CROSS-SEROTYPE IMMUNITY AND ASPECTS OF VIRULENCE IN *ICHTHYOPHTHIRIUS MULTIFILIIS*

by

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ABSTRACT

The parasitic ciliate *Ichthyophthirius multifiliis* infects the skin and gill epithelia of freshwater fish. It expresses membrane proteins referred to as immobilization antigens (i-antigens), which define serotypes that are distinguished by antibody immobilization antibodies. Antibody cross-linking of i-antigens confers serotype-specific protection, making it of interest to characterize differences in virulence and mechanisms of cross-immunity between these serotypes. In these studies, it was found that the differences in virulence between isolates are not due to their initial infection rate, but rather to the interaction between their life cycle period and the kinetics of the fish immune response. It was also found that cross-immunity exists between *I. multifiliis* serotypes, and that this protection did not involve the i-antigens. These studies lay the groundwork for the identification of parasite virulence mechanisms as well as other proteins that could be used in vaccines.

INDEX WORDS: *Ichthyophthirius multifiliis*, Ich, *Ictalurus punctatus*, channel catfish, virulence, serotype, immobilization antigens, vaccine.

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DEDICATION

I dedicate this thesis to my wife, Amanda, whose strength of love held me together throughout its preparation.

"…to an isle in the water with her would I go…" – Yeats

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TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. General Biology

1.1 Phylogeny

Based on ribosomal RNA and protein sequence data, ciliates, dinoflagellates, and apicomplexans are grouped in the subkingdom *Alveolata* (3, 96). All of its members possess alveoli, which are flattened vesicles that, together with the plasma membrane and the underlying epiplasm, compose external cellular coverings known as pellicles (in ciliates) or thecal plates (in dinoflagellates) (20, 21). Within this subkingdom, all ciliates, both free-living and parasitic, belong to the phylum *Ciliophora*. Members of this phylum are quite diverse, though they are united by several characteristics (21, 77). With few exceptions, members exhibit nuclear dimorphism. All have an infraciliature located beneath the pellicle that contains basal bodies from which cilia emerge. They exhibit homothetogenic binary fission (in contrast to mirror-image, or semetrogenic fission), meaning that the plane of cell division is perpendicular to the organism's anterior-posterior axis. Syngamy is absent in ciliates, and in conjugation, haploid gametic nuclei replace true gametes. A cytostome (mouth) is usually present and is associated with an atrial, vestibular, or buccal cavity.

Ichthyophthirius multifiliis, an obligate parasite of fish, shares the order *Hymenostomatia* (class *Oligohymenophora*) with the facultative fish parasite

Ophryoglena canenula and the entire genus *Tetrahymena*. The latter is composed of both free-living members, such as *T. thermophila*, and opportunistic parasites, such as *T. corlissi* and *T. pyriformis*. The taxonomic grouping of these organisms is supported by 18s rRNA sequencing (107). *I. multifiliis* and *O. canenula* are both members of the suborder *Ophryoglenina*, which is distinguished by the presence of the organelle of Lieberkühn, whose function remains enigmatic (70). The two are each the sole members of their respective families. The phylogeny of *Cryptocaryon irritans*, a pathogen of marine fish, has been the subject of debate. It shares considerable similarity with *I. multifiliis* in its possession of a direct life cycle involving palintomic division, and it is an obligate parasite of fish that appears as white spots on the epithelium of its host (28). However, it does not possess the organelle of Lieberkühn, and 18s rRNA sequencing does not support pairing with *I. multifiliis*, *O. canenula*, or *Tetrahymena* (30). It is likely that the similarity shared by *I. multifiliis* and *C. irritans* is the result of convergent evolution.

1.2 Life Cycle and Morphology

The life cycle of *I. multifiliis* consists of three stages: the infective theront, the parasitic trophont, and the reproductive tomont, all of which are ciliated (79). The theront is approximately 30 x 60 μm and swims in a characteristic spiral. It is phototactic and is positively chemotactic to proteins in fish serum and mucus, though the exact identities of these proteins have yet to be determined (7). Upon contact with a host, the theront releases the contents of its anterior secretory mucocysts, and this material may assist in adherence to its host (36). Once the theront adheres to the host epithelium, it may secrete enzymes to facilitate host invasion (36). A structure known as the apical

perforatorium, which is a 1.5 – 2 μm ectoplasmic protrusion, is used to physically penetrate the outer epithelial layers (28, 36). The parasite differentiates into a trophont within minutes, and attains its maximum epithelial penetration within five minutes. The organelle of Lieberkühn, which is present at the anterior end of the theront stage, is lost (28, 70). The shallow buccal cavity of the theront develops into the trophont's vestibular cavity, where the cytostome, a mouth structure, soon develops (36). The trophont increases in size until it is visible as a white spot on its host.

Although a theront will invade any part of the skin or gill epithelium, the trophont migrates toward major afferent blood vessels as infection progresses, remaining closely associated with the epithelium of its host throughout its development (34). The number of crystalline mucocysts increases significantly as the trophont develops, possibly to prepare for the secretion of a cyst wall during its reproductive phase (34, 37). The number of lipid bodies also increases significantly, and their localization within prominent vacuoles suggests a potential role as energy reserves during the non-feeding host-free life cycle stages (34).

The trophont attains a maximum size of approximately 800 μm before leaving its host. Though the exact mechanism that triggers exit from the host epithelium remains unclear, developmental maturity of the trophont is likely an important factor in this phenomenon. It is likely that the development of lipid reserves, contractile vacuoles, and mucocysts, in conjunction with a general separation of the parasite from the host epithelium, leads to host exit (35). Discharge of contractile vacuole contents may mediate the release of the trophont by placing pressure on the overlying host epithelium (35). If an infected fish dies, the infecting trophont leaves if it has attained a critical size

and level of development (28). It has been shown that if a fish has been parasitized for a longer time, a greater proportion of the trophont population will depart the epithelium upon host death (35). Also, several studies have indicated that a minimum diameter of approximately 95 μm is necessary for survival upon exit (34, 72). Thus, there is a developmental threshold, represented by size, which must be attained in order for a trophont to exit its host and survive as a tomont.

Immediately after exit from its host, the cell settles to a substrate and encysts. The cyst wall has a non-uniform thickness of 9 – 30 μm, and is thicker at the point of substrate attachment (71). It contains two distinct layers, both composed of proteinaceous fibrils (28). The inner layer is similar in density to the contents of the mucocysts that secrete it, and the outer layer is less dense and sticky, containing bacteria and other debris (37). While the cyst is essential for the initiation of division, its removal after the first division has no effect on subsequent divisions (71). It is possible that the cyst serves primarily in substrate adherence and to shield the developing tomont from bacteria and fungi. After encystment, the tomont typically undergoes equal division seven to nine times to produce 128 – 512 tomites, though the number of tomites produced depends on tomont size (28, 71). These daughter cells differentiate into theronts, acquiring an elongated shape, a buccal cavity, and an apical perforatorium (37). As division progresses, the cyst wall becomes progressively thin, and the fully-differentiated theronts bore through the cyst wall and seek hosts (71).

The *I. multifiliis* life cycle contains two critical periods that occur during initial host invasion and immediately following host exit. Upon invasion, only half of all theronts that gain host entry ultimately survive past ten minutes on the host epithelium.

In the initial forty-five minutes of infection, mean trophont diameter increases by twenty-five percent (38). Such rates of growth have not been observed at any other time point in trophont development. Therefore, the first minutes of epithelial invasion are limiting in the progression of the parasite's overall life cycle.

Another critical period occurs during host exit, due to either trophont developmental changes or host death. Few trophonts that leave their hosts one or two days post-infection survive twenty-four hours after they depart (38, 79). Parasites prematurely exiting at this point not only resemble theronts in morphology, but are able to re-infect naïve fish at low levels (99). However, nearly all trophonts that exit their hosts at three, four, or five days post-infection survive host-free for twenty-four hours (38, 79). Also, the number of theronts produced by a trophont exiting at three days post-infection is significantly smaller than the number produced by a trophont exiting one or two days later (38). Similarly, the number of theronts produced increases significantly as tomont size increases. While a tomont of 100 μm may only produce about fifty theronts, one that attains a diameter of 400 μm may produce six hundred (38). Thus, trophonts that persist on their hosts and attain a larger diameter are more likely to survive as tomonts and are able to produce a greater number of daughter cells.

Temperature is an important mitigating factor in the life cycle of *I. multifiliis*, as the duration of a life cycle period and water temperature share a positive correlation (38, 71, 79, 80). Trophonts grow to 95 μm, their critical size for survival as tomonts, in two days at 27˚C and in three days at 22˚C (71). Nearly all parasites survive host exit at three and four days post infection at temperatures of 21˚C – 26˚C (38, 79). In the laboratory, *I. multifiliis* culture propagation is conducted at 20 – 23˚C. Under these conditions,

trophonts typically leave their hosts after six to seven days of parasitism and reproduce in 18 – 24 hours (28, 49). However, at 9˚C, one life cycle period takes an average of twenty-two days, though there is no difference in parasite viability or in surface antigen expression when compared to cultures grown at 25˚C (80). These findings are consistent with the global distribution of *I. multifiliis* epizootics, which have been documented as far north as Finland when the water warmed to 14˚C (95). The elongated life cycle seen at low temperatures may allow the parasite to maintain a low-level infection within a population until conditions favor reproduction on a large scale, though this has not been directly proven in a natural population.

1.3 Diagnosis, Treatment, and Control

Severe infection with *I. multifiliis* occurs most often in stressed captive fish populations. Initially light parasite loads in closed systems usually result in high parasite loads and high mortality rates after several life cycle periods. Infected fish can often be identified by their abnormal behaviors. Fish sometimes engage in "flashing," where they swim in a twisting motion and scrape their epithelial tissues against objects (28). They eventually become lethargic, lying motionless at the bottom of the aquarium in which they are housed (49). Flared opercula are present in some cases and indicate respiratory distress caused by a heavy infection of the gill epithelium. Feeding behavior is often reduced or absent (49). Epithelial abnormalities can be indicative of infection. These often include excessive mucous, ulceration, pinpoint hemorrhages, fin tissue loss, and white spots representing the parasitic trophont (49). In heavy, persistent infections, the epidermis begins to fragment and dissociate from the lower dermal layers.

I. multifiliis is only susceptible to chemical treatment during its host-free life cycle stages (43). Therefore, treatment must be aimed at preventing the re-infection of fish. This requires frequent chemical treatments as well as water changes timed to interrupt the parasite's life cycle, reduce the number of theronts in the system, and prevent the establishment of new trophonts (92). In addition, water temperature can also be raised to approximately 26 ˚C to adversely affect the heat-sensitive theront and enhance the fish immune response (43). Water quality should also be maintained to prevent undue stress to the fish.

Numerous chemical agents are available for the treatment of fish, including formalin, malachite green, formalin-malachite green mixtures, copper sulfate, and potassium permanganate (54). Treatment for short periods with 3% sodium chloride can also be effective against external parasites (11). Of the aforementioned treatments, only formalin solution and sodium chloride are approved by the United States Food and Drug Administration for the control of protozoa in food fish (11). Quarantining incoming fish for several weeks is generally the most practical method for preventing infection of a naïve fish population.

2. Host Immunity

2.1 Innate Immunity

The first line of defense against *I. multifiliis* is the thick mucous layer that covers the fish epithelium. Mucous not only serves as a physical barrier, but it also contains numerous nonspecific immune factors that inhibit infection by parasites. In fish skin, both the classical and alternative complement pathways are present, and analogs to the

primary mammalian complement factors (C1 – C9) have been identified in fish (97). The genes encoding C3 are expressed in the skin in response to *I. multifiliis* infection, though the *in vivo* effects of complement have yet to be demonstrated in this system (91). Factors, such as transferrin, C-reactive protein, opsonins, histone-like proteins, and lysozyme are also present and may play a role in the nonspecific defense against pathogens (81, 97, 106).

Early nonspecific cellular responses to *I. multifiliis*, consisting primarily of neutrophils, occur 1 – 2 days following infection (24, 48). Leukocyte infiltration at days 3 – 4 is characterized by the presence of basophils and eosinophils, and may be triggered by either exogenous (parasite-derived) or endogenous factors (24). In previously exposed carp (*Cyprinus carpio*), eosinophilic granular cells (EGCs), which are similar to mammalian mast cells, are also present and release factors that increase vascular permeability (24, 98). As the epidermis is not a vascularized tissue, this may play a significant role in *I. multifiliis* infection, allowing for further infiltration of leukocytes. Macrophages that have Fc and C3 receptors are also present, though phagocytized material is apparently composed primarily of host debris (23, 24, 97). Though leukocytes are present in the skin surrounding established trophonts relatively early in infection, they likely do not perform any direct effector function, as the parasites themselves are free from any damage (22). However, phagocytic cells may uptake parasite antigen, cilia, and other debris for use in antigen presentation. These leukocyte responses are accompanied by the secretion of cytokines and chemokines, such as IL-1, TNF-α, and IL-8, whose levels peak approximately four days post-infection, coinciding with the secondary epidermal leukocyte infiltration (90).

It has been suggested that non-specific cytotoxic cells (NCCs), which are similar in function to mammalian natural killer (NK) cells, might play a role in defense against *I. multifiliis*. Though differences exist between NCCs and NK cells, they are similar in morphology and share several functional properties. As in mammalian systems, NCCs specifically lyse malignant or virus-infected cells through granule exocytosis, receptor-ligand binding, and soluble lytic factor release (33, 89). They also mediate other immunologic processes through cytokine secretion and possibly through direct interaction with other cells. NCCs, normally resident in the head kidney, shift to the peripheral blood in *I. multifiliis* infected fish (45). These peripheral blood NCCs also had increased cytolytic activity when exposed to transformed mammalian cells. Also, they lyse deciliated or immobilized *Tetrahymena pyriformis in vitro* (44). Recruitment of NCCs to the skin or gills in response to *I. multifiliis* infection has not been shown. Also, NCCs have not been directly implicated in the killing of protozoa *in vivo*. Another class of fish NK-like cells exists that does not display the NCC surface marker (NCCRP-1) (88). While these cells do kill their targets without prior sensitization, their anti-protozoan activity has not been studied.

2.2 Acquired Immunity

The lymphoid organs involved in the immune system of teleosts differ from those of the mammalian immune system two general ways: first, no bone marrow or lymph nodes are present; and second, a large amount of secondary lymphoid tissue is located in the kidney (113). The major lymphoid organs of teleosts include a thymus, renal lymphoid tissue (head kidney and renal kidney), and a spleen, all of which contain large numbers of leucocytes (97). The thymus is a primary lymphoid organ, and has been

shown to seed the other lymphoid organs with their cell populations early in development (93). As in mammals, it is thought to play a major role in immunologic tolerance. The head kidney serves as both a primary and secondary lymphoid organ, and has similar cell populations to the mammalian bone marrow, producing erythroid, lymphoid, and myeloid cells (112). The renal kidney contains small patches of hematopoetic tissue that are similar to that found in the head kidney, though its role in the fish immune system remains enigmatic. The spleen, a secondary lymphoid organ, contains areas of red pulp and white pulp, as well as melanomacrophage centers that may play a role in antigen storage (46). The nurse shark (*Ginglymostoma cirratum*) spleen has a complex cellular architecture (84). Its white pulp areas consist of a central T cell zone and a dendritic cell network, surrounded by naïve B cells. There is no major lymphopoetic activity, and it is likely that, as in mammals, B cells migrate to the spleen after they are activated elsewhere. A similar organization likely exists in all teleost spleens.

Analogs to both helper T cells and cytotoxic T cells have been identified in fish based on their expression of T cell receptor α and β genes (105). Fish T cells secrete cytokines upon activation and also act as helper cells for antibody responses, which is likely their dominant role in defense against *I. multifiliis*. Houghton and Matthews (1990) suggested a role for cell-mediated immunity in protection against *I. multifiliis*. Immune fish injected with corticosteroids no longer resist challenge, though their serum antibody levels remained high. Because steroid administration depletes immune cells but not antibodies, the authors argued that cellular elements must be involved in protection (52). However, it is important to note that corticosteroids also suppress immunoglobulin production by B cells (76). Also, the large size and extracellular nature of an *I. multifiliis*

trophont (100 – 1000 μm) suggests an antibody-mediated immune response. T cell costimulation of B cells may be required for a robust antibody response, but T cells probably do not act as the effectors of immunity in this situation.

