using Jump® (JMP IN Version 3.2.6)(SAS Institute, Inc., Wadsworth Publishing, Belmont, CA).


ODmuc
weight
-0.0762
mucAb
weight
-0.1547
ODmuc
serAb
-0.2402
mucAb
serAb
-0.1939
ODmuc
mucAb
0.3042
serAb
weight
0.3370

CHAPTER 3

IMMUNOGLOBULIN- CONTAINING LYMPHOCYTES WITHIN THE HEAD KIDNEY AND EPIDERMIS OF THE CHANNEL CATFISH *(ICTALURUS PUNCTATUS)*[1]

[1] Maki, Joanne, M. Ard, B. Steffens, and H. W. Dickerson. To be submitted to *Journal of Leukocyte Biology*.

ABSTRACT

Channel catfish (*Ictalurus punctatus*) respond to antigenic stimulation by producing a single class of antibody (Ab). This tetrameric immunoglobulin (Ig) is present on the surface membranes of B-lymphocytes, in sera and mucosal secretions. We are interested in identifying and characterizing *I. punctatus* antibody-secreting cells (ASC) *in situ* to better understand the organization of their immune system and more specifically determine if the skin is an immunologically active tissue. Detection of ASC in mucosal tissues, such as the skin, supports the hypothesis that fish have a common mucosal immune system. Immunohistochemical techniques were used to detect Ig-containing lymphocytes in resin-embedded tissues of normal adult fish by transmission electron microscopy (TEM). The primary detection reagent was goat anti-channel catfish Ig-specific Fab-2 fragments followed by a biotinylated rabbit anti-goat Ab and avidin-gold beads. In the head kidney, a distinct population of lymphocyte-like cells, containing large numbers of discrete cytoplasmic vesicles, was labeled. Antibody binding was limited to the cytoplasm of individual cells while surrounding lymphocytes and macrophages were negative. In the epidermis, cells having a similar morphology and labeling pattern were detected but very infrequently in comparison to the head kidney. Epidermal and dermal macrophages, capillaries and connective tissue were negative. These results demonstrate cytoplasmic Ig-containing cells in the head kidney and skin of normal channel catfish. Based on their morphology and staining pattern we conclude that they are ASC. The presence of epidermal ASC suggests that the skin may be a site of antibody secretion and thereby an immunologically active component of the channel catfish mucosal immune system.

Keywords: *Ictalurus punctatus*, channel catfish, antibody secreting cell (ASC), mucosal immunity, epidermis, head kidney, plasma cell, immunohistochemistry.

INTRODUCTION

Channel catfish (*Ictalurus punctatus*), like other teleosts, produce a single class of immunoglobulin (Ig), a tetrameric IgM-like antibody in their blood and mucus that appears to be identical in structure (10, 23, 24, 27). Although there is experimental evidence supporting the existence of a separate mucosal immune system in fish, it is currently unknown how mucus antibodies reach the epithelial surface of the gut, gills and skin (1, 2, 9, 15, 16, 20-22, 32-34, 38). To answer this question we were interested in identifying lymphocytes within tissues where mucosal antibodies are generated. To do so will provide a better understanding of the channel catfish immune system and perhaps shed light on the mechanisms by which antibodies are transported to the skin. If channel catfish skin contains antibody-secreting cells (ASC), this finding would suggest local antibody secretion and identify this tissue as an immunologic effector site.

As a first step, goat Fab-2 fragments made against purified channel catfish Ig were prepared and used to detect immunoglobulin within lymphocytes in resin-embedded tissues using standard immunohistochemical techniques and transmission electron microscopy. We chose the head kidney as the site to initially look for these cells since this tissue is considered to be the bone marrow equivalent in fish and has been shown to contain ASC in other fish species (3, 9, 19, 30, 31, 36, 39). Second, we wished to determine whether or not similar Ig-containing cells were present in the skin.

Immuno-gold labeling of channel catfish head kidney and skin revealed labeled lymphocyte-like cells of similar morphology and staining pattern. This is the first report describing the ultrastructural morphology of cytoplasmic Ig+ cells in the channel catfish. Our results not only characterize ASC in an economically important food fish species, but also support the long-standing hypothesis that fish skin is a component of a mucosal immune system. From these data one may further hypothesize that antibodies found in the cutaneous mucus arise from local production within the epidermis.

MATERIALS AND METHODS

Light Microscopy Tissue Samples:

A single adult (> 3 year of age) female channel catfish (*Ictalurus punctatus*), (~ 1.4 kg) obtained from Georgia Department of Natural Resources fish hatchery (Thomson, GA) and reared in confinement was euthanized by immersion in water containing 100-200 mg/L of tricaine methanesulfonate (MS-222)(Argent Chemicals, Richmond, WA). Head kidney and skin sections were collected and fixed as described below. Skin sections consisted of epidermal and dermal to allow for tissue orientation.

Preparation of the anti-catfish immunoglobulin Fab-2 fragments:

The primary antibody (Ab) consisted of Fab-2 fragments of a goat polyclonal Ab made against purified catfish sera. To prepare this reagent, channel catfish immunoglobulin (Ig) was purified from a pooled serum sample. Blood collected from the caudal venous sinus of 5-6 adult channel catfish was allowed to clot overnight at 4^{o}C. Sera was collected and centrifuged 10,000g for 10 min in a tabletop centrifuge (Marathon 13K/Fisher) and transferred to 1.5ml polypropylene tubes for storage at –20^{o}C. Catfish Ig was purified using

an IgM purification kit (Immunopure, Pierce, Rockford, IL) following the manufacturer's standard protocol.

Purified catfish Ig, 1.0 µg in a 1.0-ml volume of phosphate buffered saline (PBS)(136 mM NaCl; 2.7 mM KCl; 10.1 mM Na_2HPO_4; 1.5 mM KH_2PO_4)(pH 7.2)(35), was mixed with 1.0 ml of Freund's incomplete adjuvant (Sigma). The emulsified antigen was administered by intramuscular injection into a domestic mixed breed goat (*Capra hircus*) three times over a three-month interval. Blood was periodically collected from the goat via the jugular vein using a sterile needle and syringe prior to and following immunization. Serum was collected from clotted blood as described above. Goat Ig was purified from the sera using an immobilized Protein A/G column (Pierce).

Fab-2 fragments were generated by adding trypsin (Sigma)(5 µg/ml of PBS) was added to 5.0 mg of purified goat Ig after adjusting the pH of the reaction mixture to pH = 4.0 with 200 mM HCl. The mixture was incubated for 1.5 hr at 37^{o}C and centrifuged for 10 min in a bench-top centrifuge (~12,000g). The Fab-2 fragments were collected by passing the mixture over an equilibrated Protein A/G column (Pierce) after adding 100 mM NaOH to bring the reaction pH back to 7.0. The absorbance (A_{280}) of individual aliquots were determined and those containing >0.5 µg of protein were pooled and frozen at –20^{o}C.

Transmission Electron Microscopy:

Tissues were fixed in 1.0% formaldehyde, 1.0% glutaraldehyde, and 0.1% picric acid in 0.1-M cacodylate-HCl buffer for 4 h at ambient room temperature (RT) of 21-25^{o}C. Blocks were dehydrated in sequential ethanol baths and infiltrated with LR White Resin (hydrophilic acrylic resin, Polysciences, Inc., Warren, PA). Thin sections cut with a diamond knife on a Reichert Ultracut S Ultramicrotome (Leica, Inc., Deerfield, IL) and

mounted on Formvar (E.F. Fullam, Inc., Latham, NY)-carbon coated nickel grids. The grids were incubated with 0.2 M Tris-buffered saline (pH = 8.2) + 1% globulin-free bovine serum albumin (BSA)(Sigma, St. Louis, MO) for 1 hr at RT. Non-specific binding sites were blocked with normal rabbit sera (1:10) diluted in saline and sections were processed as described below.

