STUDIES ON *ICHTHYOPHTHIRIUS MULTIFILIIS* AND THE IMMUNE SYSTEM OF *ICTALURUS PUNCTATUS* WITH EMPHASIS ON EARLY DETECTION OF DISEASE, CHEMOTHERAPEUTIC AGENTS AND PRODUCTION OF BIOLOGICAL REAGENTS

by

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B.S., Kansas State University, Manhattan, KS 1980

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Division of Biology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1985

Approved by:

Major Professor

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ACKNOWLEDGEMENTS

I wish to express my appreciation to professor Bill Fortner and Harold Klaassen for their guidance and assistance in the production of the following pages and the borrowing of their equipment. A special thanks to my major professor (until he retired) Dr. Merle Hansen for allowing me to work freely and for his expert editorial assistance. Recognition is due the graduate students and technicians of Drs. Rodkey, Fortner and Broce for their advice on immunological procedures and in the handling of rabbits. The illustration (Figure 1 of paper 3) is the work of my good friend Dr. Tim Van Geem whose encouragement along with the support of my wife Daylene and son Casey saw to it this manuscript was completed.

INTRODUCTION

Recently the field of immunoparasitology and the production of immunodiagnostics has expanded at an exponential rate and has come into its own as a newer science and technology. The application of the techniques of this science to fishes is new and will aid the fisheries manager, scientist and aquaculturist in better understanding the complicated biological relationships of fish to their environment.

The predominant goal of this study was to find a simple method to enumerate the tomite or infective stage of Ichthyophthirius multifiliis immunologically without having to identify the protozoan in a fixed water sample. This would eliminate the need for protozoological training and it was hoped the method could be adapted for field use. It was speculated that finding a way to detect the presence and numbers of a microorganism in a column of water without physically seeing it would have application in several scientific fields. Detecting the numbers of tomites at a given time could provide information on the dynamics of an outbreak of ichthyophthiriasis.

Before a study of a host-parasite relationship can he undertaken the parasite and the host must, on an individual basis, be thoroughly understood by observation before conclusions can be drawn about their interactions. An excellent series of papers are available on the host-parasite relationship of I. multifiliis and the mirror carp (Cyprinus carpio) (Hines and Spira, 1973; 1974) and several reviews of the literature have been written in respect to I. multifiliis (Areerat, 1974; Beckert, 1975; Beeler, 1981; Joshi, 1966; Lyman, 1982). Since the thesis by Lyman (1982) most of the recent literature pertains to the production of a xenologous vaccine for protection against I. multifiliis (Goven, Dawn and Gratzek, 1981). Some questions about the practicality of a vaccine, assuming it could be administered en-masse, need to be answered since

fish usually succumb to ichthyophthiriasis because of stress produced by an artificial environment. Another question that needs to be snswered is what causes the trophozoite to leave the host naturally when it is one millimeter in size. Since I. multifiliis is an obligate aerobe it may be due to 0_2 diffusion since the trophozoite is restricted to the non-vascularized epidermis. Factors causing trophozoites to leave the host before maturity could be an unique and effective method of control.

The humoral immune response of catfish in respect to infection by I. multifiliis hss been studied Areerat, 1974; Beckert and Allison, 1964; Beckert, 1975; Joshi, 1966; Lyman, 1982) with in vitro immobilization of tomites being the serological test in every instance. The immobilization was assumed to be antibody mediated but not proven, plus the viability of an obligate parasite deteriorates with time if deprived of a host, therefore results of the immobilization assay can be variable.

With the previous comments in mind the following research will show the life cycle of I. multifiliis with emphasis on the surface morphology of each stage as this is whst the immune system of the catfish presumably interacts with. An attempt was made to show the classical humorsl immune response to I. multifiliis to be antibody mediated. The enzyme-linked immunosorbance assay (ELISA) was used ss a tool to enumerate tomites colorimetrically and finally dinitrochlorobenzene was used to elicit a cellular immune response in the skin of the catfish as a prophylactic measure against I. multifiliis or to make the epidermis of the fish an unsuitable environment for the parasite.

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Scanning Electron Microscopic Studies of The Life Cycle of *Ichthyophthirius multifiliis*

by

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Received June 1, 1984; accepted October 23, 1984.