Fish B cells, which are analogous to their mammalian counterparts, exhibit rearrangements at the immunoglobulin heavy chain locus as well as allelic exclusion, and they produce both secreted and membrane forms of the immunoglobulin heavy chain (75). Subpopulations of antibody secreting B cells (ASCs) have been identified in the head kidney, spleen, and peripheral blood of teleosts. Bromage *et al*. (2004) proposed a model for the distribution of these ASC subpopulations, known as plasmablasts and plasma cells, in fish (6). B cells develop into mature naïve B cells in the head kidney and disperse to peripheral tissues via the blood. When activated by antigen, these cells mature into either plasmablasts (in the peripheral blood) or short-lived plasma cells (in the anterior kidney and spleen). These cells migrate back to the head kidney, where some are maintained as long-lived plasma cells to facilitate immune memory. A population of ASCs specific for *I. multifiliis* has been shown in the head kidney and skin of immune fish (114). Antibody production against *I. multifiliis* may thus occur locally in the skin of infected fish, as well as centrally in the head kidney.

2.4 Antibodies

The primary immunoglobulin molecule in fish is an IgM-like 700-kDa tetramer. Each monomer contains two heavy chains and two light chains (66). Monoclonal antibodies have been used to identify three different populations of this molecule as well as two different light chain populations; however, none of these have been identified as predominant in serum or mucous alone (64, 68, 69). The 700-kDa molecule is associated

with a 15-kDa J-chain, which may assist in molecular assembly, but has no documented role in secretion (74). While a second IgD-like immunoglobulin has been identified in channel catfish by sequence analysis, its function remains unknown (as in mammals) (104).

Fish immunoglobulin exhibits an unusual noncovalent architecture when analyzed in denaturing, non-reducing solvents. The molecule disassociates into eight subpopulations, each composed of a different number of covalently linked "halfmers," or heavy chain-light chain pairs that disassociate based on variable disulfide bonding (42, 63, 66). A heterogenic immunoglobulin population may be produced from a single immunoglobulin gene by each individual B cell (55). Kaattari *et al*. (1998) proposed that these "redox forms" may each have different affinities for opsonization, complement-mediated lysis, and Fc receptor binding, and thus might provide an alternative to the immunoglobulin isotype diversity seen in mammals (55). However, no differences have been found between the immunoglobulin present in the skin and serum of fish.

Affinity maturation of antibodies occurs in fish, though it appears that fish antibodies have an intrinsically low affinity relative to those found in mammals (56, 63, 86). The identification of AID (activation-induced cytidine deaminase) confirms that the machinery that drives somatic hypermutation is present in the kidney, spleen, and skin of channel catfish (86). However, a study conducted by Kaattari *et al*. (2002) showed that antibody affinity reached a threshold level approximately twelve weeks after immunization (56). This might be due to either selection of specific B cells, somatic mutation, or both. The increase in affinity correlated with a decrease in the level of antigen, possibly driving selection of ASCs that produce high affinity antibodies over

those that produce antibodies of a lower affinity. A mechanism exists that limits the affinity of decavalent mammalian IgM (50). However, the affinity of bivalent IgG is allowed to exceed this value when isotype shifting occurs. Despite the higher affinity threshold of IgG, IgM antibodies can attain high avidities because they have five times the number of binding sites. Because fish antibodies are tetrameric, and thus octovalent, it is likely that slight increases in affinity result in large increases in avidity, which may necessitate a regulatory mechanism. As a result, antibody affinity may be limited in fish due to the structure of the antibodies themselves.

2.5 Mucosal Immunity

During infection and parasitism, *I. multifiliis* comes into contact with antibodies in the cutaneous mucous of its host. Though the nature of this interaction is poorly understood, several studies taken together can be used to infer a model for fish mucosal immunity. Lobb and Clem (1981) radiolabeled serum immunoglobulin purified from the sheepshed (*Archosargus probatocephalus*) and injected it intravascularly into the same fish from which it was collected. Subsequently, radiolabeled immunoglobulin was detected in the blood, but not in the cutaneous mucous (67). Thus, it is likely that mucous immunoglobulin arises locally in the skin, rather than by diffusion or transport from the blood. In another study, Lobb (1987) bath immunized channel catfish with dinitrophenylated horse serum albumin (65). This triggered a specific mucosal antibody response but no systemic response. While little increase in mucus antibody level was detected at seven weeks post immunization, mucous antibody increased dramatically at sixteen weeks following a second immunization. Serum antibody levels remained

unchanged relative to pre-immersion levels. As a result, it can be concluded that the mucosal immune response occurred independently of the systemic response.

Maki and Dickerson (2003) demonstrated a lack of correlation between the serum and mucous antibody levels in fish surface challenged with *I. multifiliis* or intercoelomicly (IC) injected with purified i-antigen (73). Serum antibody levels in all immunized fish were significantly higher than pretreatment values, and fish within both populations had similar titers that peaked fourteen weeks post-immunization. Surface challenged fish had an elevated, though variable, mucous antibody level that peaked seven weeks post exposure. In contrast, few IC immunized fish had higher mucous antibody levels after immunization, and fish having the highest serum antibody levels did not have correspondingly high mucous antibody levels. As a direct result of its surface exposure, a surface-immunized fish may produce a greater mucous antibody response, possibly due to local antigen processing and antibody secretion in the skin. Because serum and mucous antibody levels cannot be correlated, it is unlikely that mucous antibodies diffuse from the blood. These results also show that serum and mucous antibody levels peak at different time points following exposure, making a case for a difference in kinetics between the systemic and mucosal immune responses, further indicating their compartmentalization.

Passive immunization studies using antibodies against *I. multifiliis* yield results consistent with the compartmentalization model for fish mucosal immunity. Leff (1993) purified catfish antibodies from immune serum and passively transferred them by intravenous injection (57). This did not protect the recipient fish from challenge, though serum from these fish immobilized *I. multifiliis* theronts *in vitro*, indicating the presence

of serum antibodies. These results suggest that the mucosal and systemic antibody responses are conducted in relative isolation, as fish antibodies are unable to move from the bloodstream to the cutaneous mucous. Lin *et al*. (1996) conducted further passive immunity experiments that utilized murine monoclonal antibodies (mAbs) against the *I. multifiliis* i-antigens (59). Channel catfish were protected against challenge by the parasite when IgG mAbs were injected IC. These antibodies reached both the serum and mucous of the immunized fish. However, mouse IgM antibodies injected IC failed to protect fish against parasite challenge and were not detected in the serum or mucous. Because IgG but not IgM antibodies were found in the serum and mucous of immunized fish, it is possible that molecular size prevents the movement of larger antibody molecules, such as fish immunoglobulin and mouse IgM, between these compartments.

2.6 Mechanism of Acquired Immunity

Serum from *I. multifiliis* immunized fish has been shown to immobilize theronts *in vitro*, and the degree of immobilizing effect has been correlated with antibody titer (13, 14). This suggests a role for antibody-mediated immobilization in immunity to *I. multifiliis*. Hines and Spira were first to propose that antibodies in the skin blocked invasion of *I. multifiliis* (47). This is further verified by the presence of immobilizing antibodies in the skin of immune fish (110). These antibodies, which are locally secreted in the skin, render theronts unable to infect fish and kill theronts at low dilutions (109). While it is uncertain exactly how long it takes fish to develop immunity, immunoglobulin genes are transcribed in response to *I. multifiliis* infection four days after infection (91). Specific antibodies can be detected 12 days following infection of catfish in either skin or serum (111). Titers continue to increase in magnitude, as antibody in skin and serum

show peak values at 7 and 14 weeks, respectively (73). While the kinetics of immunity to *I. multifiliis* remain somewhat enigmatic, the exact mechanism of antibody-mediated immunity is well understood.

Clark *et al*. (1996) proved that antibody cross-linking of ciliary i-antigens is the likely mechanism of immunity to *I. multifiliis* (17). In these experiments, i-antigen-specific mAbs injected IC conferred full protection against *I. multifiliis* infection, while non-immobilizing mAbs were not protective. Bivalent $F(ab)_2$ fragments of the specific antibody were completely protective as well, thus indicating that Fc receptor mediated effects, such as complement, were not necessary to achieve full protection. Monovalent Fab fragments gave no protection, though the protective effect of Fab fragments could be restored by the addition of polyclonal anti-mouse IgG antibodies, indicating the importance of antibody cross-linking. Interestingly, no dead *I. multifiliis* cells were observed in the epithelium of protected fish, and the theronts with cross-linked cilia appeared otherwise viable. Thus, it is generally accepted that the mechanism of humoral immune protection against *I. multifiliis* is the cross-linking of cilia by antibodies, thus triggering rapid exit of the host. It is somewhat paradoxical that mAbs that immobilize cells *in vitro* trigger motility (host exit) *in vivo*, though it is possible that low levels of antibody trigger an avoidance response that is mediated by an uncharacterized signal transduction event (12).

3. Immobilization Antigens

While little effort has been made to characterize *I. multifiliis* on the genomic or proteomic level, the i-antigens have received special attention due to their immune-

relevance. The sequence of the gene encoding the 48-kDa i-antigen of isolate G1 (*IAG48[G1]*) has several interesting features. The gene itself encodes a proprotein of 442 amino acids and a theoretical molecular mass of 45,025 Da (18). It possesses a hydrophobic N terminal region of 20 amino acids that is a signal peptide, likely for targeting to the plasma membrane. The purified protein has been analyzed by Edman degradation, and the N-terminal amino acid corresponds with the valine residue in position 21, indicating that residues 1 – 20 are removed from the preprotein (19). Sequence analysis also predicts a P-loop (G-X_4-GKS) domain at residues 316 – 323 (18). These domains are typical of proteins that bind ATP or GTP (85). A hydrophobic C terminal region of 14 amino acids is separated by a short spacer region from three small amino acids (CAS) (94). This C terminal sequence suggests that the i-antigens are GPI anchored proteins, an assertion that is further confirmed when the proteins are examined in native form. Following removal of the anchor region by treatment with phospholipase C, the i-antigens become hydrophilic and react with antibodies that recognize determinants common to all GPI anchors (16).

The most interesting feature of this gene is the presence of tandemly repeated domains that extend throughout most of the protein's length (18). Five homologous segments exist, each of which is approximately eighty amino acids in length. Each repetitive period contains six cysteine residues that fall into register when aligned. The cysteines are regularly spaced, forming fourteen motifs of the form C-$X_{2,3}$-C. These contribute to four larger motifs of the form C-X_2-C-X_{20}-C-X_3-C-X_{20}-C-X_2-C. Similar repetitive regions can be found in the sequences of other protozoan membrane proteins, including the variant-specific surface proteins (VSPs) of *Giardia lamblia*, when

compared using BLASTp (1). The VSPs of *G. lamblia* are quite similar, as they share the same C-$X_{2,3}$-C motif structure and overlap at 29 of the 30 cysteines in the *I. multifiliis* i-antigen (18). Similar repeats are also present in the SerL, SerH, and SerJ i-antigen loci of *Tetrahymena thermophila*, though their cysteine motif structures and repeat copy numbers are slightly different at each locus (31, 32, 41). Cysteine motifs of this nature are common to proteins with metal binding properties. Coordination of zinc atoms by cysteine residues is often associated with the stabilization of protein secondary structures, such as zinc fingers and zinc knuckles, which often facilitate interactions with either DNA or other proteins (4). Interestingly, *G. lamblia* VSPs have been shown to bind zinc, a function which may be responsible for the malnutrition characteristic of giardiasis (78). The metal binding properties of the *I. multifiliis* i-antigens are unknown.

Isolates of *I. multifiliis* express between one and three i-antigen genes. Because these isolates each express different i-antigen genes, immobilization with specific monoclonal antibodies has been used to define serotypes of *I. multifiliis* (27). The epitopes responsible for immobilization are distinct for each serotype, so theronts of one serotype will be immobilized by homologous but not heterologous antibodies. Serotype-specific monoclonal antibodies also react against the i-antigens on western blots, though only under non-reducing conditions (60). This indicates that the immobilizing antibodies recognize conformational epitopes. Six serotypes (designated A – F) have been described.

The i-antigen genes from several other isolates have been sequenced. These include the 48-kDa protein from isolate G1 (serotype A) discussed earlier, which is designated *IAG48[G1]* (18). The genes for two different 52-kDa proteins from isolate

G5 (serotype D), designated *IAG52A[G5]* and *IAG52B[G5]*, have also been characterized (62). *IAG52A[G5]* encodes a proprotein of 468 amino acids with a predicted mass of 48,281 Da, while *IAG52B[G5]* encodes a proprotein of 460 amino acids with a predicted mass of 47,583 Da. Upon comparison, they share the same central elements, including hydrophobic termini, periodic cysteines, and tandem repeats, though the products of these genes only share approximately 50% similarity on the DNA sequence level (62). It is also important to note that the i-antigens cannot be PCR amplified using primers for heterologous serotypes, and they do not hybridize with DNA of heterologous serotypes (27). Thus, though unifying elements are present within this gene family, these serotypes remain quite distinct.

The i-antigen proteins of different serotypes are most similar at their N and C termini. The C-$X_{2,3}$-C motifs are preserved, as are the CP-X-G(T/A) motifs at the start of each repeat and the KKLTSGA domain near the C terminus. The central repeats show the most divergence, and their copy number is different as well, as the *IAG52A[G5]* gene has six repeats, where the other two genes mentioned have five. The two G5 i-antigens are also expressed at different levels, as *IAG52B[G5]* expression is approximately 100-fold greater than that of *IAG52A[G5]* (62). Also, four isoelectric variants of the 55kDa G5 i-antigens have been found, indicating that the protein may be somehow modified or that other i-antigen genes exist in this isolate. Exact homologues of *IAG52A[G5]* and *IAG52B[G5]* have been found in the isolate G3, which also belongs to serotype D (15). Homologues have also been identified for *IAG48[G1]* in NY1, which is another isolate of serotype A. The fact that homologous i-antigens can be found between the different isolates of each serotype suggests that there may be only a limited number of i-antigen

genes within the species. Furthermore, the ability of mAbs to react with all of the i-antigens expressed in an isolate suggests that the existence of several i-antigen genes in one isolate may be a result of gene duplication (19).

The i-antigens of *T. thermophila*, though they share structural similarities to those of *I. multifiliis*, are variably expressed. The genes encoded at some loci are expressed in coordinate fashion (SerL), and others are expressed in mutually exclusive fashion (SerH) (32, 41). Antigen switching in *T. thermophila* occurs in response to environmental changes, such as temperature (103). A common theme in parasitology is antigen-switching by pathogens in order to evade host immune responses, a phenomenon that is perhaps best documented in the surface antigens of African *Trypanosomes* (5). While the evolutionary significance of antigen switching in free-living protists, such as *Tetrahymena* and *Paramecium*, is unclear, it has been hypothesized that antigen-switching may occur as a defense mechanism against predatory microorganisms (15). While the pathogenic nature of *I. multifiliis* might also argue for the presence of antigen switching, no studies have yet identified any such phenomena (15). However, the i-antigen genes are developmentally expressed, as theronts express fifty-fold higher levels than trophonts (19). The i-antigen expression in theronts is quite high, and can comprise up to six percent of the total poly A^+ mRNA, and as much as 12% and 60% of the total protein on the theront cell and cilia, respectively (19, 26).

In addition to being expressed in abundance on the parasite's surface, the i-antigens of *I. multifiliis* are expressed at high levels in a soluble form (108). That is, they are released into the water surrounding the parasite. These soluble i-antigens are identical to the membrane-bound form based on peptide mapping, glycosylation, and gel

mobility. Also, they are able to induce the production of immobilizing antiserum. The release of the soluble i-antigen is important in that it exposes the host to parasite antigen in a manner that it can be taken up, processed, and presented to the immune system during natural infection. Soluble i-antigens are released by other ciliates as well (2).