The Fab-2 fragments were applied to blocked grids at a 1:200 dilution containing a final concentration of 7.5 μg/ml and incubated at RT for 1 hr. After rinsing in Tris buffer containing 1% BSA (Tris/BSA) the sections were incubated for 1 hr at RT with a rabbit-Fab made against goat-Fab and conjugated to biotin (Jackson ImmunoResearch Labs, Inc., West Grove, PA)(diluted 1:5,000 in saline). Grids were then rinsed in Tris/BSA and incubated with strepavidin-gold conjugate (20nm)(BB International, Cardiff, UK) used at 1:20 dilution for (1 h/RT). Control grids were prepared using tissues processed using with Protein A/G purified pre-immune goat Ig as the primary antibody. Another control consisted of incubating sections with Fab-2 fragments that had been previously mixed with purified catfish Ig at a 1:1 molar ratio and incubated at RT for 30 min. After rinsing the grids in Tris/BSA followed by additional rinsing in de-ionized water, the grids were post-stained with methanolic uranyl acetate and lead citrate. The labeled tissues were examined using a Jeol JEM 1210 transmission electron microscope (Tokyo, Japan). Three or four grids of each tissue type were viewed to find representative cell types.

RESULTS

In the head kidney, the anti-catfish Ig Fab-2 fragments labeled a single population of lymphocyte-like cells (FIG. 3.1). The gold beads adhered to discrete foci within the lymphocyte's cytoplasmic vesicles (FIG. 3.2). Other lymphocytes, identified as early B and

T-cells, based on their low nucleus to cytoplasmic ratio, were morphologically identified and were not labeled. Cells identified as macrophages or activated lymphocytes based on the presence of inclusion bodies and mitochondria were also not labeled.

In the skin, the majority of grids were negative, however, a very low number of cytoplasmic-Ig-bearing lymphocytes were observed. Labeled cells were located in the epidermis and had cytoplasmic-labeling pattern similar to those cells identified in the head kidney (FIG. 3.3). One feature that was different from the head kidney Ig-bearing cells was the exterior membrane and overall shape of the cell was distorted. A few cells that were lightly labeled by the anti-catfish Ig Fab-2 fragments were identified as being macrophage-like (FIG. 3.4). The degree of labeling and distribution of the gold-beads on the epidermal macrophage was not the same as in the other Ig+ cells and cytoplasmic vesicles were not observed. Epidermal mucus cells were numerous and were also not labeled (FIG. 3.5). These results demonstrate the presence of cytoplasmic Ig-containing lymphocytes in the channel catfish head kidney and very low numbers of the same cell type within the epidermis.

DISCUSSION

Fish leukocytes have been systematically studied at the microscopic level since the 1950's (8). Cells bearing morphological similarity to mammalian antibody secreting cells (ASC)(i.e., plasma cells) were initially described in sharks and rays (11, 12). Multiple light microscopy and ultrastructural studies investigating fish lymphocytes have revealed that cells of the piscine immune system vary greatly between species. Even within one species, structural similarities between small lymphocytes and monocytes as

well as activated macrophages and granulocytes make classification of fish lymphocytes difficult. Researchers have been urged not to rely on morphological features alone to classify fish leukocytes (11).

Putative ASC have been described at the ultrastructural level in the sea bass (*Dicentrarchus labrax*)(26, 31); brook lamprey (*Lampertra reissneri*)(17, 40); Aleutian skate (*Batharaja aleutica*)(41); carp (*Cyprinus carpio*)(32) and the African catfish (*Clarias gariepinus*)(4, 5). As a general rule, plasma cells are typically found in the head kidney of bony fish (*Teleostei*)(30). The literature describes fish ASC as being lymphocyte-like and containing eccentric nuclei with numerous cytoplasmic vacuoles. Beyond these features, fish ASC are quite heterogeneous in appearance, particularly the size and shape of their cytoplasmic vesicles. Primitive fish, such as the lamprey, have ASC with large distended membrane-bound vesicles (40, 41); while in the sea bass, ASC are more mammalian-like with extensive Golgi network and stacks of endoplasmic reticulum (26, 31).

Immunohistochemical techniques have been used to label cytoplasmic immunoglobulin (Ig)+ cells in the head kidney of carp and the spleen of the Aleutian skate (19, 41). Similar cells have been also been labeled in the intestine of brook lamprey, carp and sea bass (17, 31, 34). Cells containing immunoglobulin (Ig) have been identified in mucus and frozen sections of rainbow trout skin (*Salmo gairdneri*) and channel catfish skin by indirect immunofluorescence (38, 42). The epidermal mucus cells of rainbow trout have also been described as containing Ig (28, 29).

Early light microscopic studies of channel catfish (*Ictalurus punctatus*) peripheral blood cells did not contain descriptions of plasma cells (7, 44). However, head kidney

and spleen impression smears of a related species, the African catfish (*C. gariepinus*), contained low numbers of lymphocytes described at putative plasma cells (4). Ultrastructural studies in this same species identified cells that were morphologically similar to mammalian plasma cells and structures thought to be Russell bodies were described (5). Lymphocytes have also been demonstrated in the cutaneous mucus of channel catfish (27).

In this study, goat Fab-2 fragments were used to identify Ig within the cytoplasm of lymphocyte-like cells in the head kidney of normal channel catfish. With the completion of our initial goal, we successfully identified similar cells in the epidermis. The identification of ASC in the head kidney was expected and supports the description of this organ as a bone marrow equivalent in this species. The presence of Ig+ cells in the channel catfish skin is the first description of putative ASC in this tissue. The cutaneous mucus of channel catfish has been shown to contain antigen-specific antibody (1, 40, 41, Maki and Dickerson, in preparation). However, the origin and mechanism of mucosal antibody secretion remains unknown. Epidermal cells identified as Ig+ lymphocytes in this study may arise from capillary beds within the dermis or may reside within these tissues (30). In either case, their presence in the skin supports the concept of local antibody production. These results, identifying Ig+ cells in the head kidney and skin of channel catfish, not only provide insight as to the origin of their mucosal surface Ab, but also support the long-standing hypothesis that fish skin is a component of a mucosal immune system.

REFERENCES

1. **Ainsworth, A. J., C. D. Rice, and L. Xue.** 1995. Immune responses of channel catfish, *Ictalurus punctatus* (Rafinesque), after oral or intraperitoneal vaccination with particulate or soluble *Edwardsiella ictaluri* antigen. J of Fish Dis. **18:**397-409.
2. **Bradshaw, C. M., A. S. Richard, and M. M. Sigel.** 1971. IgM antibodies in fish mucus. Proc Soc Exp Biol Med. **136:**1122-1124.
3. **Boomker, J.** 1979. The haemocytology and histology of the haemopoietic organs of South African freshwater fish. I. The haemopoietic organs of *Clarias gariepinus* and *Sarotherodon mossambicus*. Onderstepoort J Vet Res. **46:**217-22.
4. **Boomker J**. 1981a. The haemocytology and histology of the haemopoietic organs of South African freshwater fish. III. The leucocytes, plasma cells and macrophages of *Clarias gariepinus* and *Sarotherodon mossambicus*. Onderstepoort J Vet Res. **48(4):** 185-193.
5. **Boomker J.** 1981b. The haemocytology and histology of the haemopoietic organs of South African freshwater fish. IV. Ultrastructure of some cells of *Clarias gariepinus* and *Sarotherodon mossambicus*. Onderstepoort J Vet Res. **48(4):** 195-205.
6. **Breazile, J. E., H. J. Mass, J. Wollscheid, L. L. Zinn.** 1982. A light and electron microscopic study of the leukocytes of channel catfish (*Ictalurus punctatus*). Zbl Vet Med C Anat Histol Embryol. **11:**107-116.
7. **Cannon, M. S., H. H. Mollenhauer, T. E. Eurell, D. H. Lewis, A. M. Cannon, and C. Tompkins**. 1980. An ultrastructural study of the leukocytes of the channel catfish, *Ictalurus punctatus*. J of Morphol. **164:**1-23.