INTRODUCTION

The holotrichous ciliate Ichthyophthirius multifiliis Fouquet, 1876 is a worldwide obligate ectoparasite of freshwater teleosts (Psperna, 1972; Nigrelli et al., 1976). Epizootics of ichthyophthiriasis rarely occur in freely flowing water (Kozel, 1976; Allison and Kelley, 1963) hut can devastate populations of cultured and feral fishes in close confinement, with younger fish being most susceptible (Pillay, 1968). The life cycle has three stages termed tomite, trophozoite and cyst.

The small 15 x 40 μ fusion tomite, also termed theront, is the infective stage and can survive only 96 hours without a host (Suzuki, 1935). The narrowed anterior end of the tomite possesses a characteristic filamentous projection from a conical depression in the pellicle. This filament differs from the somatic infraciliature in its 3 times greater length and usually rigid orientation (Kozel and Dohra, 1978).

Cytochemical techniques have demonstrated a capsule-like structure, also located near the anterior end of the tomite, which contained acid phosphatase and non-specific esterases. The capsule and anterior filament may be part of a perforatorium as cells partially burrowed into host tissues have never been observed to possess a trailing filament (Kozel, 1980). Beckert (1975) considered the tomite to he able to drill its way into the host epithelium with its pointed anterior end and its cilia.

After burrowing into the epidermis of the teleost the tomite becomes spherical and develops its oral apparatus. It is now called a trophozoite. The parasite cilia dislodge surrounding host tissue and the trophozoite ingests the debris through its cytostome. The buccal cavity is a rigid cup-like organ located near the anterior end and is equipped with long cilia that beat continually inward (MscLennan, 1935). The trophozoite grows at a linear rate,

dependent on temperature, and leaves the host when it reaches a diameter of 100-1000 μ (MacLennan, 1942).

The freed trophozoites swim in slow spirals, compared to the tomite, and eventually adheres to surfaces of its environment. A fine gelatinous exocyst is exuded, the oral apparatus is resorbed and schizogony begins. The first 2-4 daughter cells of the schizont forms an endocyst of fine anastomosing fibrils that reinforce the exocyst. The endocyst may be formed in more than one layer in larger schizonts (MacLennan, 1937). The endocyst(s) and exocyst are not uniform in thickness and for the ultra structure of these membranes and of the schizonts, the reader is referred to Ewing et al. (1983) and Brown and Gratzek (1980). A large cyst may release up to 1000 infective tomites to complete the cycle.

The purpose of this paper is to present scanning electron micrographs of the entire life cycle of I. multifiliis and the ultra structure of several surface features.

MATERIALS AND METHODS

A culture of I. multifiliis was maintained in the laboratory by serial passage on non-immune 8 to 20 cm channel catfish (Ictalurus punctatus) kept in 80 liter aquaria. Water temperature was maintained at 22°C and aeration provided 7-8 ppm dissolved oxygen. The water pH (6-8) was controlled by adding three millequivalents/liter of $NaHCO_3$ as a buffer and adjusting with one molar NaOH or HCl.

Trophozoites were collected by rubbing infected fish by hand in 18 cm diameter syracuse dishes filled with aquaria water filtered through 0.45 micrometer filters and termed biologically conditioned water (BCW). This filtered water served as a culture medium for I. multifiliis allowing the parasite to complete its life cycle.

When a number of trophozoites had come off the catfish they were transferred by Pasteur pipet through two washes of distilled deionized water, 10 minutes in each wash. This procedure removed fish mucus and cellular debris which adhered to the trophozoites. The trophozoites were returned to BCW and from this culture medium different stages were selected for observation and fixation.

The procedure for preparation of trophozoites and schizonts is modified from Marszalek and Small (1969). Trophozoites or cysts were transferred from the BCW with a 3 ml syringe into a Nucleopore filter holder that had a 5 micrometer polycarbonate membrane in place. Distilled water was flushed through the filter with the syringe leaving enough water in the holder to cover the microorganisms. Three milliliters of 2.5 percent glutaraldehyde in distilled water were forced through the filter and the protozoans held at 4°C for 30 minutes. After fixation the trophozoites and cysts were washed twice with distilled water in the filter holder. Ethyl alcohol was used as a dehydration medium beginning with a 70 percent solution and increasing, in 10 percent increments, to 100 percent. The protozoans were kept in each solution 15 minutes and were not removed from the filter holders.