4. Vaccinology

4.1 Whole Parasites as Vaccines

Live parasites confer protection against subsequent *I. multifiliis* infection. Fish exposed to sublethal doses of theronts do not become infected upon subsequent challenge and their serum immobilizes theronts *in vitro* (47). Fish treated following potentially lethal challenge also become protected and have specific antibody titers (13). It is also possible to immunize fish by injection of live parasites. Theronts injected IC establish infection in the body cavity, where they grow for twenty-one days before they become surrounded by granulomatous tissue and die (29). Fish immunized in this manner become immune to surface challenge. While live parasites are capable of inducing a strong immune response, obvious impracticalities exist in developing a vaccine containing live *I. multifiliis*. First, fish immunized with sublethal doses of theronts could be infected much more severely if infection inadvertently occurs within a closed system. In this case, a very light infection could easily overwhelm a fish population and cause severe mortality given sufficient time. Second, the production of *I. multifiliis* in quantities sufficient for mass immunization would be extremely difficult, as it cannot be grown *in vitro* or stored for future use. A fish host is required for completion of its life

cycle, so the production of parasites is labor- and time-intensive, and requires that many fish be sacrificed.

Immunization has also been performed, with varying degrees of success, using several different cellular preparations of *I. multifiliis*. IC injection with theront lysates, formalin-fixed theronts, and theront cilia each gave no protection against lethal challenge, as did immunization with formalin fixed theronts by immersion. Formalin-fixed trophonts protected approximately half of immunized fish, and live theronts conferred total protection (8). Similar results were seen in subsequent studies, as surface immunization and IC injection with live theronts both conferred complete protection. IC injection with sonicated trophonts, at levels similar to the former study, conferred 90% survival, though sonicated trophonts did not protect by immersion (111). These studies are difficult to reconcile, as the former study found no protection from a trophont lysate, yet the latter found protection using a trophont sonicate administered by IC injection. Even more striking, both studies obtained protection using no adjuvant, which is usually required for a strong antibody response against an *I. multifiliis* preparation (100). These studies demonstrate the feasibility of several different vaccination strategies that utilize killed cells or cellular homogenates.

4.2 Subunit Vaccine

Because the i-antigens have been shown to be central in immunity to *I. multifiliis*, they have also been studied for use in subunit vaccines. Immunization with 10 μg of affinity purified i-antigen in complete Freund's adjuvant (CFA), followed by a boost in incomplete Freund's adjuvant (IFA) conferred 72% survival following lethal challenge 84 days after their first injection (102). This level of protection was not significantly

different from that seen in fish immunized with live theronts. Serum and mucous antibody levels in these two treatment groups were also elevated beginning two weeks after their initial immunization, and were maintained at high levels nine months after the fish were challenged.

Fish were also protected in a similar manner when immunized IC with the i-antigen and CpG oligodeoxynucleotides (CpG ODN) when compared to fish immunized with i-antigen in CFA (100). However, fish in this study only received one injection, and protection was reduced to 33.3% and 40%, respectively in these groups. Interestingly, fish in this study that were injected with i-antigen and non-CpG ODN were not protected and died at the same time as fish immunized with BSA and CpG ODN. These studies demonstrate that the i-antigen and adjuvant can elicit immunity comparable to exposure to live parasites. Also, fish receiving the i-antigen in a non-CpG ODN were not protected against challenge, demonstrating that the i-antigen elicits protection only when presented in the context of immunostimulators, such as adjuvants or live parasites. The i-antigen used in vaccination was affinity purified using an antibody column, a manner which would be too time consuming to produce a commercial vaccine. Also, the protein loaded over the affinity column was extracted from the membrane of *I. multifiliis* theronts, which themselves are difficult to produce in mass quantity. While the i-antigens are able to confer a robust immune response, further work should focus on production of these antigens in a manner conducive to large-scale vaccination.

4.3 Serotypes and Vaccine Development

Immunization with purified i-antigen was shown to confer serotype-specific protection (101). Fish immunized twice with either G5 or NY1 i-antigen in Freund's

adjuvant were subsequently challenged with live theronts of either serotype at doses lethal to controls. Individuals immunized with G5 i-antigen were 70 % protected when challenged by G5 theronts, while those challenged with NY1 parasites died in a similar manner to controls immunized with BSA. 33.3% of fish immunized with NY1 i-antigen were protected when challenged with NY1, and were killed when challenged with G5. Antibody titers showed that fish immunized with the i-antigens of one serotype developed specific antibody titers against that serotype only, showing that the serum antibodies themselves were serotype-specific (101). As a result, subunit vaccines containing only one i-antigen would not provide a breadth of protection against natural *I. multifiliis* infection.

While protection conferred by i-antigens is serotype-specific, studies using live parasites have shown that immunity to natural infections is not dependant upon serotype. Fish immunized by sublethal infection with G1.1 (Serotype B) were shown to carry a lighter parasite load than naïve fish when challenged with G2 (Serotype C). Similar results were obtained when G2 immunized fish were challenged with G1.1 (58). A second study demonstrated cross-serotype protection in fish immunized IC with live G3 (Serotype D) theronts and challenged with G4 (Serotype C). Similar protection was seen in fish immunized with G4 and challenged with G3 (53). The latter study showed that immune fish challenged with a heterologous serotype had no surface parasite load and complete survival, though all naïve fish receiving a similar challenge became infected and died. Also, immune fish challenged with a heterologous strain had absent or very low immobilization titers against the challenge strain when tested 58 days after challenge, even though their titers against the immunization strain remained high. These

experiments argue for the presence of antigens that are expressed on parasites of multiple serotypes. Also, they show that elements other than antibody immobilization may play a role in protection, as cross-challenged fish do not immobilize parasites of the challenge strain. It is possible that the antibody populations of fish immunized with one serotype and then challenged with another become enriched for these cross-reactive, but non-immobilizing antigens. Other immune mechanisms, such as complement or cellular responses, may also offer protection through mechanisms other than immobilization.

4.4 DNA Vaccine

Due to the difficulty in producing large amounts of i-antigen, DNA vaccination offered an attractive alternative to subunit vaccination. In order to accomplish this, an i-antigen gene from isolate G5 (*IAG52A[G5]*) was modified to reflect a traditional genetic code. In ciliates, UAA and UAG code for the amino acid glutamate, rather than for termination (9, 51). Thus, these codons were replaced with CAA and CAG, which reflect the codon preference of channel catfish. The synthetic gene is thus referred to as *IAG52A[G5/CC]*. Also, two truncated forms of the modified i-antigen gene, lacking either the N-terminal signal sequence or the C-terminal GPI anchor addition site were also generated. All of these were cloned into a commercial eukaryotic expression vector. Channel catfish were vaccinated intramuscularly (IM) with these constructs at one of three dose levels. All three constructs protected against challenge in a manner that did not appear to be dose-dependant, inducing 63 – 100 % protection relative to controls (100). Serum antibody titers were elevated, though they decreased substantially after two weeks and were much lower than those of fish injected with the i-antigen protein.

Subsequent work has shown that the modified *IAG52A[G5/CC]* can be expressed in *E. coli* and mammalian COS-7 cells when cloned into the appropriate expression vectors (61). However, when expressed in COS-7 cells, the protein was not correctly targeted to the plasma membrane, and it is possible that signal peptides and GPI anchor addition sequences differ in these species. However, the successful expression of these proteins, along with the specific antibody response seen in channel catfish, shows that these genes would likely induce an immune response in a variety of fish species and thus might be useful in vaccine development. Further work in this area could focus on the addition of other sequences to the vaccine construct, such as CpG motifs and cytokine sequences to increase the antibody titer seen in response to immunization. In addition, it would be beneficial to consider other routes of immunization, such as oral immunization, rather than IM injection. However, DNA vaccines present an attractive alternative to subunit and whole parasite vaccines because they are more easily mass produced.

4.5 Heterologous Expression

Because *I. multifiliis* cannot be generated in large amounts, a source of recombinant i-antigen was sought for further vaccine trials. However, several issues prevent the expression of *I. multifiliis* proteins in standard expression systems, such as *Escherichia coli* and *Saccharomyces cerevisciae*. Based on the limited sequence analysis conducted on *I. multifiliis*, its genome appears to be A + T biased. In other ciliates, A + T bias can be as high as 62% in coding regions and 89% in noncoding regions (83). Similar A + T bias is seen in the i-antigen genes, especially in the third position of codons, where 84.9% A + T bias has been observed, features which are consistent with the i-antigen genes of other ciliates (25). A + T rich sequences are inherently less stable, making their

expression in conventional systems difficult (39, 82). Codon usage is also unusual in ciliates, as UAA and UAG encode glutamine instead of terminating translation (9, 51). Because of these unique features of ciliate genetics, the identification of an alternative expression system was necessary. *T. thermophila*, a common model for eukaryotic cell biology, was an attractive candidate for such expression because it is both well studied and closely related to *I. multifiliis*.

T. thermophila has two copies of the β-tubulin gene (BTU1 and BTU2). In *T. thermophila* strain CU522, a K350M substitution in the BTU1 gene renders the cells sensitive to paclitaxel, a microtubule-stabilizing agent (40). Genes of interest can be inserted into plasmids where they are flanked by the 5' and 3' untranslated regions of BTU1. Plasmids are introduced into the macronucleus of *T. thermophila* by biolistic bombardment, where they integrate into the BTU1 locus by homologous recombination (10). Stable transformants can then be selected for by their resistance to paclitaxel. While the BTU1 promoter can drive high level expression of heterologous genes, a thirty-fold greater expression can be achieved by inserting the cadmium-inducible metallothionein (MTT1) promoter immediately upstream of the gene to be expressed (87). *I. multifiliis* i-antigen genes have been successfully expressed in this system and retain the same serologic properties as the native protein. These recombinant i-antigens are localized to the cell surface of *T. thermophila* when the entire i-antigen sequence is expressed and are recognized by *I. multifiliis* antibodies on western blots (39). Also, *T. thermophila* cells are immobilized by the i-antigen specific monoclonal antibodies, respective of their transformed phenotype (100).

T. thermophila was transformed with several different *I. multifiliis* i-antigens. One transformant, expressing the 48-kDa i-antigen gene from isolate G1, is referred to as MTTG1. Another expressing a 52-kDa i-antigen from isolate G5 (*IAG52A[G5]*) is referred to as MTTG5. These *T. thermophila* transformants were used for the immunization of channel catfish. These fish developed immobilizing antibody titers and were protected against challenge only by theronts expressing i-antigens of the same serotype (100). That is, MTTG1 immobilized NY1 theronts (G1 and NY1 are both of serotype A) and protected against NY1 challenge, but did not immobilize G5 or protect against G5 challenge. Similar results were seen in fish immunized with MTTG5. The antibody titers against *I. multifiliis* of homologous serotype were comparable to those seen in fish immunized with live theronts, though approximately one million live *T. thermophila* were injected into each fish, in contrast with only five thousand theronts.

This study addresses several important concerns regarding the generation of a vaccine in transformed *T. thermophila*. First, the recombinant i-antigen was functionally equivalent to native protein, and thus can be used as a source of protein for vaccines. Transformants expressing a form of the i-antigen lacking the C-terminal GPI anchor addition site have been generated, and have been shown to secrete recombinant i-antigen into the culture medium (16). It is possible that subunit vaccines could be generated using this recombinant protein. Also, whole recombinant *T. thermophila* cells, either killed or live, could be used for immunization, though further study will be required to establish the efficacy of these vaccines. Second, it is clear that serotype distinction is an important consideration in the development of a recombinant vaccine. It is possible that i-antigen genes from several serotypes could be included in a vaccine or that another

surface antigen that confers a wider breadth of protection could be identified. However, the i-antigen genes expressed in this manner show promise as effective vaccines that could be applied in a commercial setting.

5. Summary

According to the United States Department of Agriculture, the aquaculture industry is the fastest growing agricultural segment in the United States. An understanding of the mechanisms responsible for fish health is crucial in meeting the global demand for food fish. The fish immune system is poorly characterized relative to that of mammals, yet numerous similarities exist. Fish have a strong innate immune system, functionally distinct immune cell populations, central lymphoid organs, as well as the ability to develop specific antibodies and immune memory. The mucosal compartment of the immune system is of special interest, as the mucus is in such close contact with the surrounding environment. The fish mucosa and epidermis are distinct immune tissues themselves, harboring innate components, macrophages and granular cells, as well as cytotoxic and antibody secreting cells.

In *I. multifiliis*, antibody binding targets a class of GPI anchored membrane proteins known as i-antigens, which are similar to those found in other protozoa. Antibody cross-linking of these antigens causes immobilization of the parasite *in vitro* and host exit *in vivo*. In addition, monoclonal antibodies binding to distinct conformational epitopes can be used to define strain differences in *I. multifiliis* known as serotypes. Each serotype is immobilized by a unique panel of monoclonal antibodies, but is not immobilized not by those of heterologous serotypes. Because *I. multifiliis* causes

severe mortality in captive fish populations and is the target of a specific humoral immune response by its host, *I. multifiliis* has been the subject of a vaccine development program. The studies described in the subsequent chapters address specific issues that have arisen from this program, namely the differences in virulence between serotypes and cross-serotype protection.

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CHAPTER 2

DIFFERENCES IN VIRULENCE BETWEEN TWO ISOLATES OF *ICHTHYOPHTHIRIUS MULTIFILIIS* THAT REPRESENT DIFFERENT IMMOBILIZATION SEROTYPES[1]

[1]Swennes A.G., J.G. Noe, R. C. Findly, and H. W. Dickerson. Submitted to *Diseases of Aquatic Organisms*.

ABSTRACT

Isolates of the parasitic ciliate *Ichthyophthirius multifiliis*, representing two immobilization serotypes, were shown to infect naïve channel catfish (*Ictalurus punctatus*) with differing virulence. The isolates, NY1 and G5, of serotypes A and D respectively, display different surface immobilization antigens. The virulence of the two isolates was compared using tail fin infections to quantitate parasite numbers and by analysis of the survival of infected fish. It was found that NY1 was more virulent than the G5, as it caused significantly greater mortality among infected fish. NY1 infection caused greater mortality because it reproduced faster than G5 and overwhelmingly reinfected fish before they could mount a protective immune response. In contrast, the longer cycle of G5 allowed some fish time to develop immunity to reinfection. This report represents the first experimental evidence for differences in virulence between isolates of *I. multifiliis*.

INTRODUCTION

The ciliate *Ichthyophthirius multifiliis* is an obligate parasite of freshwater fish that infects surface epithelia of the skin and gills. The life cycle of *I. multifiliis* consists of three stages: an infective theront, a parasitic trophont, and a reproductive tomont (Figure 2.1) (12, 13). Free-swimming theronts invade the epithelium of fish and rapidly differentiate into trophonts that grows as large as 800 μm in diameter. Following a period of feeding and growth lasting days, the trophonts leaves their hosts to become encysted tomonts. Within eighteen to twenty-four hours the tomonts divide up to ten

times to produce as many as one thousand daughter cells, referred to as tomites, which bore out of the surrounding cyst, forming infective theronts.

I. multifiliis expresses membrane proteins referred to as immobilization antigens (i-antigens), that are analogous to those found in the related ciliates *Tetrahymena* and *Paramecium* (5). The i-antigens of *I. multifiliis* are GPI-anchored surface glycoproteins ranging in size from 40-70 kDa (3). I-antigens have been used to define serotypes of *I. multifiliis*, which are distinguished by monoclonal antibodies (mAbs) that immobilize cells bearing homologous but not heterologous i-antigens (6). To date, five such serotypes have been characterized. The isolates used in this study, NY1 and G5, represent serotypes A and D, respectively. The NY1 isolate has three i-antigens that resolve as 56-, 46- and 42-kDa proteins by SDS-PAGE under reducing conditions, while the G5 isolate has a single i-antigen of 55-kDa (15). Unlike *Tetrahymena* and *Paramecium*, *I. multifiliis* does not appear to undergo antigen-switching in response to environmental changes, such as temperature or osmolarity (2). Because serotype distinction remains constant throughout parasite life, it is useful in comparative studies within the species.