8. **Catton, W. T**. 1951. Blood cell formation in certain teleost fishes. J Hematol. **6:**39-60.
9. **Chiller J. M., H. O. Hodgins, V. C. Chambers, and R. S. Weiser.** (1969) Antibody Response in Rainbow Trout (*Salmo gairdneri*): I. Immunocompetent cells in the spleen and anterior kidney. J Immunol. **102:**1193-1201.
10. **Diconza, J. J., and W. J. Halliday.** 1971. Relationship of catfish serum antibodies to immunoglobulin in mucus secretions. Aust J Exp Biol Med Sci. **49:**517-519.
11. **Ellis, A. E.** 1977. The leukocytes of fish: A review. J Fish Biol **11:**453-491
12. **Esteban, M. A., J. Munoz, and J. Meseguer**. 2000. Blood cells of sea bass (*Dicentrarchus labrax* L.). Flow cytometric and microscopic studies. Anat Rec. **258:**80-89.
13. **Fletcher, T. C., and P. T. Grant.** 1969. Immunoglobulin in the serum and mucus of the plaice (*Pleuronectes platessa*). Biochem J. **115:**65p.
14. **Fletcher, T.C., and A. White.** 1973. Antibody production in the plaice (*Pleuronectes platessa* L.) after oral and parenteral immunization with *Vibrio anguillarum* antigens. Aquaculture. **1:**417-428.
15. **Fujii, T.** 1982. Electron microscopy of the leucocytes of the typhlosole in ammocoetes, with special attention to the antibody-producing cells. J of Morph. **173:**87-100.
16. **Fujimaki, Y., and M. Isoda.** 1990. Fine-structural study of leukocytes in the goldfish, *Carassius auratus*. J Fish Biol. **8:**139-142.

17. **Imagawa, T., Y. Hashimoto, Y. Kon, and M. Sugimura.** 1991. Immunoglobulin containing cells in the head kidney of carp (*Cyprinus carpio* L.) after bovine serum albumin injection. Fish and Shellfish Immunol. **1:**173-185.

18. **Lobb, C. J., and L. W. Clem.** 1981. The metabolic relationships of the immunoglobulins in fish serum, cutaneous mucus and bile. J Immunol. **127:**1525-1529.

19. **Lobb, C. J., and L. W. Clem.** 1981. Phylogeny of immunoglobulin structure and function. XI. Secretory immunoglobulins in the cutaneous mucus of the sheepshead, *Archosargus probatocephalus*. Dev Comp Immunol. **5:**587-596.

20. **Lobb, C. J., and L. W. Clem.** 1981. Phylogeny of immunoglobulin structure and function-XII: Secretory immunoglobulins in the bile of the marine teleost *Archosargus probatocephalus*. Mol Immunol. **18(7):**615-619.

21. **Lobb, C. J.** 1985. Covalent structure and affinity of channel catfish anti-dinitrophenyl antibodies. Mol Immunol. **22(8):**993-999.

22. **Lobb, C. J.** 1986a. Structural diversity of channel catfish immunoglobulins. Vet Immunol Immunopath. **12:**7-12.

23. **Lopez-Ruiz, A., M. Esteban, and J. Meseguer.** 1992. Blood cells of the gilthead bream (*Sparus aurata* L.): light and electron microscopic studies. Anat Rec. **234:**161-171.

24. **Meseguer, J., M. A. Esteban, and B. Agulleiro.** 1991. Stromal cells, macrophages and lymphoid cells in the head-kidney of sea bass (*Dicentrarchus labrax* L.). Arch Histol Cytol. **54:**299-309.

25. **Ourth, D. D.** 1980. Secretory IgM, lysozme and lymphocytes in the skin mucus of the channel catfish, *Ictalurus punctatus*. Dev Comp Immunol. **4:**65-74.

26. **Peleterio, M., and R. Richards.** 1985. Identification of lymphocytes in the epidermis of the rainbow trout, *Salmo gairdneri* (Richardson). J Fish Dis. **8:**161-172.

27. **Peleterio, M. C., and R. H. Richards.** 1988. Immunocytochemical studies on immunoglobulin-containing cells in the epidermis of rainbow trout, *Salmo gairdneri*. Richardson: influence of bath immunization. J Fish Biol. **32:**845-858.

28. **Press, C. McL.,** 1998. Chapter 2. Immunology of Fishes. pp. 3-62. *In* Pastoret, P. P., P. Greibel, H. Bazin, A. Govaerts (eds.) Handbook of Vertebrate Immunology. Academic Press, San Diego, CA.

29. **Romano, N., L. Abelli, L. Mastrolia, and G. Scapigliati.** 1997. Immunocytochemical detection and cytomorphology of lymphocyte subpopulations in a teleost fish *Dicentrarchus labrax*. Cell Tissue Res. **289:**163-171.

30. **Rombout, J. W., L. J. Blok, C. H. Lamers, and E. Egberts.** 1986. Immunization of carp (*Cyprinus carpio*) with a *Vibrio anguillarum* bacterin: indications for a common mucosal immune system. Dev Comp Immunol. **10(3):**341-351.

31. **Rombout, J. H., W. M., N. Taverne, M. van de Kamp, and A. J. Taverne-Theile.** 1993. Differences in mucus and serum immunoglobulin of carp (*Cyprinus carpio* L.). Dev Comp Immunol. **17:**309-317.

32. **Rombout, J. H., A. J. Taverne-Thiele, and M. I. Villena**. 1993. The gut-associated lymphoid tissue (GALT) of carp (*Cyprinus carpio* L.): an immunocytochemical study. Dev Comp Immunol. **17:** 55-66.

33. **Sambrook, J., E. F. Fritsch, and T. Manniatis.** 1989. *In* Molecular Cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, MA, US.

34. **Smith, A. M., N. A. Wivel, and M. Potter.** 1970. Plasmacytopoiesis in the pronephros of the carp (*Cyprinus carpio*) Anat Rec. **167:**351-370.

35. **Smith, A. M., M. Potter, and B. Merchant.** 1967. Antibody-forming cells in the pronephros of the teleost *Lepomis macrochirus*. J Immunol. **99:**876-882.

36. **St. Louis-Cormier, E. A., C. K. Osterland, and P. D. Anderson.** 1984. Evidence for a cutaneous secretory immune system in rainbow trout (*Salmo gairdneri*). Dev Comp Immunol. **8:**71-80.

37. **Temmink, J. H. M., and C. J. Bayne** (1987) Ultrastructural characterization of leukocytes in the pronephros of carp (*Cyprinus carpio* L.). Dev Comp Immunol. **11:**125-137.

38. **Tomonaga, S., H. Zhang, K. Kobayashi, and R. Fujii.** 1992. Plasma cells in the spleen of the aleutian skate, *Bathyraja aleutica*. Arch Histol Cytol. **55:**287-294.

39. **Torroba, M., A. Chiba, A. Vicente, A. Varas, E. Sacedon, E. Jimenez, Y. Honma, and A. G. Zapata.** 1995. Macrophage-lymphocyte cell clusters in the hypothalmic ventricle of some elasmobranch fish: Ultrastructural analysis and possible functional significance. Anat. Rec. **242:**400-410.

40. **Xu, C.** 1995. Ph.D. Dissertation. The University of Georgia.

41. **Wang, X., and H. W Dickerson.** 2002. Surface immobilization antigens of the parasite ciliate *Ichthyophthirius multifiliis* elicit protective immunity in channel catfish. Clin Diag Lab Immunol. **9(1):**176-181.

42. **Weinreb, E. L.** 1963. Studies on the fine structure of teleost blood cells. I. Peripheral blood. Anat Rec. **147:**219-225.