The Nucleopore filter holders containing the parasites in absolute ethanol were placed in a critical point dryer. Three 13 mm filter holders will fit in the same critical point dryer. After drying in CO_2 the filters were removed from their holders, attached to SEM stubs with double sticky tape and coated with gold-palladium alloy (Horridge and Tamm 1969). All specimens were examined in an ETEC Auto-Scan U electron microscope.

Tomites that had hatched in the syracuse dishes were prepared in the same manner as trophozoites with the following exceptions. Twenty-five millimeter filter holders and filters were used instead of 13 mm and the filters were

coated before use with poly-L-lysine, Sigma type IV (Lyon and Kramer, 1977). Ethanol dehydration was deleted and the 25 mm filters were transferred with plastic membrane forceps to liquid nitrogen cooled isopentane and then to a freeze dryer according to Maugel et al. (1980).

Trophozoites and tomites were deciliated in a 2.5 mM solution, in BCW, of the anesthetic, dibucaine hydrochloride (Thompson et al., 1974). These stages were placed in a filter holder as before and the culture medium flushed through the filter with the anesthetic solution. The trophozoites and tomites were kept in anesthetic for 10 minutes and then the anesthetic was removed by flushing with distilled deionized water. The deciliated protozoans were then prepared for SEM in the same manner as their ciliated counter parts.

RESULTS

Scanning electron micrographs of the tomite or infective stage of I. multifiliis are shown in Figures 1 and 2. The apical filament is approximately three times longer than the somatic cilia and can be seen at the narrow end of the fusiform tomite.

Figure 3 shows the cytostome of a tomite deciliated by dibucaine hydrochloride. The stubs of the cilia left on the pellicle appear to be hollow. The free swimming trophozoite contains folds and sunken areas before and after fixation (Fig. 4). Higher magnification showing the buccal cavity and cilia is presented in Figure 5. The cilia are knobbed or clubbed on the ends and have the ability to adhere to objects. Some of this adhesiveness is lost when the trophozoites are placed in distilled water.

The schizont stage has a smooth surface morphology. To observe the daughter cells, the schizonts were removed from the filters and rolled on double sticky tape until the encystment membranes were ruptured revealing the interior (Fig. 6). The first two or four daughter cells, depending on the size of the

trophozoite, secreted their own membranes or endocysts within the trophozoites' membrane or exocyst (Fig. 7). Rupture of the trophozoite exocyst without rupture of the daughter cell membranes did not effect the development of tomites. Rupture of the endocysts at the 8 cell or greater stage resulted in death of the schizonts.

Deciliation of I. multifillis by dibucaine hydrochloride revealed the surface morphology of the pellicle usually without damaging it. Figure 8 shows a trophozoite denuded of cilia and beginning to secrete the exocyat from folds in its pellicle. Cilia removed from the trophozoite can be seen protruding from holes in the filter. Pseudopodia like processes are adhering the trophozoite to the filter. Figure 9 shows the oral apparatus to be preaent in the schizonts. This was a mature cyst with over 600 schizonts present.

The apical filament and conical depression considered to be part of the perforatorium of the tomite could be viewed following partial deciliation. Figure 10 shows the conical depression at the anterior end of a partially deciliated tomite. The apical filament can be seen arising from this depression.

DISCUSSION

Wagner (1960) citing Mugard places I. multifillis in the suborder Hymenostomata and the family Ophryoglenidae. The primary criterion for this taxonomic positioning is the subterminal cytostome and the adjacent ciliary structurea. Evidence presented here shows that in tomites, deciliated by dibucaine hydrochloride, the cytostome was found to be at the midpoint of the long axis of the fusiform stage. An extensive review of the literature to that date, plus several observations on the biology of this parasite are also given by Wagner.

It has been stated (Kozel and Dobra, 1978) that development of the oral apparatus precedes development of the apical filament and that the apical filament is not observed in tomites prior to excystment. A cytostome was observed in this study in the tomites and schizonts of I. multifiliis. The mouth or buccal cavity does not become functional until the tomite has entered a host (MacLennan, 1935).

The apical filament has not been shown to he a cilium; since TEM of this structure, showing the 9 plus 2 microtubular arrangement, is not available. Its surface morphology and the fact that it is removed by dibucaine hydrochloride suggest to us that it is a cilium. Tomites that have not encountered a host and are nearing the end of their energy reserves become spherical and rotate slowly on the bottom of the culture vessel resembling small trophozoites. The apical filament is not found on these specimens. Partially deciliated tomites, 4 to 6 hours post excystment, would also display this behavior and the apical filament would be missing.