The i-antigens of *I. multifiliis* have been investigated as potential vaccine antigens. Fish immunized intracoelomically (IC) with G5 i-antigen in Freund's adjuvant were protected against surface challenge by parasites of the same serotype at a dose that was lethal to non-immunized control fish (16). Vaccination elicited i-antigen-specific serum and mucous antibody titers that were similar to those seen in fish exposed to live theronts. Subsequent studies revealed that immunization with purified i-antigen confers serotype-specific protection, as fish immunized with G5 i-antigen only were protected

against challenge by G5 theronts and their serum only immobilized G5 theronts (15). Challenge of these fish with NY1 theronts resulted in mortality similar to that seen in non-immunized fish. Analogous results were seen in fish immunized with NY1 i-antigen. This establishes a major role for serotype distinction in immunity to *I. multifiliis*, as the i-antigens confer a strong, but serotype-specific protection.

These immunization studies also suggested that the two isolates differed in their virulence (15). The experiments reported here were carried out to determine the basis for this difference. Fish were quantitatively infected on their caudal fins with the NY1 or G5 isolate and the number of infecting trophonts was determined at days 5, 7 and 9 following exposure. Mortality incurred by fish infected with NY1 or G5 during subsequent rounds of infection were compared by survival analysis. *I. multifiliis* isolate G5 (serotype D) infected channel catfish at an initially higher level than the NY1 isolate (serotype A), but all NY1 infected fish died during the course of the study while half of G5 infected fish survived. This indicates that the NY1 isolate was more virulent than the G5 isolate in these experiments.

MATERIALS AND METHODS

Parasite culture. Clonal isolates of the two serotypes of *I. multifiliis* used in this study have been previously described (15). NY1 (serotype A) and G5 (serotype D) were cultured separately on channel catfish (*I. punctatus*). Live tomonts were harvested from infected fish using previously described techniques (7). Harvested tomonts were kept at room temperature (22 ˚C) for 24 hours, and theronts were used immediately to infect fish.

Immobilization assays. Prior to experimental infection, immobilization assays were conducted to ensure that each culture represented a single serotype. These assays were performed using previously described techniques (6). Serotype-specific monoclonal antibodies were serially diluted in microtiter plates containing 50% phosphate buffered saline in carbon filtered tap water. mAb 10H3 was used for specific identification of serotype A, while mAb G361 was used in the same manner for serotype D (9). Five hundred theronts were added to each well and their motility was observed after thirty minutes by microscopy under low magnification. Theronts from each clonal culture were incubated separately with both homologous and heterologous antibodies. Cultures were considered to be composed of only one serotype when all organisms were completely immobilized by dilutions of one mAb, but were not immobilized by the other mAb. Both isolates were immobilized by their respective antibodies at dilutions of up to 1:25,600.

Experimental fish. Outbred channel catfish juveniles with no history of exposure to *I. multifiliis* were obtained from a local hatchery. The fish were held in 627-liter aquaria with biological filtration and were treated with oxytetracycline and formalin prior to infection. Nitrite levels and pH were monitored daily and water temperature was maintained at 22 ± 1 °C.

Quantitative caudal fin exposure. For each infection, thirty naïve channel catfish with a mean weight of 25.28 ± 5.887 g were divided into two groups of 15 fish, each of which was placed in a 76-liter aquarium. These groups were infected with either the *I. multifiliis* NY1 isolate (15 fish) or the G5 isolate (15 fish) as follows. Sets of five fish were anesthetized with a 0.02% solution of Finquel MS-222 (Argent Chemical Laboratories, Redmond, WA). Once anesthetized, fish were covered in plastic wrap to

maintain their body moisture, and their caudal fins were placed in plastic multi-channel pipette troughs (Labcor Products, Frederick, MD) containing 25,000 theronts (5,000 theronts per fish) suspended in 50 mL of carbon filtered tap water. Fish were infected for five minutes before being returned to the aquaria. Infections were initiated with both serotypes on the same day to ensure standardization of temperature and water quality. Parasites on caudal fins were counted at five, seven, and nine days after exposure using a dissecting microscope (Olympus) at 10x magnification. Photographs were taken and used to measure and compare parasite sizes at each time point. The number of days to death of each fish was also recorded and compared. Three replicate infections (designated I, II, and III) were performed.

Statistical Analysis. The parasite numbers at five, seven, and nine days post infection were compared between the two isolates. Upon residual analysis, the data was found to be non-normally distributed. A log transformation normalized the data and allowed for subsequent analysis using the Tukey-Kramer multiple comparison procedure to compare parasite numbers at each time point ($\alpha = 0.01$). Kaplan-Meier curves were generated in order to illustrate the mortality caused by the parasites after infection. Survival analysis using a log-rank test was also conducted to verify the significance of the curves.

RESULTS

I. multifiliis G5 initial infection was greater than NY1

The level of infection for each fish was determined by counting the number of trophonts on the caudal fin. Five days after infection, channel catfish infected with G5

theronts had on average a significantly greater parasite load than fish infected with NY1 theronts, carrying a mean of 82.6 ± 95.7 trophonts per fish for G5 compared to 11.0 ± 11.5 for NY1 (Table 2.2). However, the number of trophonts observed per fish was variable both within a replicate and between replicates, even though the serotypes used were derived from single cell isolates (Figures 2.3, 2.4, 2.5). In replicates I and III, fish infected with the G5 isolate had a mean parasite load of 126.9 ± 98.4 or 119.3 ± 94.7, respectively, but in replicate II only 4.0 ± 5.5 trophonts were observed per fish. For NY1, a total of 4.7 ± 6.1 or 6.9 ± 8.7 trophonts were counted in replicates II and III, in contrast to replicate I where fish were infected with 21.4 ± 11.3 trophonts. Only in replicate II was an almost identical level of initial infection achieved for the two isolates (G5: 4.0 ± 5.5; NY1: 4.7 ± 6.1).

Seven days after infection, G5 infected fish continued to have a significantly greater parasite load than fish infected with NY1 (Table 2.2). Fish infected with G5 in replicates I or III carried 60.3 ± 70.5 or 95.9 ± 82.8 trophonts per fish compared to NY1 infected fish that had 1.9 ± 2.5 or 4.1 ± 3.3 trophonts per fish, respectively. In replicate II, in which the initial infection levels were similar between the two isolates, the number of NY1 trophonts decreased to 1.2 ± 1.5 per fish, while G5 numbers remained similar to day 5. For G5, the number of trophonts per fish observed at days 5 or 7 was also similar in replicate III, but in replicate I decreased two fold, from 126.9 ± 98.4 to 60.3 ± 70.5. In contrast, the number of NY1 trophonts observed on fish at day 7 showed a 2 to 10 fold decrease from that observed at day 5 in all three replicates, resulting from trophonts leaving the fish to form tomonts.

The infective cycle for *I. multifiliis* NY1 was shorter than G5

Reinfection of fish results from trophonts actively leaving the fish and developing into tomonts, which release infective theronts approximately 18 to 24 hours later. Consequently, fish can be reinfected with progeny of the initial infection starting about 18 hours after the trophont leaves the fish. In these experiments, infective theronts released into the tank reinfected the entire fish, not just the caudal fin as in the initial infection. For G5, the infection level observed at day 9 following initial infection was less than observed at days 5 or 7 for all three replicates (Table 2.2, Figure 2.3). This indicates that in these experiments G5 trophonts left the fish sometime after day 7 and reinfection was not observed by day 9 after infection. The day 9 pattern of infection with NY1 was strikingly different from that observed for G5 (Table 2.2, Figure 2.4). For NY1 the level of infection observed at day 9 increased relative to day 7 in replicates I and II, rising from 1.9 ± 2.5 to 16.1 ± 37.1 or 1.2 ± 1.5 to 38.1 ± 27.0, respectively. Little change in the level of infection was seen in replicate III. These data indicate that NY1 trophonts are leaving fish by day 7 and that reinfection can generally be observed by day 9, which demonstrates that the NY1 infection cycle takes about 7 days to complete. In contrast, it was not until day 9 that the number of trophonts decreased on G5 infected fish, indicating that the G5 infection cycle took approximately 10 days under these conditions.

G5 infection resulted in greater inflammation

The heavier infection by G5 trophonts appeared to elicit a more pronounced inflammatory response than NY1 at day 5 of infection, which was qualitatively assessed as epithelial erythema (Figure 2.5A,B). At day 5 the NY1 and G5 trophonts were similar

in size (approximate mean = 400 μm, range = 200 – 700 μm, n = 100), but the gross morphology of the caudal fin in fish infected with G5 clearly differed from that of fish infected with NY1 in which no epithelial erythema was observed. At day 7, inflammation was observed in fish infected with either isolate, although fewer NY1 trophonts were observed. Trophonts were larger than at day 5, averaging approximately 700 μm for both isolates (range = 300 – 1100, n = 100) (Figure 2.5C,D).

At day 9 inflammation resulting from infection again differed between the two isolates and appeared to be more pronounced in fish infected with G5, as epithelial erythema continued to be visible in fish infected with G5, but was not in fish infected with NY1 (Figure 2.5E,F). The inflammatory response appeared to correlate with the differences in the timing of the life cycle between the two isolates, as the G5 trophonts remaining on the fish continued to increase in size from 700 μm observed at day 7 to about 800 μm (range = 500 – 1100, n = 35). In contrast, NY1 trophonts averaged only 300 μm in size (range = 200 – 500, n = 25), smaller than those observed at day 5 or day 7 of infection. At day 9 no large trophonts were observed on NY1 infected fish and, conversely, no small trophonts were found on fish infected with G5. The difference in size of the infective trophonts between the two isolates at day 9 supports the idea that G5 trophonts represent the initial infection, while the smaller NY1 trophonts represent the second round of infection.

Infection with the *I. multifiliis* NY1 isolate resulted in complete mortality

The severity of the infections was determined by measuring fish mortality for a period of 42 days following the initial infection. The data are significantly different, as all 45 fish infected with NY1 died by day 19, but 23 of 45 fish infected with G5 remained

alive at 42 days post-infection as illustrated by a Kaplan-Meier survival curve (Figure 2.6). Prior to day 10 one NY1 and five G5 infected fish died, most likely as a result of stress from handling during initial infection and not from infection per se. The curves for the two serotypes cross at day 14 post-infection. A log-rank test was performed which assumed that the study ended at day 14, allowing for the analysis of all deaths occurring before day 14 independently of the deaths occurring after this point. This demonstrated that for the first 13 days of infection the difference in survival between NY1 and G5 was not significant ($P = 0.0941$). In contrast, as clearly indicated by the survival curves, a similar analysis beginning at day 14 showed that fish infected with the G5 isolate had a significantly higher survival rate than fish infected with the NY1 isolate ($P < 0.0001$), as all 44 remaining NY1 infected fish died by day 19, but only 17 of the remaining 40 G5 infected fish died by day 42.

The Kaplan-Meier survival curve for the second replicate infection illustrates most clearly the differences in the course of infection between these two isolates (Figure 2.7). In this replicate, the number of trophonts on infected fish at day 5 was almost identical, yet the effects of the infection were strikingly different. The initial infection with G5 led to the death of one fish at day 5 and a second at day 7, most probably as a result of handling during the initial infection. The remaining G5 infected fish survived until day 25 post infection, when 8 fish died between days 26 and 34, towards the end of the third cycle of infection. The remaining 5 fish survived through day 42. All 15 fish infected with NY1 died on day 14, which corresponded with the start of the third cycle of infection for NY1.

DISCUSSION

Quantitative tail fin infection of channel catfish with the *I. multifiliis* isolate G5 (serotype D) resulted in a heavier initial infection than with the NY1 isolate (serotype A) in two of three replicates and a similar level of infection in the third replicate. Although fish exposed to NY1 were initially infected with lower numbers of *I. multifiliis*, all NY1-infected fish died by day 19 of infection, but 23 of 45 fish infected with G5 survived through 42 days. The higher mortality following infection and reinfection demonstrates that NY1 is more virulent than G5. Our results suggest that this differential virulence was not a function of the initial level of infection, but rather, resulted from the interplay between the different times required for the two *I. multifiliis* isolates to complete their infective life cycle and the time required for the fish to develop protective humoral immune responses.

This is best illustrated by replicate II, in which the initial infection, measured at day 5, was essentially identical for NY1 (4.7 ± 6.1 trophonts per fish) and G5 (4.0 ± 5.5 trophonts per fish), and thus the course of the infection represents the response of fish to similar initial parasite loads. At day 7 the number of trophonts observed on G5 infected fish remained similar to day 5 (5.3 ± 5.3 trophonts per fish), while the number of NY1 trophonts decreased (1.2 ± 1.5 trophonts per fish). This indicated that NY1 trophonts have begun to exit the fish by day 5 to form tomonts, which release infective theronts starting about 18 hours after they leave the fish. At day 9 post infection, the results of this second cycle of infection are apparent on NY1 infected fish, as the number of trophonts increased to 38.1 ± 27.0 trophonts per fish, which is about 7 times greater than observed on day 5 of infection. This demonstrates the increased level of reinfection

expected, but is a substantial under-representation of the true extent of secondary reinfection, as only trophonts on the caudal fin were counted. Unlike the initial infection in which only the caudal fin was infected, the entire fish became reinfected during all further cycles of infection. In contrast, the number of trophonts observed on G5 infected fish at day 9 post-infection decreased to 0.4 ± 0.8 trophonts per fish, which indicates that most trophonts have exited the fish, but reinfection has not yet occurred.

During the natural course of infection, as modeled in these experiments, *I. multifiliis* leaves and reinfects fish over a period of several days, and the estimated times required to complete the life cycle are averages. A life cycle of 7 days for NY1 predicts that the second round of infection would occur over days 7 to 8 and the third round of infection over days 14 to 15. Each tomont has the potential to produce as many as 10^3 infective theronts (10). Assuming each tomont produced 500 theronts and 10% succeeded in reinfecting fish, then an initial infection of 5 NY1 theronts per fish could result in reinfection with 250 theronts on days 7 to 8 and with 12,500 theronts per fish over days 14 to 15 in these closed systems. A dose of 5,000 NY1 theronts per fish causes total mortality in naïve fish, and it likely that this dose was greatly exceeded 14-15 days after infection (15). It was at this point that 42 of the 45 fish infected with NY1 died. This demonstrates that the exponential increase in the magnitude of reinfection following 3 rounds of infection, occurring over 14 days, even starting with a modest infection as in replicate 2, can overwhelm the fish, leading to complete mortality.

In contrast to NY1, the number of trophonts found on fish infected with G5 was similar on days 5 and 7 in two replicates and decreased on day 7 in one replicate. At day 9 post infection, fewer trophonts were observed than on days 5 or 7 for all three

replicates. If G5 trophonts left the fish by day 9, their progeny could be expected to reinfect fish in the second cycle beginning on day 10, and the third cycle of infection could begin around day 20. In replicate 2, the majority of deaths in fish infected with G5 occurred on days 26 to 29 (7 fish), near the end of round three of infection, and in replicate 3, the majority of deaths in fish infected with G5 occurred on day 18 (8 fish), near the end of round two of infection. No G5 infected fish died in replicate 1.

Fish sublethally infected with *I. multifiliis* are protected against subsequent infection (8). Numerous studies have demonstrated the prominent role of humoral immunity in this protection, as anti-*I. multifiliis* serum antibody titers rise following infection, and naïve fish can be completely protected against lethal challenge by injection of immobilizing mAbs, which trigger rapid parasite exit through i-antigen cross-linking (1, 4, 9). In addition, antibodies secreted from the skin of immune fish immobilize theronts *in vitro* and render them unable to infect naïve fish (17, 18). Consequently, the time required for fish to develop a robust humoral immune response to *I. multifiliis* in relation to the time that fish are exposed to exponentially increasing numbers of theronts is critical to the ultimate outcome of the infection. Induction of adaptive immune responses to *I. multifiliis* has been observed within days following initial exposure. The earliest response correlated with infection is an increase in the level of total IgM mRNA, detected by RT-PCR, in skin or head kidney samples four days after infection of rainbow trout with *I. multifiliis* (14). Antibodies specific for *I. multifiliis* can be detected within 12 days following infection of catfish in either skin or serum (19). Specific antibody titers continue to increase in magnitude, as antibody in skin and serum show peak values at 7 and 14 weeks, respectively (11). Thus, specific antibody responses arise by 12 days,

but exactly when specific antibodies first appear in skin or serum following infection is not known.