43. **Williams, R.W., and M. C. Warner.** 1976. Some observations of the stained blood cellular elements of channel catfish, *Ictalurus punctatus*. J Fish Biol. **9:**491-497.

Figure 3.1. Production of the goat anti-catfish immunoglobulin Fab-2 fragments. Serum immunoglobulin (Ig) was purified from pooled channel catfish (*Ictalurus punctatus*) using IgM purification Kit (Pierce). The purified Ig (1.0 μg) was mixed 1:1 with Freund's incomplete adjuvant. A mixed breed domesticated goat (*Capra hircus*) was immunized three times via the intramuscular route. Blood was periodically collected from the jugular vein and aliquots of sera were passed over a Protein A/G column (Pierce) to purify total goat Ig. This antibody was incubated with trypsin to produced Fab-2 fragments. Proteins were electrophoresed by SDS-PAGE under reducing conditions and bands were visualized by staining with Coomassie Rapid Stain.

Gel Lane #	Protein (5.0-10 μg/lane)
1	Protein Marker
2	Whole channel catfish sera
3	Whole channel catfish sera
4	Purified catfish immunoglobulin
5	Empty lane
6	Purified catfish Ig
7	Protein Marker
8	Empty lane
9-13	Purified goat Ig
14-15	Trypsin-digested Fab-2 fragments of goat Ig
16	Protein marker

Production of Goat α Catfish Ig Ab

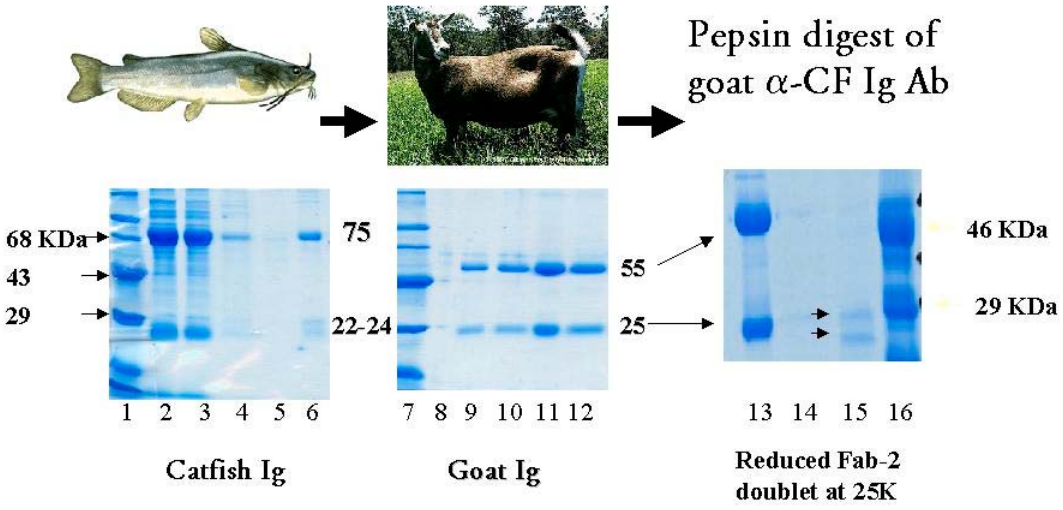


Figure 3.2. Immunohistochemical labeling of immunoglobulin within a channel catfish head kidney lymphocyte. Labeling is limited to cytoplasmic vesicles. Total magnification = 31,800. Bar = 50 microns.

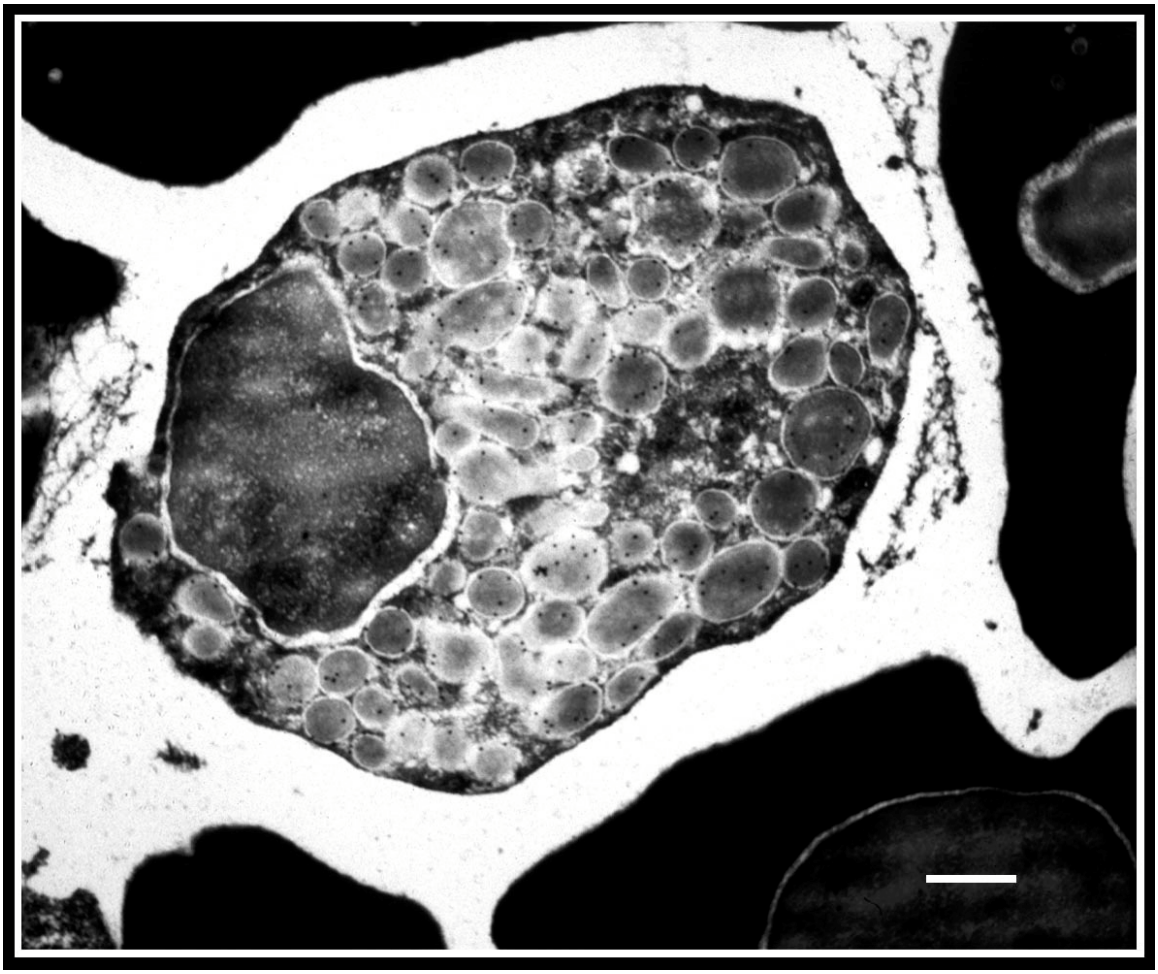


Figure 3.3. Higher magnification of immunoglobulin within a channel catfish head kidney lymphocyte. Labeling is limited to cytoplasmic vesicles. Total magnification = 79,500. Bar = 50 microns.