MacLennan (1937) removed the exocyst of the trophozoite and found this did not interfere with schizogony. He also observed trophozoites that did not produce an exocyst, but only an endocyst, would divide once and then die. It was proposed that those organisms unable to produce the exocyst were also unable to osmoregulate properly after leaving the host. We attempted to culture trophozoites free of debris in distilled, deionized water since trophozoites lose much of their adhesiveness in this medium. The trophozoites would divide to the 2 or 4 cell stage and then stop although still alive 12 hours later. The trophozoites may not have been able to produce the exocyst in this medium. The cyst wall is thought to be produced by the secretory mucocysts opening to the exterior of the cell (Ewing et al., 1983).

By using BCW from aquaria in which ichthyophthiriasis was present on the fish, it was assumed all the elements necessary for schizogony were present. Although some debris would be present after rubbing the fish in BCW this could be removed from the trophozoites by forcing a jet of BCW across adhered specimens to dislodge it. These trophozoites were then captured and placed in uncontaminated BCW or distilled, deionized water. Chloromycetin sodium succinate at 103 mg/L and 0.025 mg/L amphotericin B may be added to the culture medium to control bacteria and fungus if necessary (Groven et al., 1981).

Attempts to use Parducz's method (Parducz, 1967) or to lyophilize this organism in water, acetone or ethanol solvents met with little success. Surrounding debris adheres to all forms of I. multifiliis. Use of the two previous methods resulted in specimens with material stuck to them thus obscuring the parasite.

Contaminants of the I. multifiliis culture included the protozoans Dileptus spp., Colpoda spp. and Tetrahymena pyriformis. Dileptus spp. and T. pyriformis look very much like tomites of I. multifiliis at 35X, but swim in a different manner. The procedure used for specimen preparation whether critical point or freeze dried, worked well for these species also. These protozoans did not present the difficulties in preparation for SEM that I. multifiliis did.

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Figure 1. The tomite or infective stage of I. multifiliis. The apical filament is at the narrow end of the fusiform tomite. Bar = 10μ

Figure 2. Closer view of the apical filament, (arrow) note the similarities to the somatic ciliature. Bar = 1μ

Figures 3. The oral apparatus of the tomite located at the midpoint of the long axis of the tomite. Arrows indicate the cytostome or opening into the cytoplasm. Bar = 1μ

Figure 4. A free swimming trophozoite after removal from a catfish host and prior to encystment. Bar = 10μ