The time frame for reported increases in antibody titers is well correlated with the protection observed in these experiments. Although *I. multifiliis* antibodies can be produced by day 12, the results of these experiments clearly demonstrate that the early phase of the humoral response was not sufficient to prevent complete mortality caused by the massive third round of infection at day 14 by NY1. The response, however, apparently was able to provide some protection against G5 with its longer cycle of infection and higher level of inflammation, as deaths did not occur until day 18, near the second round of infection, or until days 26 to 29, near the end of the third round of infection.

The difference in virulence observed between these two isolates of *I. multifiliis* is best explained as resulting from the interplay between the time needed by fish to develop protective immune responses following infection and the time required for *I. multifiliis* to progress through its life cycle. The death of fish infected with *I. multifiliis* is believed to be a result of loss of gill function caused by the high numbers of parasites found in gills. For NY1, the infective cycle took 7 days, and compared to G5 induced less severe epithelial erythema, an indicator of host inflammatory responses, during the first round of infection. The third round of NY1 infection occurred around day 14, by which time specific antibodies should be present, but as this is still early in the development of protective humoral immune responses, the magnitude of this third cycle of infection was sufficient to overwhelm the fish. In contrast, G5 had a both a longer 10 day infective life cycle and induced a more pronounced epithelial erythema, presumably indicative of a

more pronounced immune response. As shown in replicate 2, in which NY1 and G5 initial infections were essentially identical, these differences allowed some fish in the G5 infected population to survive, presumably because they had time to generate specific antibodies at levels sufficient to protect against the third and subsequent rounds of infection.

An important observation stemming from these results relates to the design of vaccine trials in catfish. Such trials regularly use 2 week cycles of immunization and challenge. These results suggest that at two weeks following infection or immunization, when fish are only beginning to produce antigen specific antibodies, the antibody levels are sufficient to provide only modest protection against infection. This work suggests that a longer three week cycle of immunization and challenge may prove a more effective method for assessing vaccine efficacy in fish.

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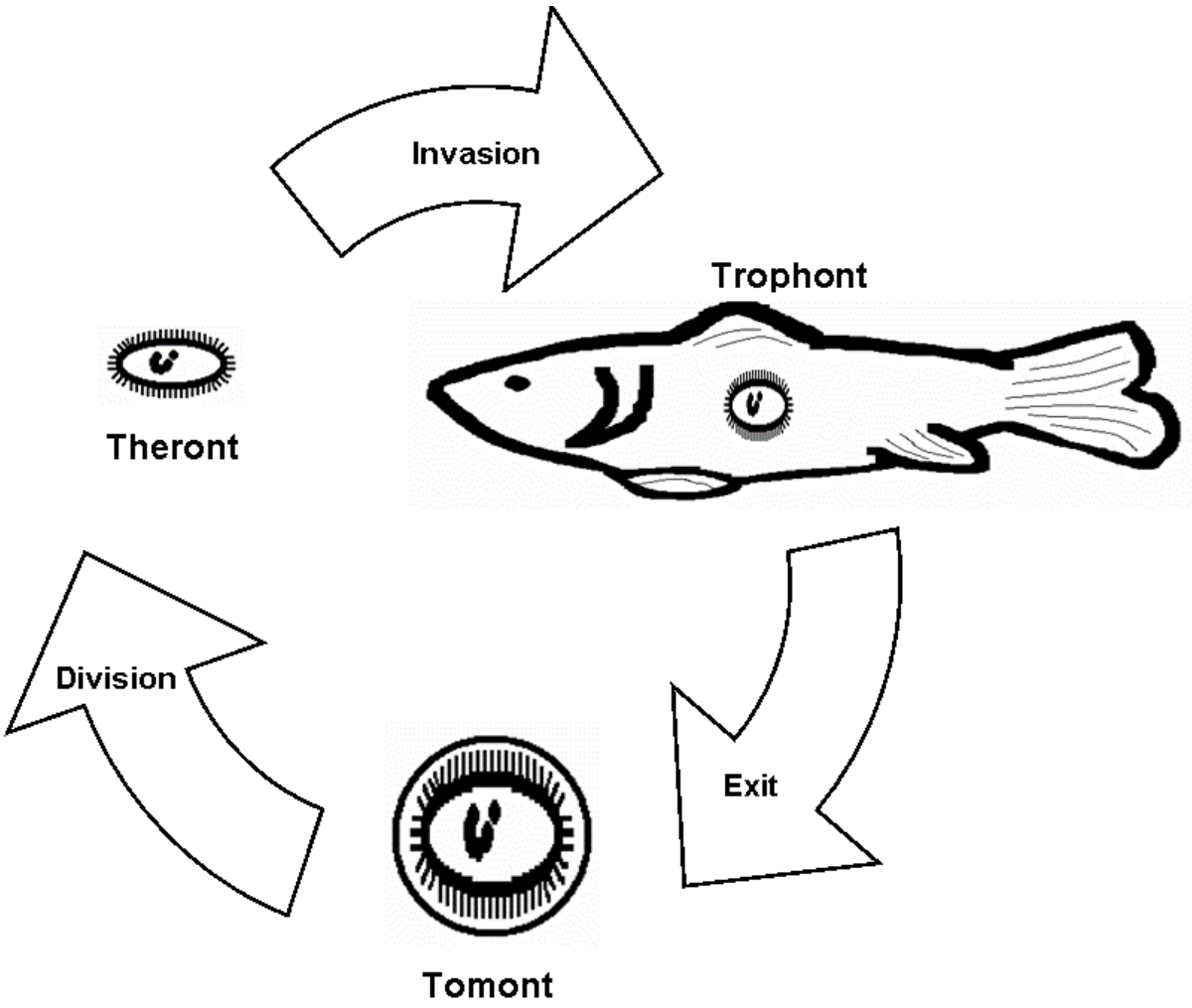

Figure 2.1. The life cycle of *I. multifiliis* has three functional stages represented by infective theronts, parasitic trophonts, and reproductive tomonts. At 22 ˚C, theronts invade the epithelia of fish within minutes of contact with the fish. Theronts immediately differentiate into trophonts that grow in the fish epithelium for seven to ten days, and then leave the fish. The free-living tomonts encyst within one hour. The encysted tomont undergoes rapid cell division, producing as many as one thousand daughter cells over a twenty-four hour period. These exit the cyst as infective theronts and initiate the next round of infection.

Table 2.2. Mean numbers of *I. multifiliis* trophonts observed on caudal fins are shown at five, seven, and nine days post-infection. The two isolates were compared using the Tukey-Kramer multiple comparison procedure. Rows marked by an asterisk indicate means that are significantly different.

Five days post infection

	NY1	**G5**	**P-value**
Replicate I	21.4 ± 11.3	126.9 ± 98.4	< 0.0001*
Replicate II	4.7 ± 6.1	4.0 ± 5.5	0.9610
Replicate III	6.9 ± 8.7	119.3 ± 94.7	< 0.0001*
Replicates I, II, III	11.0 ± 11.5	82.6 ± 95.7	< 0.0001*

Seven days post infection

	NY1	**G5**	**P-value**
Replicate I	1.9 ± 2.5	60.3 ± 70.5	< 0.0001*
Replicate II	1.2 ± 1.5	5.3 ± 5.3	0.1052
Replicate III	4.1 ± 3.3	95.9 ± 82.8	< 0.0001*
Replicates I, II, III	2.4 ± 2.7	54.0 ± 72.0	< 0.0001*

Nine days post-infection

	NY1	**G5**	**P-value**
Replicate I	16.1 ± 37.1	15.7 ± 12.7	0.0922
Replicate II	38.1 ± 27.0	0.4 ± 0.8	< 0.0001*
Replicate III	2.9 ± 2.5	36.8 ± 38.6	0.0007*
Replicates I, II, III	19.4 ± 30.1	16.6 ± 26.1	0.8378

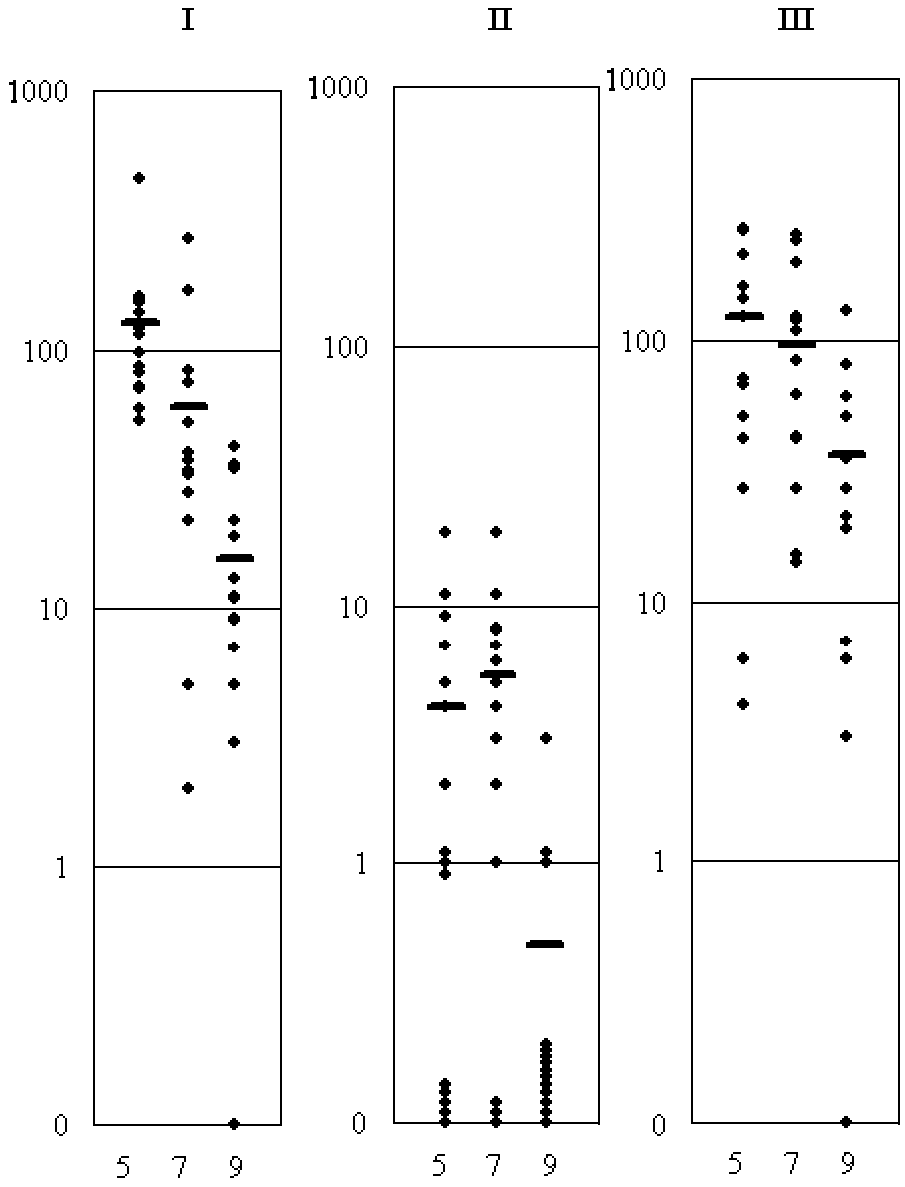

Figure 2.3. Numbers of trophonts on the caudal fins of fish infected with *I. multifiliis* isolate G5 at five, seven, and nine days post infection. Each point represents a single infected fish. The three replicates (I, II, and III) are shown separately for each serotype.

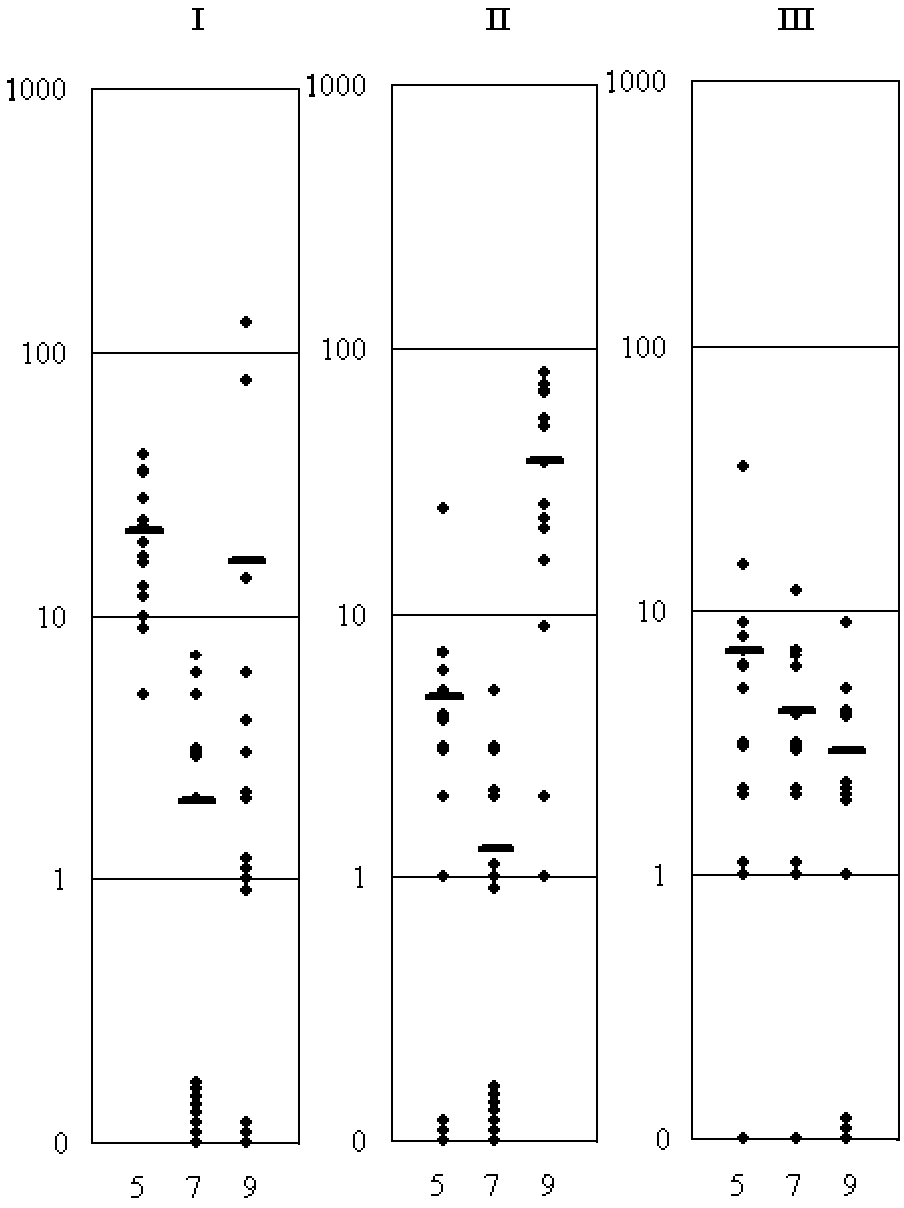

Figure 2.4. Numbers of trophonts on the caudal fins of fish infected with *I. multifiliis* isolate NY1 at five, seven, and nine days post infection. Each point represents a single infected fish. The three replicates (I, II, and III) are shown separately for each serotype.