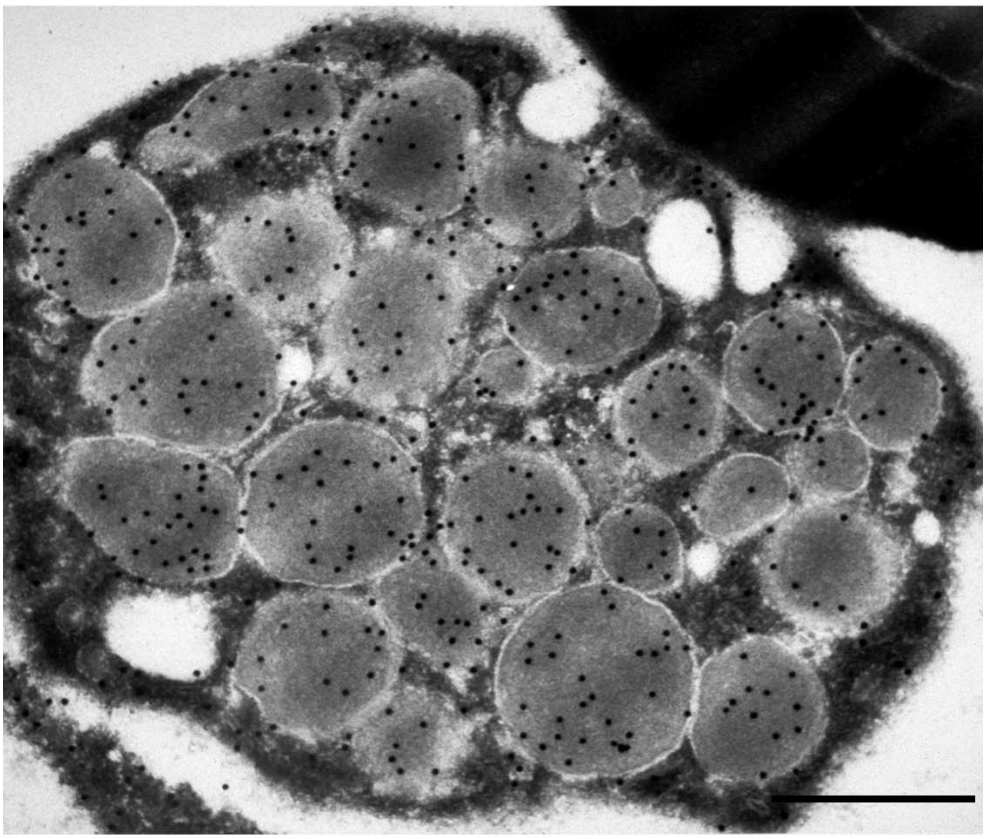


Figure 3.4. Immunohistochemical labeling of immunoglobulin within the cytoplasm of a lymphocyte-like cell in channel catfish epidermis. Total magnification = 20,000. Bar = 50 microns.

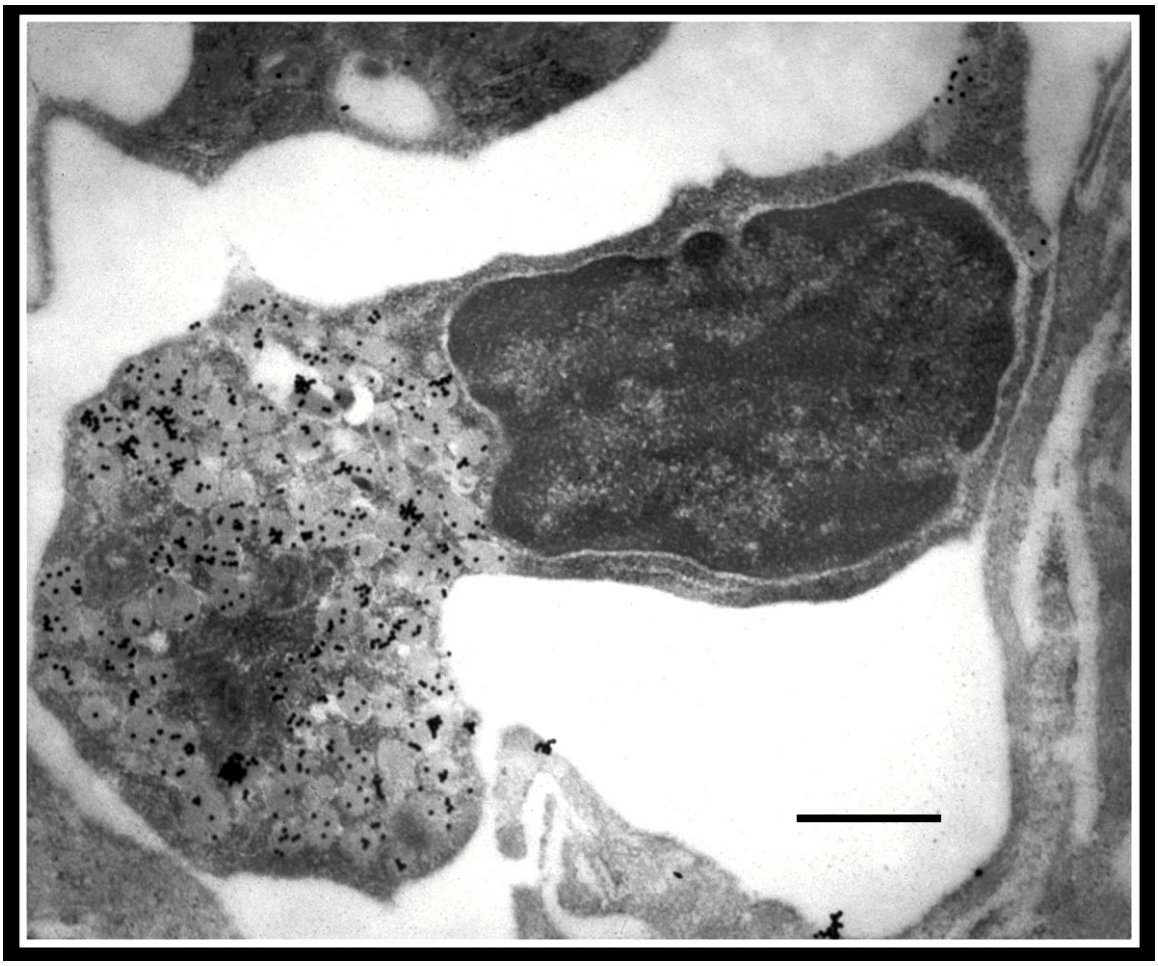
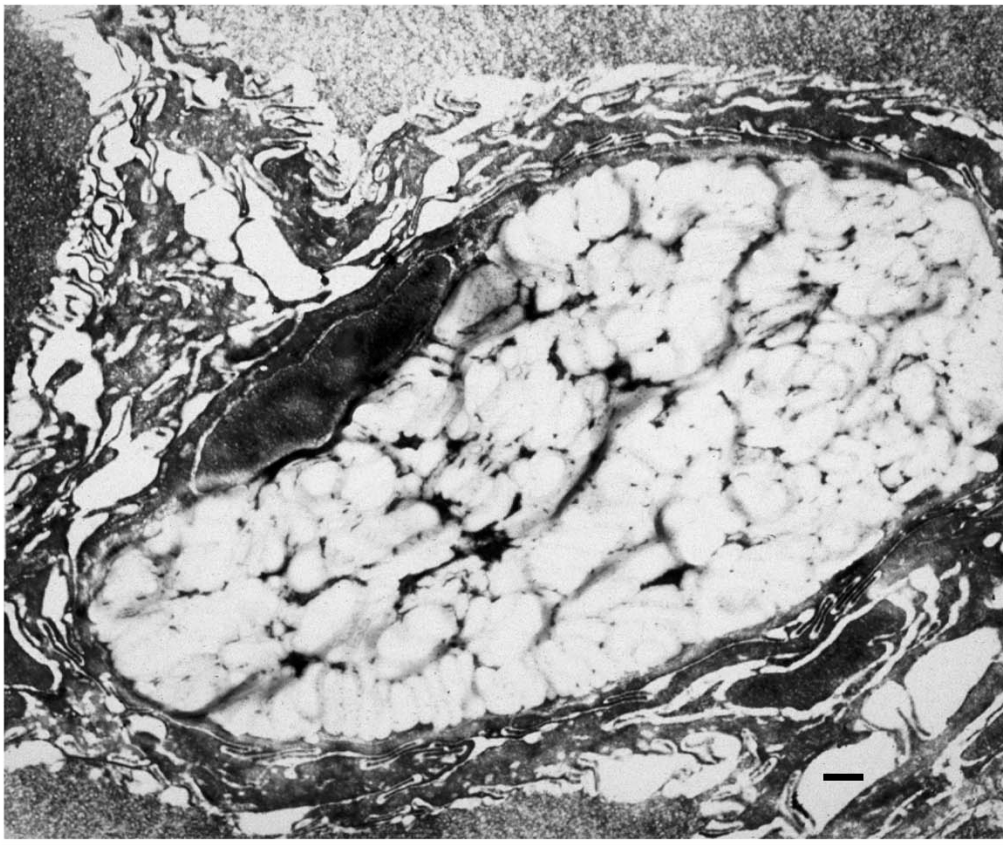


Figure 3.5. A channel catfish epidermal mucus cell demonstrating a lack of labeling by the goat anti-catfish Ig Fab-2 fragments. Total magnification = 6,000. Bar = 50 microns.