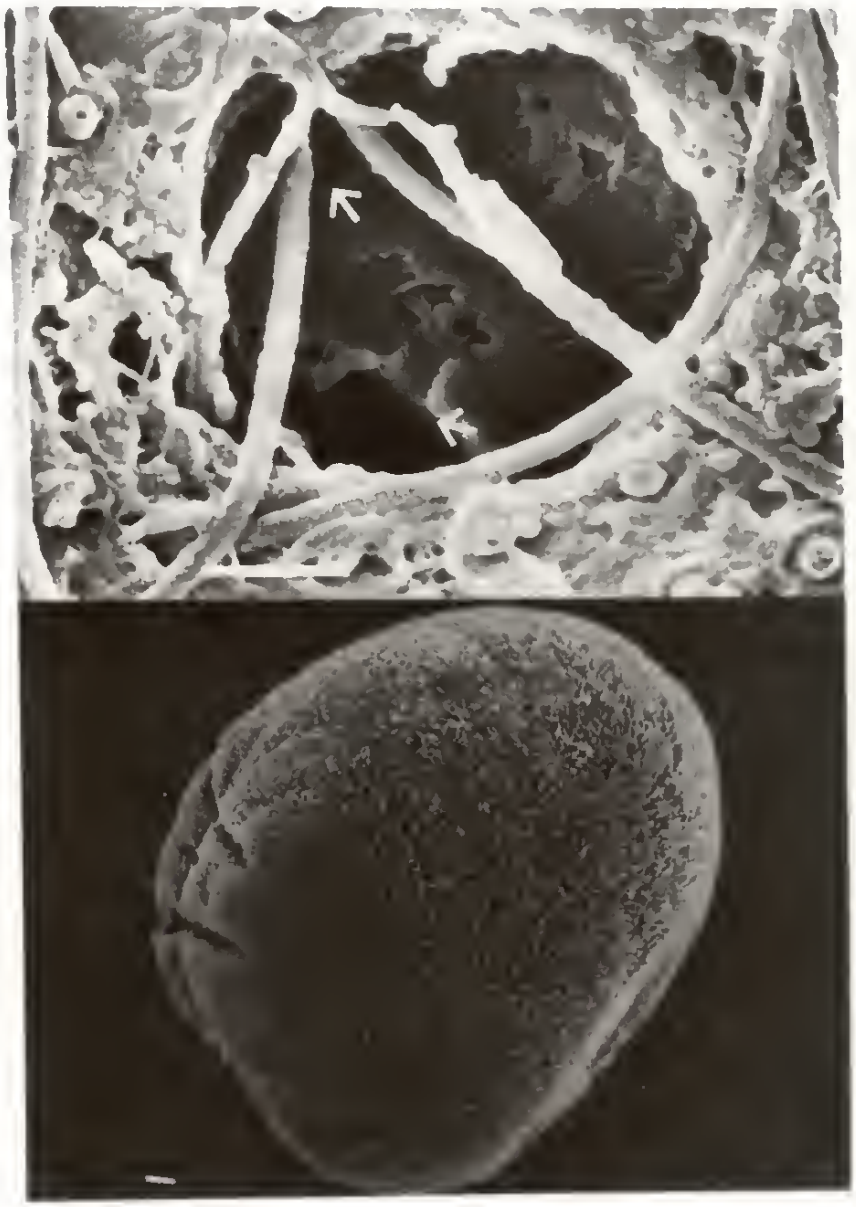


Figure 5. Buccal cavity or vestibulum of the trophozoite and associated ciliature. Bar = 10μ

Figure 6. Cyst stage of the life cycle of I. multifiliis with a section of the encystment membrane removed. Bar = 100μ