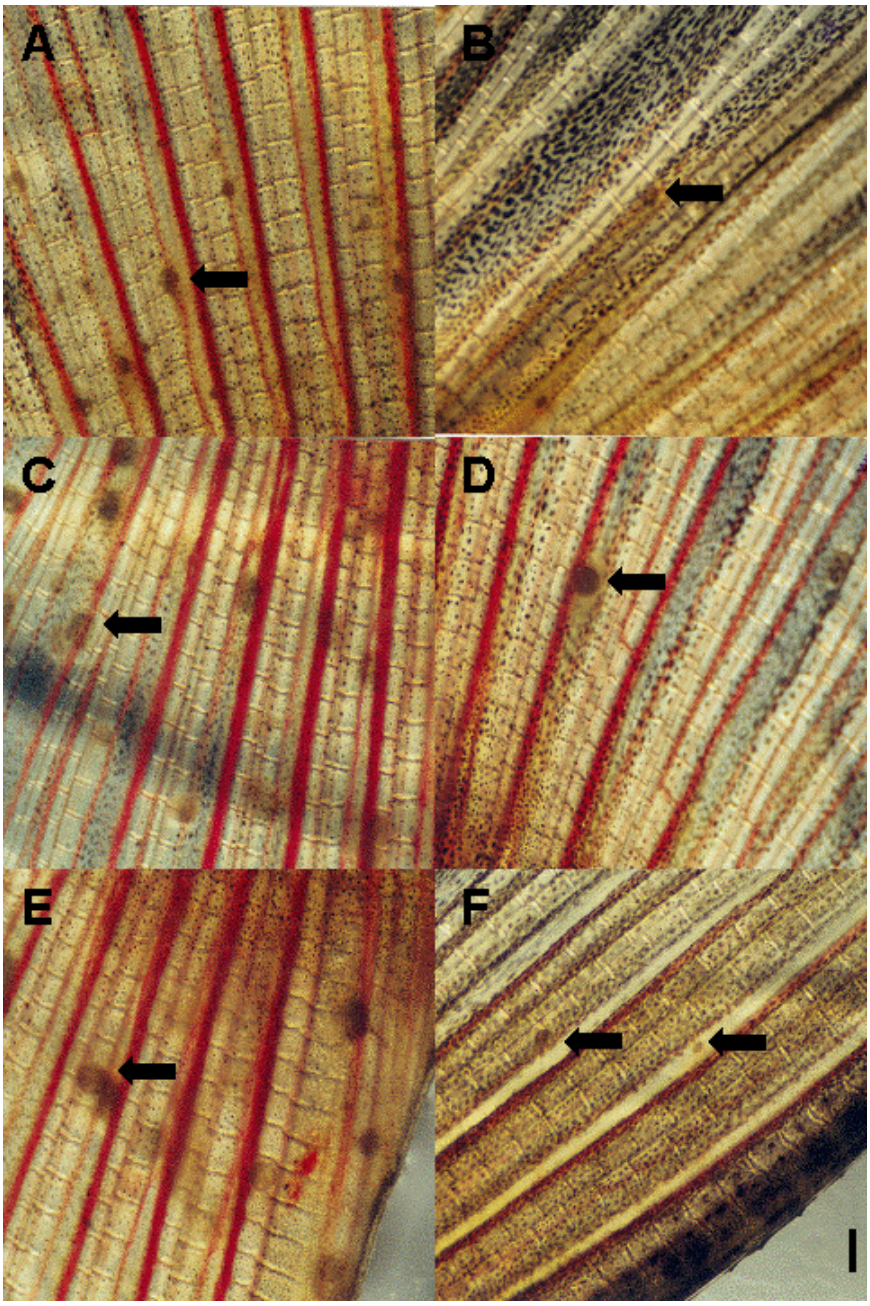

Figure 2.5. *I. multifiliis* trophonts (arrows) are shown in the caudal fin epithelium at five (A and B), seven (C and D), and nine (E and F) days post-infection. Fish in panels A, C, and E were infected with isolate G5. Fish in panels B, D, and F were infected with isolate NY1. Scale bar = 1 mm.

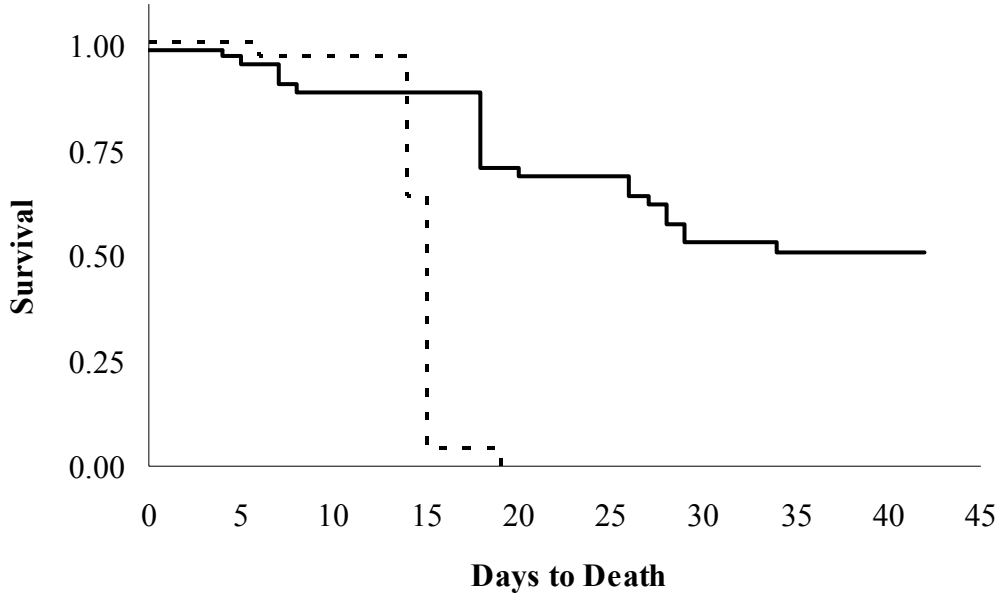

Figure 2.6. Kaplan-Meier survival curves of fish infected with *I. multifiliis* isolates G5 (solid line) and NY1 (dotted line). Fish from the three infection replicates are shown together.

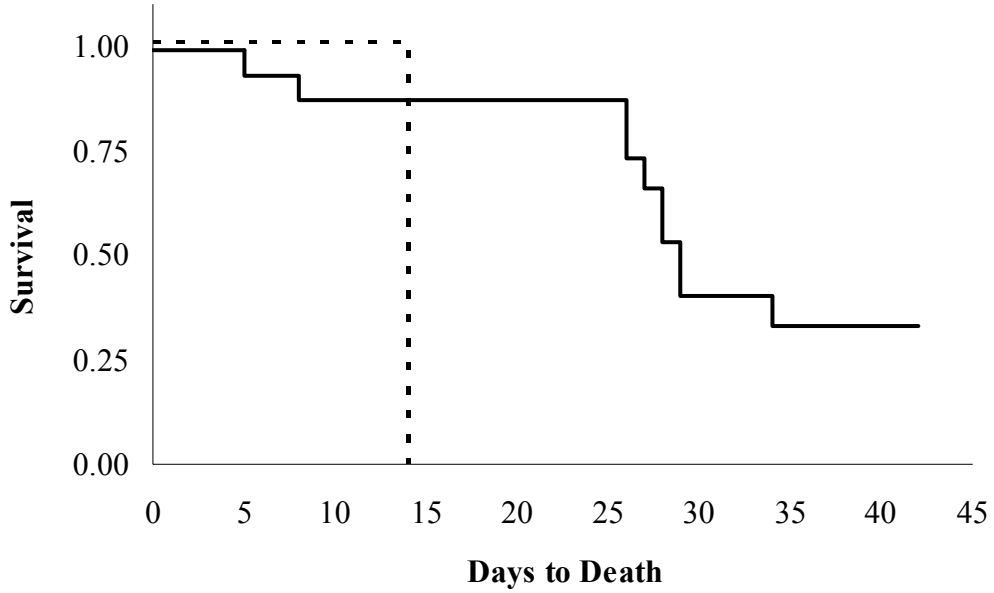

Figure 2.7. Kaplan-Meier survival curves of fish from infection II infected with *I. multifiliis* isolates G5 (solid line) and NY1 (dotted line).

CHAPTER 3

CROSS-IMMUNITY TO DIFFERENT IMMOBILIZATION SEROTYPES OF *ICHTHYOPHTHIRIUS MULTIFILIIS*[1]

[1]Swennes A.G., R. C. Findly, and H. W. Dickerson. To be submitted to *Fish and Shellfish Immunology*.

ABSTRACT

Immunization with either of two serotypes of the parasitic ciliate *Ichthyophthirius multifiliis* conferred protection against challenge infection by both homologous and heterologous serotypes. The isolates used in this study, G5 and G12, representing serotypes D and F, display different surface immobilization antigens and are thus distinguishable by immobilization with specific antiserum or monoclonal antibodies. To demonstrate cross-serotype protection, fish were immunized with live theronts of one serotype and then challenged by infection with the other serotype. Immunized fish were protected against homologous or heterologous challenge, but displayed elevated immobilization titers only against the immunizing strain. Trophont homogenates were evaluated as vaccines, and while immobilization titers were elevated, protection was not seen. A strong positive correlation was seen between immobilization titer and survival, reinforcing the importance of antibodies in protection against *I. multifiliis*. No potential cross-protective antigens were identified on western blots probed with sera from immune fish. These results demonstrate that cross-immunity occurs between *I. multifiliis* serotypes, but which proteins are involved in this protection is not clear.

INTRODUCTION

The ciliate *Ichthyophthirius multifiliis* is an obligate parasite of freshwater fish that infects surface epithelia of the skin and gills. Its life cycle consists of three stages: an infective theront, a parasitic trophont, and a reproductive tomont, all of which are ciliated. *I. multifiliis* express a family of membrane proteins referred to as immobilization antigens (i-antigens) that are analogous to those found in *Tetrahymena*

and *Paramecium* (6). In contrast with other ciliates, each isolate expresses only one set of i-antigens and no evidence of antigen switching has been found (2). The i-antigens of *I. multifiliis* are a family of GPI-anchored surface glycoproteins ranging in size from 40-70 kDa which have been used to define serotypes of *I. multifiliis* (3, 7). Isolates G5 and G12 represent serotypes D and F, respectively. G5 expresses one i-antigen protein that resolves as a single 55-kDa band by reducing SDS-PAGE and is immobilized by monoclonal antibody (mAb) G3-61 (14). G12 represents a newly discovered serotype and is not immobilized by mAb G3-61 or G5 antiserum.

Antibody binding to the i-antigens plays an important role in protection against *I. multifiliis*. Passive transfer of immobilizing IgG mAbs confers complete protection against infection through cross-linking of the cilia, triggering parasite exit from the host epithelium (4, 12). Because antibodies targeting the i-antigens confer strong protection through a well-characterized mechanism, i-antigens have been investigated as vaccine antigens. Fish immunized intercoelomicly (IC) with 10 µg of affinity purified G5 i-antigen in Freund's adjuvant were protected against challenge infection by the same serotype at a dose that was lethal to non-immunized control fish (15). Vaccination elicited i-antigen-specific serum and mucous antibody titers that were similar to those seen in fish exposed to live theronts. Immunization with purified i-antigen conferred serotype-specific protection, as fish immunized with G5 i-antigen were only protected against challenge by G5 theronts and their serum only immobilized G5 theronts (14). Challenge of G5 vaccinated fish with NY1 theronts, representing a different serotype, resulted in mortality similar to that seen in non-immunized fish. Analogous results were seen in fish immunized with NY1 i-antigen and challenged with G5 theronts (14). This

established serotype distinction as important in *I. multifiliis* infection, as the i-antigens confer strong, but serotype-specific protection.

While the i-antigens play an important role in immunity to *I. multifiliis*, studies have also documented cross-serotype protection (10, 11). Fish immunized IC with live G3 parasites resisted both homologous (G3) and heterologous (G4) challenge, though high immobilization titers (1:2000 and higher) were present only against the strain used in immunization (10). Similar results were seen in fish immunized with G4. Because fish immunized with live *I. multifiliis* are protected against infection by theronts expressing either homologous or heterologous i-antigens, other protective antigens must also be expressed. The identification of these antigens is of interest to vaccine development efforts, as they should confer protection against numerous serotypes.

Vaccination studies to determine whether protection could be obtained from killed *I. multifiliis* preparations have also been undertaken using either theront or trophont preparations. Trophont preparations are of particular interest, as this is the stage found on the fish. Theront lysates in Freund's adjuvant, formalin-fixed theronts, and theront cilia yielded no protection against lethal challenge, but significant protection was seen in fish immunized with formalin-fixed trophonts (1). Sonicated trophonts at a similar dose also provided some protection, and these fish displayed elevated antibody titers in their skin and serum by both ELISA and immobilization (16). Because protection against challenge by homologous serotype has been observed after vaccination with trophont preparations, this study tested the efficacy of vaccination with trophonts against both homologous and heterologous challenge.

MATERIALS AND METHODS

Parasite culture. *I. multifiliis* clonal isolates G5 and G12 were cultured separately by serial passage on channel catfish (*Ictalurus punctatus*). Isolate G5 has been previously described and is immobilized by mAb G3-61, specific for serotype D. G12 has not been previously characterized, and is designated as serotype F. This isolate is not immobilized by mAb G3-61. Theronts were isolated using previously described techniques (8). Trophonts were dislodged from heavily infected fish by hand in glass dishes, transferred by Pasteur pipette to oil centrifuge tubes on ice, and concentrated by centrifugation.

Theront and trophont homogenates. Theronts or trophonts were concentrated by centrifugation in oil centrifuge tubes at 300 x g for three minutes and were further concentrated in eppendorf tubes at 4000 x g (for theronts) or 6000 x g (for trophonts) for five minutes. The supernatant was discarded, and the pellet was resuspended in 0.1% NP-40 in PBS. A general-use protease inhibitor cocktail, containing ABESF, E-64, bestatin, leupeptin, aprotinin, and sodium EDTA (Sigma-Aldrich, St. Louis, MO), was added at 10 μL per 10^6 theronts or 20 μL per 0.25 mL of packed trophonts. The cells were transferred to an ice cold Dounce homogenizer (Wheaton Science Products, Millville, NJ) and were lysed using forty strokes of the tight pestle. The lysate was spun at 10,000 x g for ten minutes at 4 °C to remove insoluble elements. The pellet was discarded and the supernatant was stored at -20 °C. Protein concentration was determined by protein assay (Bio-Rad Laboratories, Hercules, CA).

Vaccination procedure. Outbred channel catfish with a mean weight of 12.2 ± 1.8 g were obtained from a local hatchery and randomly assigned to seven groups, each

containing forty fish. Each group was divided into two subgroups of twenty fish, and subgroups were randomly assigned to 76-liter aquaria. For vaccination, fish were anesthetized with a 0.02% solution of Finquel MS-222 (Argent Chemical Laboratories, Redmond, WA) and injected intercoelomicly (IC) with homogenized trophonts or live theronts. Fish receiving homogenized trophonts were injected with 50 µg of protein (equivalent to 20 trophonts) in an equal volume of complete Freund's adjuvant (CFA). The antigen was emulsified in the adjuvant by repeated passage between two glass syringes connected by a 22-gauge needle. Fish receiving live theronts were injected IC with 5000 live cells in carbon filtered water. All immunizations were given in a total volume of 150 µL. Theront immunized fish were treated with 25 ppm formalin for ten days to clear any inadvertent surface infection. All immunized fish were boosted nineteen days later in the same manner, except antigen injections were given in incomplete Freund's adjuvant (IFA).

Antiserum and immobilization assays. Serum was collected from randomly selected fish from each treatment group before treatment (preimmune), fourteen days after their first and second injections (eight fish from each group), and following challenge infection (six fish from each subgroup). To obtain serum, fish were anesthetized with a 0.02% solution of Finquel MS-222 (Argent Chemical Laboratories, Redmond, WA) and bled from the caudal vein with a 23-gauge needle. Blood was allowed to clot for one hour at room temperature or overnight at 4 °C, and serum was recovered by centrifugation at 1500 x g for ten minutes. Serum was heat inactivated at 56 °C for thirty minutes, and stored at -20 °C. Serum from individual fish was tested by immobilization assay against both serotypes used in the study, and reported

immobilization titers are the inverse of the highest dilution where all theronts were immobilized (7). Subsequently, preimmune serum as well as post-challenge serum from fish immunized with live theronts was pooled and used to probe western blots.