CHAPTER 4

OCCURRENCE OF *ICHTHYOPHTHIRIUS MULTIFILIIS* WITHIN THE PERITONEAL CAVITIES OF INFECTED CHANNEL CATFISH, *ICTALURUS PUNCTATUS*[1]

[1] Maki, Joanne L., Corrie C. Brown, and Harry W. Dickerson. Reprinted with permission from *Diseases of Aquatic Organisms*.

ABSTRACT

Ichthyophthirius multifiliis is a ciliated protozoan parasite that infects the skin and gills of freshwater fish. This report describes the unusual finding of *I. multifiliis* within the peritoneal cavities of experimentally infected channel catfish (*Ictalurus punctatus)*. Twenty catfish fingerlings were exposed to *I. multifiliis* theronts using a standardized protocol. Five infected fish and two control fish were killed at various time points after infection and their tissues examined. Formalin-fixed, paraffin-embedded sections were processed for light microscopy and immunohistochemical detection of *I. multifiliis* immobilization antigen. Trophonts were observed in skin and gill sections of all exposed fish. Parasites were associated with epithelial hyperplasia, focal areas of cellular disruption and necrosis. In addition to these usual sites of infection, individual trophonts were unexpectedly found within the peritoneal cavities of four fish. Staining for parasite antigen facilitated their detection within abdominal adipose tissue or adjacent to intestines. This discovery is interesting as it suggests *I. multifiliis* may be found in tissues other than the skin and gills during the course of a normal infection**.**

Keywords: *Ichthyophthirius multifiliis*, Ich, Protozoa, Ciliate, *Ictalurus punctatus*, Channel catfish, Pathology, Immunohistochemistry

INTRODUCTION

The ciliated protozoan parasite, *Ichthyophthirius multifiliis*, infects freshwater fish worldwide causing an economically important disease referred to as “ich” or “white spot”. Infected fish are diagnosed by macroscopic observation of trophonts within the host’s skin and gills. Fish may overcome mild infections and develop a parasite-specific immunity; however high morbidity and mortality often occur in facilities where fish are maintained at high stocking densities such as aquaria or ponds.

I. multifiliis is considered a pathogen of mucosal surfaces and has not been shown to penetrate into the deeper tissues of its host. Pathological lesions associated with *I. multifiliis* have been well characterized. In the skin and gills, infections cause localized lymphocytic infiltration, focal necrosis and varying degrees of epithelial proliferation. In severe cases, sloughing of the epidermis has been observed (1, 5). Other than these common sites, *I. multifiliis* has been reported within the cerebral cavity, circumorbital clefts and nasal pits of a naturally infected carp hybrid (6). Additionally, under artificial conditions, *I. multifiliis* can survive and grow within the peritoneal cavities of channel catfish. This observation was made during experiments in which fish were injected with live parasites by the intraperitoneal route (3).

Fish used in this study were exposed to infective *I. multifiliis* theronts following an established protocol as part of an experiment to develop *in situ* parasite antigen detection methods (2). Histological and immunohistochemical staining of various tissues

collected from twenty *I. multifiliis*-exposed fish led to the unexpected discovery of trophonts within the peritoneal cavities of four fish. This report describes these atypically located parasites and discusses plausible routes of entry.

MATERIALS AND METHODS

Twenty catfish fingerlings (12-18 grams each) were infected using a standard challenge protocol (2). Fish were exposed to 5,000-7,000 infective theronts/fish in glass jars containing 500 ml of aerated, charcoal-filtered water for one hour. The fish, parasites and water were placed in a 76-liter aquarium and maintained at 20-25^{o}C for 11 days. Unexposed control fish were maintained under similar environmental conditions. Water quality (pH and NO_2) was monitored daily.

Five infected fish and 2 control fish were killed and examined at 1, 5, and 10 days after *I. multifiliis* exposure. Tissues (i.e., gill, skin, head kidney, renal kidney, spleen, liver and intestines) were fixed in 10% neutral buffered formalin for 15-20 hr and embedded in paraffin following standard protocols. Replicate 3μm sections were cut and mounted on glass slides. One set was stained with hematoxylin and eosin, and a second set was processed for immunohistochemical detection of *I. multifiliis* surface immobilization antigen (i-Ag) as follows.

Sections were deparaffinized and covered with citrate antigen retrieval buffer (Vector Labs Burlingame, CA). Antigenic sites were exposed by heating the sections in a 1.1 kW microwave set on full power for 5 minutes. Nonspecific antibody binding sites were blocked with 2% normal goat serum diluted in phosphate buffered saline (137 mM NaCl, 12 mM NaH_2PO_4, 2 mM KH_2PO_4 and 0.2 mM KCl) plus 0.005% Tween 20

(PBST) (Sigma, MO). The slides were drained and sections incubated for 2 hr at 37°C with a Protein A-purified anti-*I. multifiliis* i-Ag rabbit polyclonal antibody (1:3,000 in PBST) (prepared by X. Wang in the Dickerson laboratory). Following two five minute washes in PBST (0.2-0.5 ml per slide), sections were incubated for 1 hr at 37°C with a biotinylated goat anti-rabbit antibody (Vector Labs) diluted 1:250 in PBST. Slides were drained, washed twice with PBST and the reactive sites detected with an enzyme complex of avidin-biotin-alkaline phosphatase (ABC Elite, Vector Labs) and substrate DAB (3'3-diamino-benzidine,Vector Labs). Slides were incubated with the enzyme complex for 1 hr at 37°C, rinsed with PBST as described above and the substrate applied. Color development was evident within 8-20 minutes and the reaction stopped by immersing the slides in water. Sections were dried overnight at 22-25°C, counter-stained with hematoxylin and mounted with glass coverslips.

RESULTS

I. multifiliis infection was confirmed in 100% of fish within five days of exposure. By seven days, the fish were anorectic and depressed. At 10 days, they were euthanized due to a severe, debilitating second-round infection. Gross morphological lesions observed in these fish included pale gills, pale livers and empty gastrointestinal tracts. Control fish remained free of parasites and collected tissues were normal.

I. multifiliis trophonts were easily identified in skin and gill sections collected five and 10 days post-exposure. In the skin, they primarily occurred as single parasites; however, multiple trophonts were occasionally observed in close proximity to each other (FIG. 5.1A). Infected fish skin had areas of epidermal hyperplasia, disrupted cellular

integrity and necrotic foci as well as areas that appeared normal. Alarm cells, a common feature of normal catfish skin, were disrupted, distorted or contained cytoplasmic vacuoles. Skin collected from fish 10 days after infection showed the greatest degree of pathology. In the gills, trophonts were observed at the base of primary filaments as well as within gill epithelia (FIG. 5.1B). By 10 days, primary filaments were severely blunted and epithelial hyperplasia occluded the majority of secondary filaments. Immunohistochemical staining for parasite antigen allowed visualization of parasite external membranes (FIGS. 5.1A and 5.1B). Histopathological changes were not observed in the head kidney, renal kidney, spleen or livers of infected fish.