Parasite challenge. Immunized fish were challenged 65 (G5 immunized fish) or 66 (G12 immunized fish) days after their initial immunization. Doses of 30,000 live G5 theronts and 5,000 live G12 theronts per fish were determined to be lethal challenge doses for naïve fish, and reflect the differences in virulence seen between isolates. Fish from each tank were placed in 2 L of carbon filtered tap water with aeration and incubated for two hours with their appropriate challenge dose. Fish were then returned to their aquaria along with the water used in the challenge procedure. Water quality was monitored daily and mortalities were recorded for each group. Individuals surviving past 26 days post-challenge, which showed no signs of external infection, were considered to have survived challenge. Immobilization assays were performed using sera from six fish randomly selected from each group containing survivors at 26 days post-challenge (91 – 92 days post-immunization).

Western Blotting. One dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on *I. multifiliis* theronts and trophonts. Homogenized theronts or trophonts (10 μg protein per lane) were resolved on 4% stacking and 10% separating gels under non-reducing conditions. Bands were visualized by silver stain (Pierce Biotechnology, Rockford, IL). For western blots, proteins were transferred to nitrocellulose membranes and blocked in 3% BSA in tris-buffered saline (TBS) with 0.05% tween 20 (TBST) overnight at 4 °C and washed in TBST. These membranes were incubated overnight at 6 °C in catfish antiserum diluted 1:10 in TBS

using 1 mL per 8 cm^2 of membrane. Filters were washed and incubated with alkaline phosphatase-conjugated MAF-13, a mouse monoclonal antibody against the heavy chain of catfish immunoglobulin, for two hours in TBS, washed, and reacted with alkaline phosphatase substrate for six minutes (Pierce Biotechnology, Rockford, IL). Western blots were also probed with preimmune sera and the secondary antibody alone to distinguish non-relevant binding patterns. Proteins were separated under non-reducing conditions because catfish IgM does not bind to i-antigens under reducing conditions. Consequently, molecular weights of proteins cannot be accurately estimated.

Statistical Analysis. Immobilization titer was compared between the study groups using the Tukey's HSD to generate 95% homogenous subsets. Survival analysis of the challenge groups was conducted using Log-Rank tests. Fish surviving at twenty-six days post-challenge were considered survivors and were censored. Correlation of immobilization titer and survival was performed using the Pearson correlation procedure. All tests were performed using SPSS statistical analysis software (SPSS, Chicago, IL) at 95% confidence ($\alpha = 0.05$).

RESULTS

Live parasites protected against homologous and heterologous challenge

Immunization with live G12 theronts IC completely protected against challenge infection by G5 or G12 theronts, while non-immunized controls had complete mortality. The G12 immunized fish had a mean immobilization titer of 790 prior to challenge (Table 3.1). This value decreased to 427 after G12 challenge and 373 after heterologous challenge with G5 theronts (Table 3.2). Fish immunized IC with live G5 theronts were

also protected against challenge by G5 or G12, although not completely, as 80% of these fish challenged with G5 theronts were protected against challenge, and 65% of those challenged with G12 theronts were protected (Figure 3.3). While protection was not complete in these groups, survival was significantly higher than that of non-immunized controls, which had complete mortality ($P < 0.001$). After two immunizations, G5 immunized fish had a mean titer of 2080 (Table 3.1). Although this titer was higher than observed in G12 immunized fish, the fish were not completely protected from challenge infection. Following challenge, these titers decreased to 1493 and 1387 for G5 and G12 challenged fish, respectively (Table 3.2).

Sera from G12 immunized fish did not immobilize G5 theronts post-challenge, and sera from G5 immunized fish did not immobilize G12 theronts (Table 3.2). These results show that clearance of the heterologous serotype following challenge does not result from development of antibodies that can immobilize the parasite through binding to heterologous i-antigens. Rather, it suggests that antibodies developed against proteins other than i-antigens are critical to protection. It also shows that challenge infection does not lead to significant antibody recognition of the heterologous i-antigens.

Fish recognize the i-antigens of their immunization strain on western blots

Western blots were used to identify *I. multifiliis* antigens potentially involved in cross-serotype protection. Sera from fish immunized with live G5 or G12 theronts that survived challenge with either G5 or G12 were used to probe total cell proteins from G5 and G12 theronts and trophonts. Pooled sera from fish immunized with G5 and challenged with either G12 or G5 recognized the G5 i-antigen in lanes containing G5 theronts or trophonts, though the i-antigen was barely detectable in trophont preparations

(Figure 3.4). Sera from G5 immunized fish did not recognize the G12 i-antigen. Pooled sera from fish immunized with G12 and challenged with G5 or G12 recognized only the G12 i-antigens, which appear as two bands in lanes containing G12 theronts or trophonts, though again the band is faint in G12 trophont preparations (Figure 3.4). G12 immunized fish did not recognize the G5 i-antigen. The i-antigen bands were visible in theront preparations, yet nearly invisible in trophont preparations because total i-antigen mRNA, and presumably i-antigen expression, increases by approximately 50-fold between the trophont and theront stages of the parasite's life cycle (5). A low molecular weight band was identified on blots reacted with G5 antiserum from fish receiving either homologous or heterologous challenge, which was of nearly comparable intensity to that of the G5 i-antigen (Figure 3.4). The identity of this protein has not been determined, but it is unlikely to play a role in the cross-serotype protection seen in this study, as serum from fish immunized with G12 did not recognize this protein.

Homogenized trophonts did not elicit protection

Immunization with homogenized trophonts did not elicit protection against challenge by either G5 or G12 theronts, although homologous immobilization titers were elevated in all groups (Figure 3.5). Fish immunized with 50 μg of homogenized G12 trophonts had a mean G12 immobilization titer of 305 after immunization (Table 3.1). Fish immunized with G12 trophonts and challenged with G12 had a median days to death (MDD) of 14 days, and showed no difference in survival when compared to non-immunized controls challenged with G12, which had a MDD of 15 days ($P = 0.157$). G5 challenged fish also had a MDD of 14 days, but this was significantly longer than non-immunized controls challenged with G5, which had an MDD of 11 days ($P < 0.001$).

Groups immunized with 50 μg of homogenized G5 trophonts had a mean G5 immobilization titer of 100 after immunization (Table 3.1). The G5 challenge group had a MDD of 15.5, which was significantly different from non-immunized controls challenged with G5, which had a MDD of 11 days ($P < 0.001$). Those challenged with G12 had a MDD of 15 days, which was identical to that of non-immunized controls challenged with G12 ($P = 0.068$). Again, protection was not observed (Table 3.2).

Correlation between immobilization titer and survival

A strong positive correlation (0.829) was seen between immobilization titer two weeks after the second vaccination and survival in groups immunized and challenged with the same serotype. This indicates that immobilization titer is very predictive of survival when immunization and challenge are performed using only one serotype. A strong positive correlation (0.705) was also observed between mean homologous immobilization titer and percent survival of all of the groups in this study, including those challenged with heterologous isolates. This suggests that homologous titer is indicative of the potential for protection against either homologous or heterologous challenge, even though the immobilization phenomenon occurs due to the presence of antibodies against one serotype-specific i-antigen epitope and heterologous challenge groups must respond against other antigens to develop protection.

DISCUSSION

Fish immunized with live G12 or G5 theronts were protected against lethal challenge infection by live theronts of either homologous or heterologous serotype. G12 immunized fish were completely protected against G12 or G5 challenge. Eighty percent

of G5 immunized fish survived homologous challenge and 65% survived heterologous challenge that was lethal to all non-immunized controls. In all cases, the survival level in these groups was significantly higher than that of controls in which all non-immunized fish died following challenge infection. Thus, these results demonstrate that cross-protection occurs between *I. multifiliis* serotypes. The protection was accompanied by elevated homologous immobilization titers in each immunized group, which were not observed in controls.

The titers seen in this study are similar to those of previous reports. Several previous studies have shown fish immunized with live theronts to be completely protected against challenge by the same isolate. One study found complete protection after immunization with 20,000 theronts once by IC injection, though titers were not tested (1). A more recent study also found complete protection after the same dose of live theronts, and found serum immobilization titers of 190 and 120 at 12 and 21 days post immunization (16). In another study, fish injected with 8000 live theronts and boosted with 10,000 live theronts five weeks later had immobilization titers of 160 (at 2 weeks), 240 (at 7 weeks), 460 (at 9 weeks), and 460 (at 11 weeks) following initial immunization (15). However, in our trials, essentially no response was detected 14 days post-primary immunization, but 14 days after the second immunization average titers of 790 were observed in G12 immunized fish and 2080 in G5 immunized fish. These titers decreased to approximately 400 in G12 immunized fish and 1400 in G5 immunized fish 26 days after challenge, though the reason for the decrease is unclear.

The higher titers seen in this study compared to other reports are likely due to differences in immunization protocols, although the precise reasons are unclear. Fish in

this study received two injections of 5000 theronts nineteen days apart. In contrast, a previous study immunized fish with 8000 – 10,000 theronts in two injections spaced five weeks apart, resulting in lower antibody titers than seen in this report (15). However, even in this study, a 2.6-fold difference in titer was observed between G5 and G12 immunized fish, although fish were from the same population and vaccinated under identical conditions with the same number of theronts. In a previous study, fish immunized once with 11,000 live theronts by surface exposure or 5 μg of i-antigen in IFA by IC injection did not begin to show significantly elevated serum antibody titers against *I. multifiliis* total membrane protein until five weeks post-immunization (13). In this study, it is possible that antibody titers were sampled at a time when they were close to their ultimate peak values following two immunizations. The decrease seen in post-challenge titers relative to their pre-challenge values supports this assertion.

Previous studies have shown that immobilizing antibody responses induced by immunization with i-antigen produce serotype-specific protection (14). Thus, these titers represent an antibody response against a single antigen that is not involved in cross-serotype protection. The positive relationship seen between immobilization titer and survival suggests that immobilization titer is indicative of the potential for protection against homologous and to a lesser extent against heterologous challenge. In this manner, it can be used as an indicator of the intensity of the overall humoral immune response. Nevertheless, fish that have high antibody titers do not necessarily show higher rates of survival than fish with lower titers. This is demonstrated in fish immunized with live G5 theronts, which were 80% protected against homologous challenge and 65% protected against heterologous challenge. These fish had the highest homologous

immobilization titer values in the study. However, they were protected to a lesser, though not significantly different level than fish immunized with G12 theronts. G12 immunized fish had lower immobilization titers but were completely protected from infection. This illustrates that, while high immobilization titer is indicative of an increased potential for survival, it is not entirely predictive of the level of survival that is seen upon challenge.

Though homologous immobilization titer correlated with protection in this situation, protection must be mediated by antibodies recognizing many antigens in addition to the i-antigens. Numerous studies have described an important role for antibodies in protection against *I. multifiliis* infection. Passive transfer of i-antigen specific monoclonal antibodies confers complete protection from challenge (12). These antibodies immobilize the parasite *in vitro* and cause host exit *in vivo* (4). In addition, numerous studies (including this report) have observed a correlation between i-antigen specific antibody titers and protection from challenge (14-16). While other mechanisms may be involved to a lesser extent in protection, the importance of antibodies in this case is logical because *I. multifiliis* is a large motile extracellular parasite. This indicates that other antigens are also likely the targets of antibody-mediated protection against *I. multifiliis*.

In these experiments, no additional antigens were identified by western blots which might play a role in cross-protection. It is most likely that the sensitivity of the western blots was not sufficient to identify other antigens, either because of low levels expression or because of the intrinsic low affinity of fish IgM antibodies. A low molecular weight band was identified in G5 theronts that were reacted with serum from

G5 immunized fish (Figure 3.4). However, this band was not evident in any G12 immunized fish. Clearly, other methods to characterize cross-protective antigens of *I. multifiliis* are needed.

Fish immunized with 50 µg of homogenized trophonts did not survive challenge infection. This study is the first to test the protective ability of killed *I. multifiliis* trophont preparations against cross-challenge. Previous studies have suggested that trophont preparations can provide some protection against homologous challenge. Catfish immunized once IC with 200 formalin-fixed trophonts and challenged 21 days later were 51% protected from lethal challenge (1). The dose of 200 trophonts was equivalent to 500 µg of protein. It is not clear whether the difference in dose or the method of vaccination accounts for the difference in protection. In another study, catfish were immunized once IC with 20 sonicated trophonts per gram of fish weight and challenged 23 days later, resulting in 90% protection (16). Approximately 300 trophonts were used in immunization. The level of protection seen in this study was higher than in other studies, though immobilization titers were similar, achieving a value of 120 at day 21. This is similar to the G5 group in this study, which had a titer value of 100 at day 33, but lower than the G12 group in this study, which had a mean titer of 305 at day 33. Although immobilization titers were similar in this experiment, no protection was observed. The reasons for the differences are not clear.

Cross-protection is an important consideration in the development of a vaccine, as a vaccine should protect against challenge by any serotype of *I. multifiliis*. That both homologous and heterologous protection can be elicited by immunization suggests that broadly protective antigens may exist and that their identification and characterization

should be useful in vaccine development efforts. Efforts are already underway to produce vaccines through overexpression of various i-antigens in *Tetrahymena thermophila* (9). However, the serotype-specific nature of i-antigen mediated protection underscores the need for serotype-independent antigens for use in vaccines.

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Table 3.1. Pre-challenge homologous immobilization titers are shown. Homologous titers are the mean inverse serum immobilization titer of eight fish selected at random from each group tested against live theronts of the immunization serotype. Lower case letters following titers indicate 95% homogenous subsets by Tukey's HSD. No fish had antibody titers to the heterologous serotype prior to challenge.

Immunization	**Homologous titer (range) after 1st immunization**	**Homologous titer (range) after 2nd immunization**
Non-immunized	0 (0)[a]	0 (0)[a]
Homog. G5 trophonts	0 (0)[a]	100 (0 – 320)[a]
Homog. G12 trophonts	10 (0 – 40)[a]	305 (40 – 640)[a,b]
Live G12 theronts	3 (0 – 20)[a]	790 (80 – 2560)[b]
Live G5 theronts	3 (0 – 20)[a]	2080 (1280 – 2560)[c]

Table 3.2. Percent survival and median days to death (DTD) of all challenge groups are shown. Survival P-values indicate the significance of that group's survival curve when compared to the homologous challenge control by Log-Rank test, and asterisks indicate groups with significantly longer survival than controls. Post-challenge immobilization titers are the mean inverse serum titer of six fish selected at random from each group tested against live theronts of both G5 and G12. Lower case letters following titers indicate 95% homogenous subsets by Tukey's HSD when compared to the other titer values in that column.