Four of twenty *I. multifiliis*-infected fish intestines were found to contain trophonts. One fish examined five days after infection and three fish examined 10 days after infection, had single parasites embedded within abdominal cavity adipose tissue (n=1), associated with intestinal serosa (n=1) or adjacent to mesenteric blood vessels (n=2). One parasite was found in a hematoxylin and eosin stained section while the other three were found in sections stained for parasite antigen. Other than their location, these parasites appeared similar in morphology and antigen staining pattern as those found in the skin and gills (FIG 5.2A and 5.2B). Histopathological evidence of a host response to *I. multifiliis* within the peritoneal cavities was limited. Cells of host origin were observed in contact with a trophont found between two loops of intestine suggesting a mild host inflammatory response (FIG 5.2B).

DISCUSSION

Finding *I. multifiliis* trophonts within the peritoneal cavities of recently infected channel catfish suggests that this ciliated protozoan parasite may occur in tissues other than skin and gills during the course of a normal infection. This report adds to the single previous finding of *I. multifiliis* within the cerebral space of a heavily infected carp hybrid. In this study, host-origin cells were observed in association with one trophont located between two loops of intestine. The presence of a mild inflammatory response supports these results as not being a processing or sectioning artifact. Even so, additional histological observations are necessary to determine the frequency of this phenomenon.

I. multifiliis trophonts are typically large enough to observe in fish skin and gills without immunohistochemical enhancement. In this study, infected fish were killed to develop *in situ* methods to detect *I. multifiliis* proteins secreted during tissue migration. Unexpectedly, these methods also facilitated the discovery of trophonts in the peritoneal cavity, a previously unknown site of parasite entry. In retrospect, it is likely that three of these atypically located parasites would not have been found without parasite antigen-specific staining.

Demonstrating *I. multifiliis* trophonts within the peritoneal cavities of catfish does not reveal their method of entry. Three possible routes are postulated. One would be through the esophageal wall, another would require penetration of the pneumatic duct, a thin-walled structure connecting the esophagus to the swim bladder (4). A third route would be a retrograde migration from the anus into the rectum and through the intestinal wall. Penetration of any of these structures would conceivably result in a trophont

entering the peritoneal cavity. To date, however, *I. multifiliis* has not been shown to penetrate submucosa or tissues other than skin or gill epithelia.

ACKNOWLEDGEMENTS

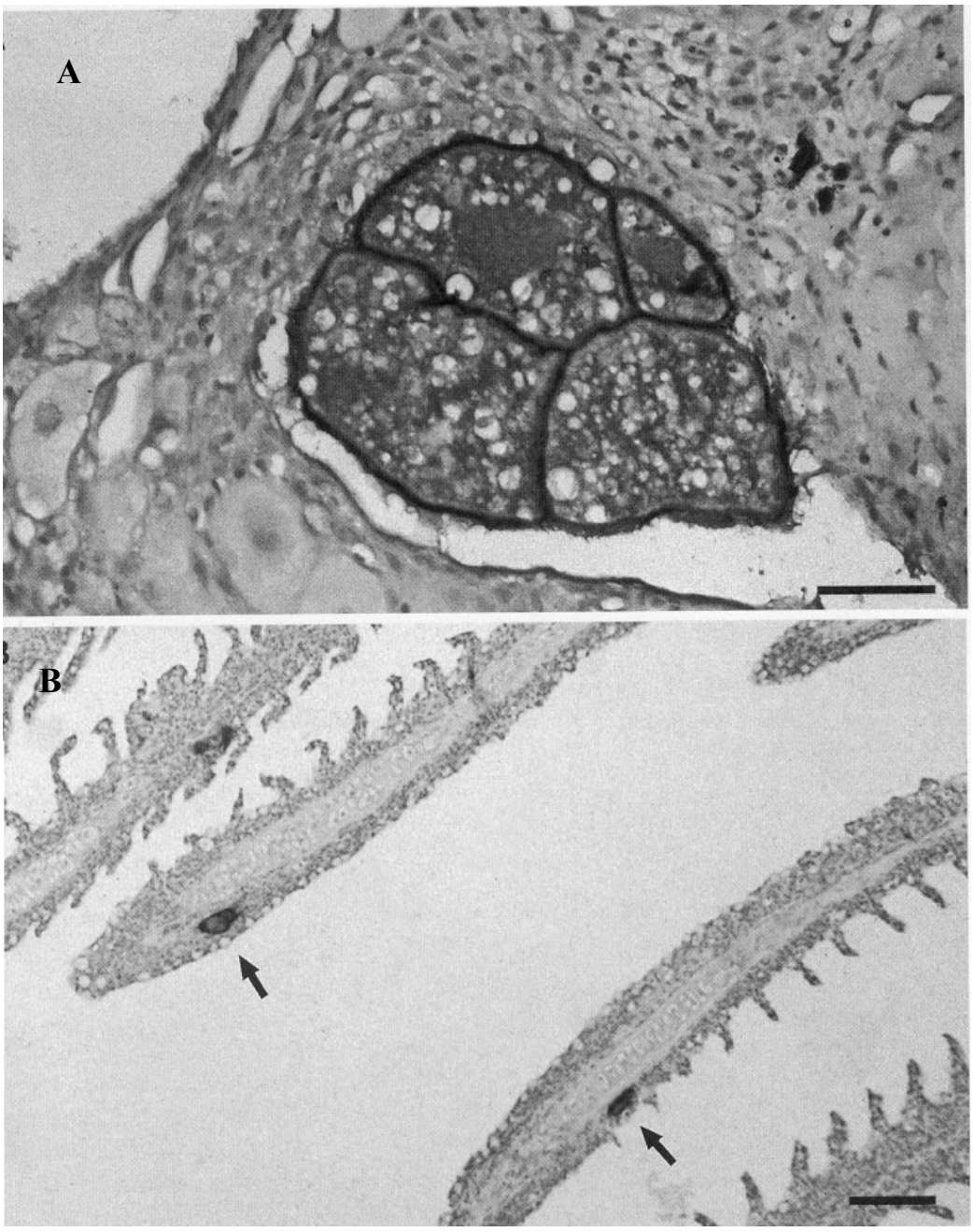
This research was funded by a National Institutes of Health Mentored Clinical Scientist grant #AI01429 (J. L. Maki) and US Department of Agriculture National Research Initiative Grant #98-35204-6812 (H. W. Dickerson). The authors would like to thank Melissa Scott and Chris Herron for their technical assistance.