Immunization	Challenge Strain	Percent Survival	Median DTD	Survival P-value	G5 titer (range) post-challenge	G12 titer (range) post-challenge
Non-immunized	G12	0	15.0	N/A		
Non-immunized	G5	0	11.0	N/A		
Homog. G5 trophonts	G12	0	15.0	0.068		
Homog. G5 trophonts	G5	0	15.5	< 0.001*		
Homog. G12 trophonts	G12	0	14.0	0.157		
Homog. G12 trophonts	G5	0	14.0	< 0.001*		
Live G12 theronts	G5	100	N/A	< 0.001*	0 (0)[a]	373 (320 – 640)[a]
Live G12 theronts	G12	100	N/A	< 0.001*	0 (0)[a]	427 (160 – 640)[a]
Live G5 theronts	G12	65	18.0	< 0.001*	1387 (640 – 2560)[b]	0 (0)[b]
Live G5 theronts	G5	80	11.5	< 0.001*	1493 (1280 – 2560)[b]	0 (0)[b]

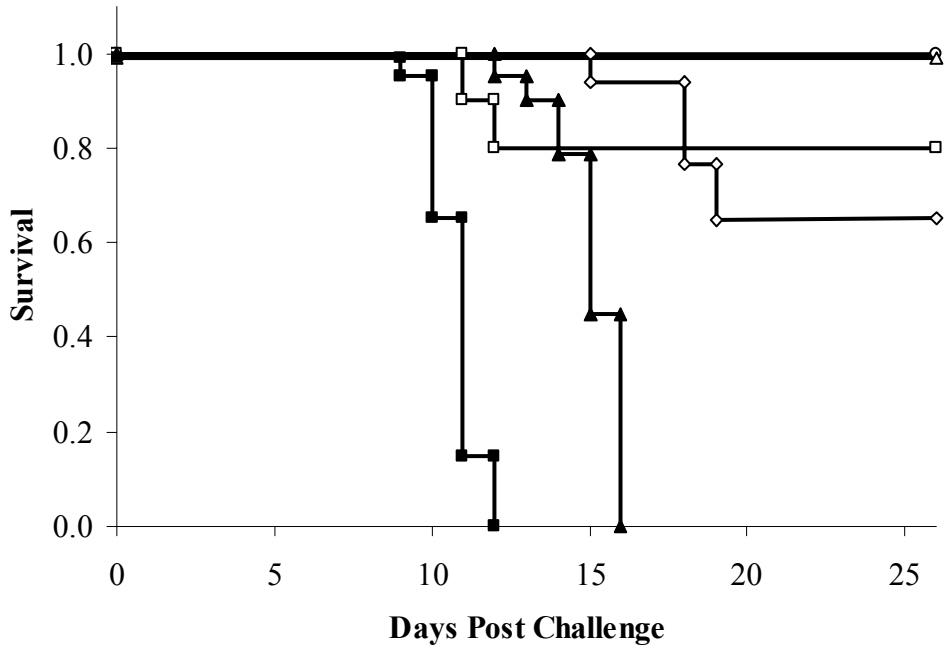

Figure 3.3. Kaplan-Meier survival curves of fish immunized with live *I. multifiliis* theronts. Fish immunized in this manner with G12 and subsequently challenged with G12 (open triangle) and G5 (open circle) as well as fish immunized with G5 and challenged with G5 (open square) and G12 (open diamond) are shown. Non-immunized controls challenged with G12 (solid triangle) and G5 (solid square) are also shown.

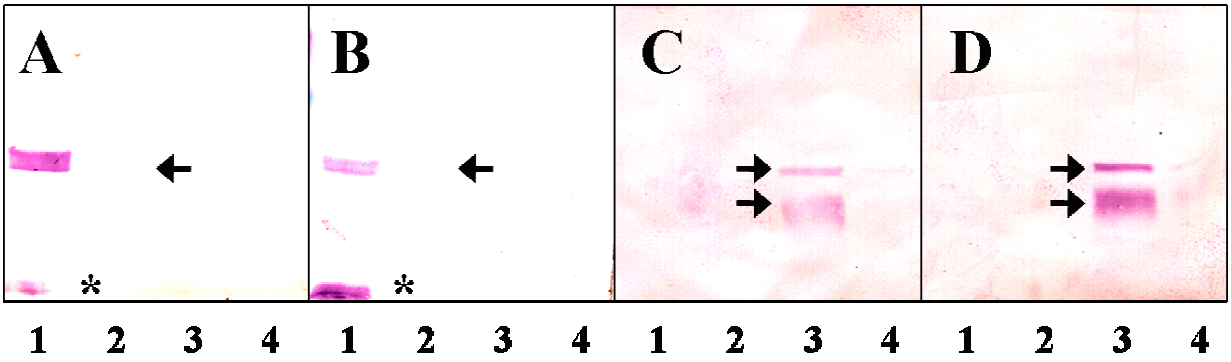

Figure 3.4. Western blots probed with antiserum from cross-protected fish are shown. Membranes were probed with pooled antiserum from G5 immunized fish challenged with G12 (A) or G5 (B) and fish immunized with G12 and challenged with G5 (C) or G12 (D). Arrows indicate the i-antigens of G5 (A and B) and G12 (C and D), and stars indicate the low molecular weight protein seen in G5 theronts. Each lane was loaded with 10 µg of non-reduced homogenized G5 theronts (lane 1), G5 trophonts (lane 2), G12 theronts (lane 3), or G12 trophonts (lane 4).

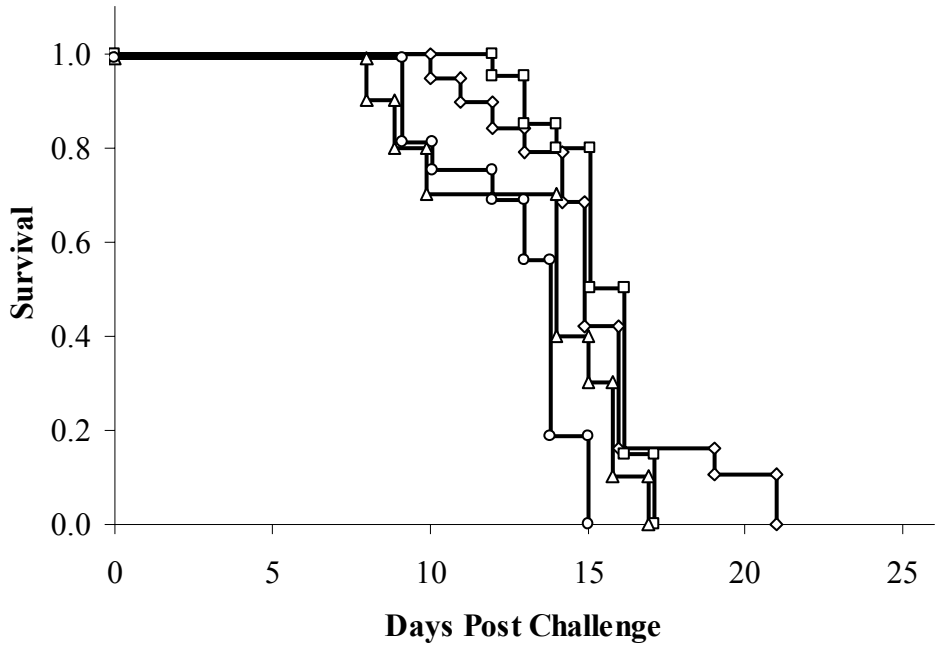

Figure 3.5. Kaplan-Meier survival curves of fish immunized with homogenized *I. multifiliis* trophonts. Fish immunized in this manner with G12 and subsequently challenged with G12 (triangle) and G5 (circle) as well as fish immunized with G5 and challenged with G5 (square) and G12 (diamond) are shown. Non-immunized controls were omitted for clarity.

CHAPTER 4

CONCLUSIONS

The ciliate *Ichthyophthirius multifiliis*, an obligate parasite of freshwater fish, infects the surface epithelia of the skin and gills. As in related ciliates, it expresses 40 – 70 kDa surface glycoproteins referred to as immobilization antigens (i-antigens). Antibody binding to these proteins and the resulting cross-linking of cilia cause immobilization of the parasite. This phenomenon has been used to characterize serotypes of *I. multifiliis*, which are immobilized by specific antiserum or monoclonal antibodies. Passive transfer of immobilizing antibodies confers complete protection against challenge with live parasites, causing forced exit of the parasite from its host. Immunization with purified i-antigen confers complete protection against *I. multifiliis* infection through this mechanism, and has thus been the subject of a vaccine development effort. However, i-antigens confer serotype-specific protection, so it has been of interest to characterize the differences that exist between serotypes as well as mechanisms of cross-serotype immunity.

Anecdotal evidence has suggested that isolates of different *I. multifiliis* serotypes infect naïve fish with different levels of virulence. To test this, fish were quantitatively infected with identical doses of two different parasite isolates, initial parasite numbers were noted, and survival was monitored. One isolate infected fish at an initially higher level and caused a higher degree of inflammation in the host, though approximately half

of fish infected with this isolate survived infection. Another isolate infected fish at much lower levels initially, but replicated faster and overwhelmed its hosts, causing complete mortality. Upon further analysis, it was found that the differences seen between these isolates were not a result of their initial infection rate, but rather of the interaction between their life cycle period length and the kinetics of the fish immune response. This study is the first report describing differences in virulence within the species.

Cross-immunity between different serotypes of *I. multifiliis* is an important consideration in the development of a vaccine, because a vaccine would ideally offer protection against challenge by many different isolates. Fish in this study were immunized with one serotype and challenged with live theronts of both the immunization strain and an isolate of heterologous serotype. These fish were all protected from challenge infection at a level that was significant over non-immunized controls. A strong positive correlation was also seen between immobilization titer and protection among the treatment groups. Further analysis by immobilization assay and western blot showed that protection against heterologous challenge was not mediated by antibodies specific to the i-antigens. While cross-serotype protection has been demonstrated to some extent in prior work, this study is the first to definitively show that protective mechanisms independent of the i-antigens exist.

The studies described here could lead to further characterization of the differences seen between *I. multifiliis* isolates. Further work could focus on the identification of virulence factors that might be responsible for the differences between isolates or the identification of other proteins that could be used in vaccines. Future studies could also focus on the fish mucosal and cutaneous immune system, the mechanics of which are not

well understood. Ultimately, the results described here will aid in the development of a more effective, broadly protective vaccine against this economically important pathogen.

APPENDIX

Fish immunized with 50 μg of homogenized *Ichthyophthirius multifiliis* trophonts of either G12 or G5 were not protected against homologous or heterologous challenge. One previous study found 51% protection against lethal challenge infection by immunizing once with 200 formalin-fixed trophonts (1). Another study found that fish immunized once with 20 sonicated trophonts per gram of fish weight were 90% protected against challenge (2). This dose was approximately equal to 314 trophonts. Two hundred homogenized trophonts was determined to be approximately equal to 500 μg of trophont homogenate by protein assay (Bio-Rad Laboratories, Hercules, CA). Thus, it was of interest to determine if cross-serotype protection could be elicited by immunization with a higher dose of trophont antigen.

Two groups of forty naïve channel catfish were immunized with 500 μg of G5 or G12 homogenized trophonts in complete Freund's adjuvant (CFA) by IC injection. Because the trophont homogenates were too dilute to be administered in one injection, two injections, each containing half of the immunization dose in 150 μL, were performed on consecutive days. The immunization procedure was repeated after two weeks using incomplete Freund's adjuvant (IFA). Sera was collected from these fish and used to obtain immobilization titers. Fish were challenged with 30,000 G5 or 5000 G12 theronts following their second injection. For detailed immobilization and challenge protocols, please the Materials and Methods section of Chapter 3.

Fish immunized with 500 µg of homogenized G12 trophonts were protected against challenge by G12 and G5 theronts, at levels that were significant over non-immunized controls ($P < 0.001$). Of fish immunized with this dose of G12 trophont antigen, 31% were protected against G12 challenge infection. In contrast, all fish challenged with the G5 strain were protected against challenge infection (Figure 5.1). The mean pre-challenge immobilization titer of fish immunized with G12 trophont antigen was 560 (Table 5.2). Following G12 challenge infection, fish had a mean G12 titer of 853, while the G5 challenged subgroup had a mean G12 titer of 200 (Table 5.3). Fish immunized with 500 µg of homogenized G5 trophonts and challenged with G12 parasites were 73% protected, which is significant over non-immunized fish challenged with G12 ($P < 0.001$). However, this immunization did not protect against G5 challenge infection, as no fish in that subgroup survived challenge infection, though their survival time was significantly longer than controls ($P < 0.001$) (Figure 5.1). The mean pre-challenge titer of fish immunized with G5 trophont antigen was 197 (Table 5.2). After challenge infection, G12 challenged fish had a G5 immobilization titer of 960 (Table 5.3).

In this report, vaccinated fish challenged with the heterologous isolate showed a higher level of survival. While cross-protection may be attainable by immunizing with trophont homogenates rather than live theronts, it would be logical to assume that fish challenged with the homologous isolate would be better protected than those challenged with the heterologous isolate, as was seen in theront immunized groups (see the Results section of Chapter 3). While these data in this study were collected carefully, it is clear that this experiment should be repeated in order to verify these results.

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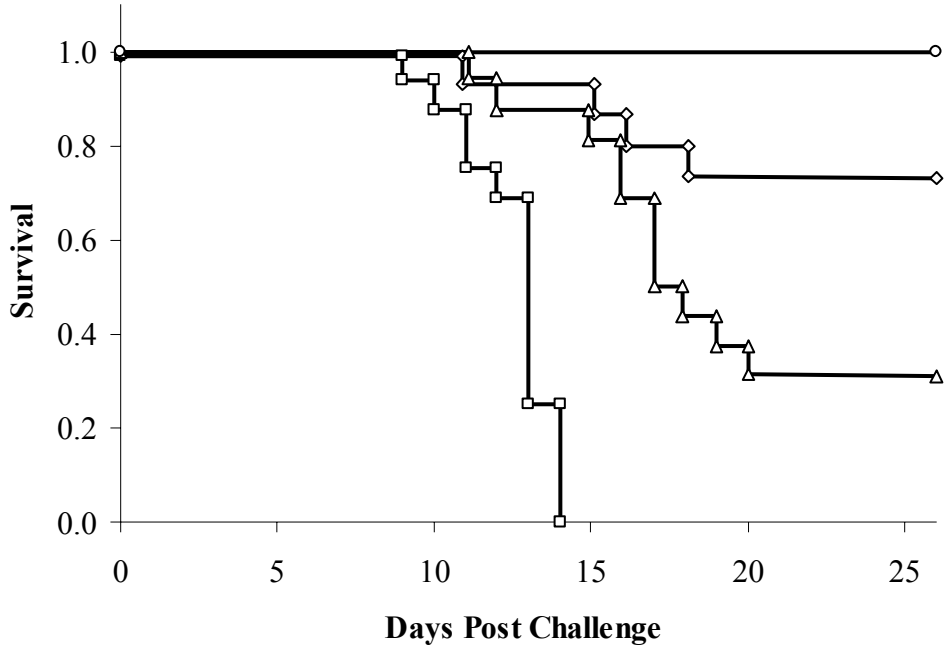

Figure 5.1. Kaplan-Meier survival curves of fish immunized twice with 500 µg of homogenized *I. multifiliis* trophonts. Fish immunized in this manner with G12 and subsequently challenged with G12 (triangle) and G5 (circle) as well as fish immunized with G5 and challenged with G5 (square) and G12 (diamond) are shown.

Table 5.2. Pre-Challenge homologous immobilization titers and survival analysis data are shown. Homologous titers are the mean inverse serum immobilization titer of four fish selected at random from each group tested against live theronts of the immunization strain. Lower case letters following titers indicate 95% homogenous subsets by Tukey's HSD when compared to other values in that column. No fish had antibody titers to the heterologous strain prior to challenge.

Immunization	**Homologous titer (range) after 1st immunization**	**Homologous titer (range) after 2nd immunization**
Non-immunized	0 (0)[a]	0 (0)[a]
Homog. G5 trophonts (500 µg)	5 (0 – 20)[a]	197 (20 – 640)[a,b]
Homog. G12 trophonts (500 µg)	23 (0 – 40)[b]	560 (160 – 1280)[a,b]

Table 5.3. Percent survival and median days to death (DTD) of all challenge groups are shown. Survival P-values indicate the significance of that group's survival curve when compared to the homologous challenge control by Log-Rank test, and asterisks indicate groups with significantly longer survival than controls. Post-challenge immobilization titers are the mean inverse serum titer of six fish selected at random (except the group marked #, which is the mean of three fish) from each group tested against live theronts of both G5 and G12. Lower case letters following titers indicate 95% homogenous subsets by Tukey's HSD when compared to the other titer values in that column.

Immunization	**Challenge Strain**	**Percent Survival**	**Median DTD**	**Survival P-value**	**G5 titer (range) post-challenge**	**G12 titer (range) post-challenge**
Non-immunized	G12	0	15.0	N/A		
Non-immunized	G5	0	11.0	N/A		
Homog. G5 trophonts (500 µg)	G5	0	13	< 0.001*		
Homog. G12 trophonts (500 µg)[#]	G12	31	17	< 0.001*	0 (0)[a]	853 (640 – 1280)[a]
Homog. G12 trophonts (500 µg)	G5	100	N/A	< 0.001*	0 (0)[a]	200 (80 – 320)[b,c]