REFERENCES

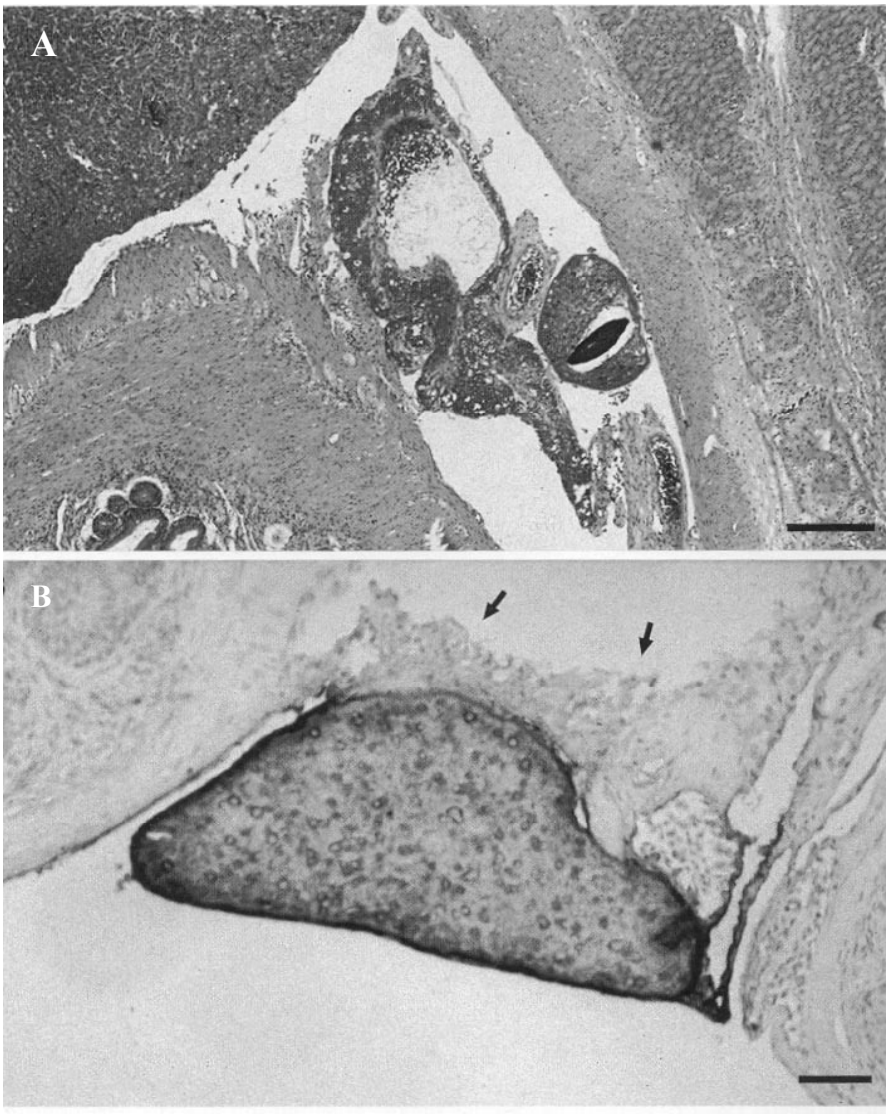
1. Cross M. L., and R. A. Matthews. 1993. Localized leukocyte response to *Ichthyophthirius* establishment in immune carp, *Cyprinus carpio. Vet Immunol Immunopathol*. 38:341-358.
2. Dickerson H. W., D. L. Dawe, J. B. Gratzek, J. Brown, and S. W. Pyle. 1981. Induction of *Ichthyophthirius multifiliis* Fouquet Infections in Channel Catfish, *Ictalurus punctatus* Rafinesque: Standardization of the Procedure. Fish Biologics: Serodiagnositics and Vaccines, *Dev Biol Stand*.49:331-336.
3. Dickerson H. W., A. L. Lohr, and J. B. Gratzek.1985. Experimental intraperitoneal infection of channel catfish, *Ictalurus punctatus* (Rafinesque), with *Ichthyophthirius multifiliis* (Fouquet). *J Fish Dis*. 8:139-142.
4. Grizzle J. M., W. A. Rogers. 1976. The Digestive System. *In*, Anatomy and Histology of the Channel Catfish. Agriculture Experimental Station, Auburn University, Auburn, AL, pp. 27-28.

5. Hines R. S., and D. T. Spira. 1974. *Ichthyophthirius multifiliis* (Fouquet) in the mirror carp *Cyprinus carpio* L., III. Pathology. *J Fish Bio.* 6:189-196.
6. Ventura M. T., and I. Paperna. 1985. Histopathology of *Ichthyophthirius multifiliis* infections in fishes. *J Fish Bio.* 27:185-203.

Figures 4.1A & 4.1B. Light photomicrographs of *Ichthyophthirius multifiliis* trophonts within the epidermis and gills of channel catfish stained for the parasite immobilization antigen (i-Ag). Sections were blocked with normal goat antisera and stained with a rabbit anti-I-Ag antibody (1:3,000) (X. Wang). A goat anti-rabbit biotin secondary (1:250) was applied followed by an avidin-biotin-alkaline phosphatase complex (ABC Elite, Vector Labs) and counter-stained with hematoxylin. (A) Multiple trophonts within the epidermal layer of the skin (750x)(scale bar = 20 μm). (B) Individual trophonts in gill epithelium (arrows) (220x)(scale bar = 50 μm).


A
B

Figures 5.2A & 5.2B. Light micrographs of *Ichthyophthirius multifiliis* trophonts within the peritoneal cavity of two channel catfish. (A) A trophont is located adjacent to intestines and near mesenteric blood vessels (H&E) (226x) (scale bar = 53 μm). (B) Immunohistochemical staining of a trophont between 2 loops of intestine. Host-origin cells, suggestive of an inflammatory response, are adjacent to the parasite (arrows) (475x) (scale bar = 21 μm).


A
B

CHAPTER 5

CONCLUSIONS

The channel catfish has served as a comparative immunology research model for more than thirty years. It has also been used as a representative host species to investigate the mechanisms of protective immunity in fish against the virulent protozoan parasite, *Ichthyophthirius multifiliis*. Even though this protozoan parasite was identified as a virulent pathogen of fish well over one hundred years ago, it remains one of the most economically important infectious diseases in aquaculture. Control methods beyond chemical additives are lacking and an effective vaccine is needed.

Previous work conducted in our laboratory has identified and characterized the immunodominant antigens of *I. multifiliis* at the molecular level. These i-antigens (a highly related group of GPI-anchored proteins) have been purified, sequenced and selected genes cloned into expression vectors. Recombinant *Tetrahymena* expressing these i-antigens, under the control of a cadmium-inducible promoter, have shown great promise as potential subunit as well as live recombinant vaccines.

The channel catfish infected with *I. multifiliis* has also been developed as cutaneous immunity model. The presence of parasite-specific antibody in the skin and cutaneous mucus of fish has been shown to be an important component of the protective immune response. My work has taken this host/parasite system one step further by investigating potential mechanisms and kinetics of antigen-specific cutaneous antibody production in channel catfish.

Parasite-specific antibodies in sera and cutaneous mucus of channel catfish were determined after parasite exposure or primary immunization with purified i-antigens using an ELISA incorporating detergent-extracted *I. multifiliis* membranes. This study is the first to demonstrate that levels of cutaneous mucus antibodies against *I. multifiliis* are independent of concentrations found in sera of the same fish. Fish immunized by two different routes had increasing sera antibody levels while cutaneous mucus antibody levels did not follow the same kinetics. Although results of this experiment did not identify the mechanism of cutaneous antibody production, it is apparent that mucosal antibodies against *I. multifiliis* do not simply diffuse from sera to the surface of the fish.

In a second experiment, immunohistochemistry and transmission electron microscopy were used to determine if immunoglobulin-containing cells occur within channel catfish skin. Before answering this question, goat anti-catfish immunoglobulin Fab-2 fragments were used to label a single population of lymphocytes in the head kidney (i.e., the equivalent of mammalian bone marrow) that contained large cytoplasmic vesicles. These cells were similar in appearance to plasma cells described in other fish species and were determined to be channel catfish ASC. This is the first report describing the morphological appearance of channel catfish ASC at the ultrastructural level. These same methods were used to identify a very low number of similar cells in normal channel catfish epidermis. The demonstration of putative ASC in channel catfish skin supports the hypothesis of a separate mucosal immune system in this species as well as suggests a capacity for local antibody production.

In a third experiment, *I. multifiliis* trophonts were detected within the peritoneal cavities of channel catfish infected by surface exposure. These results suggest that this

ciliated protozoan parasite may infect tissues other than skin and gills during the course of a normal infection. To date, *I. multifiliis* has not been shown to penetrate submucosa or other tissues, but the detection of trophonts within the peritoneal cavity requires a reexamination of the current concept that this is parasite of external mucosal surfaces. Taken together these data support the hypothesis that channel catfish have a separate mucosal immune system and the skin is an active participant in the immune response (i.e., an effector site). These data also support the role of antibody in protection against *I. multifiliis* and emphasize the importance of the mucosal immune response against this virulent fish pathogen.