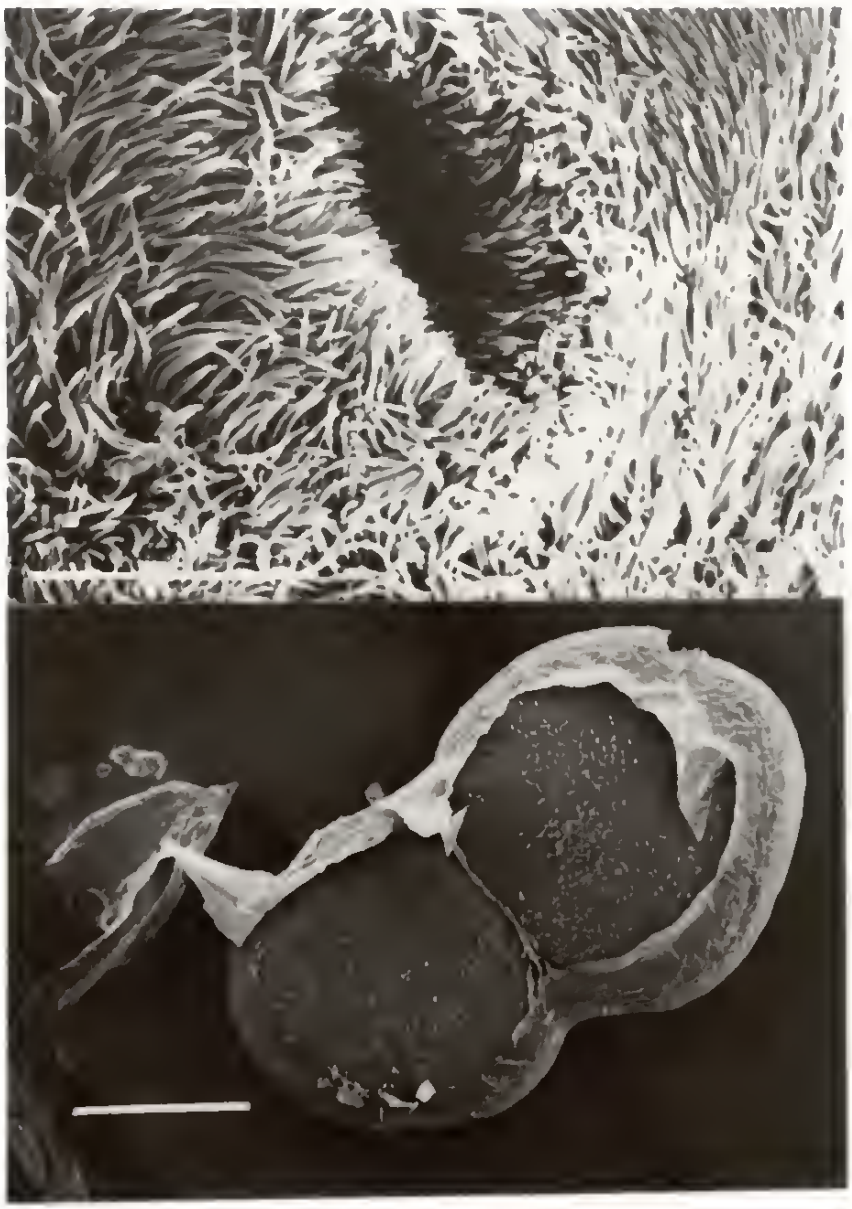


Figure 7. View of the endocyst between daughter cells showing redundancy of these membranes (arrows) in a large cyst. Bar = 10μ

Figure 8. Deciliated trophozoite beginning to produce an exocyst from folds in the pellicle. Arrow shows cilia removed from this organism protruding from the holes in the filter membrane. Bar = 10μ

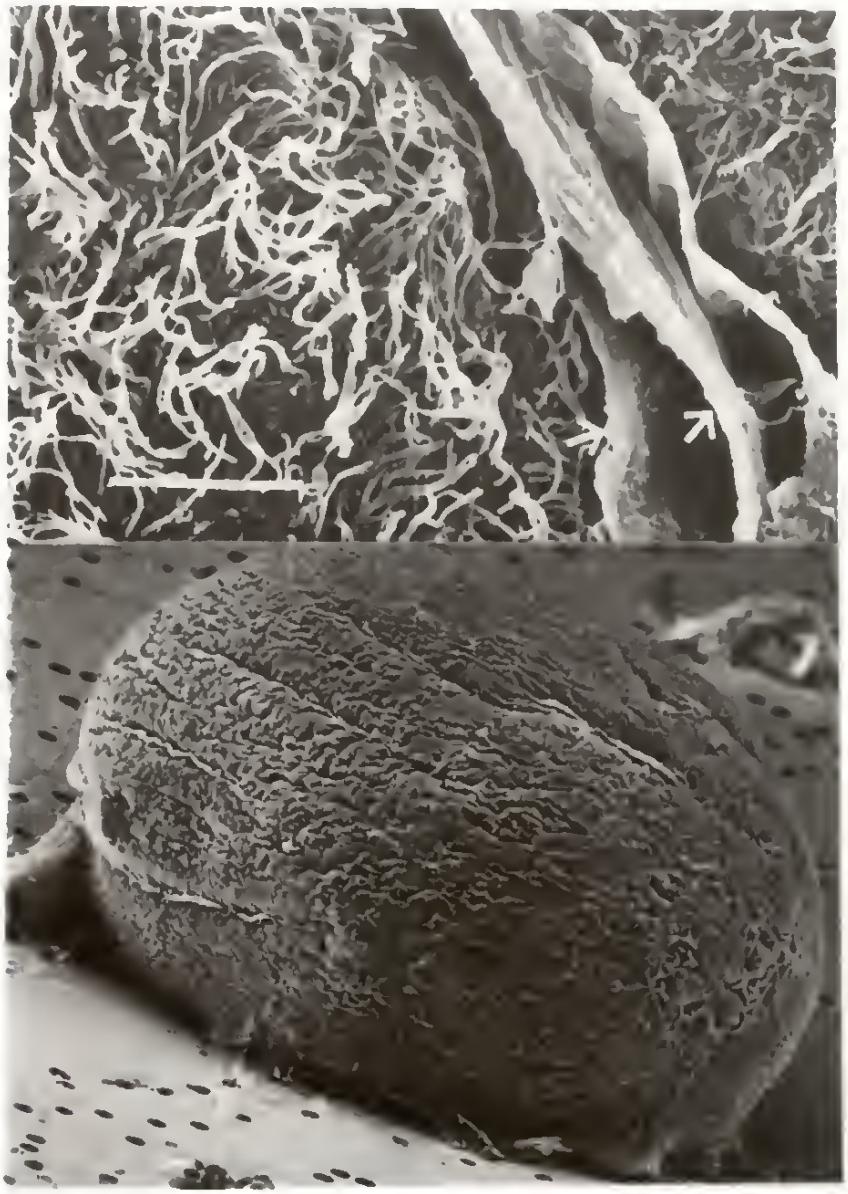
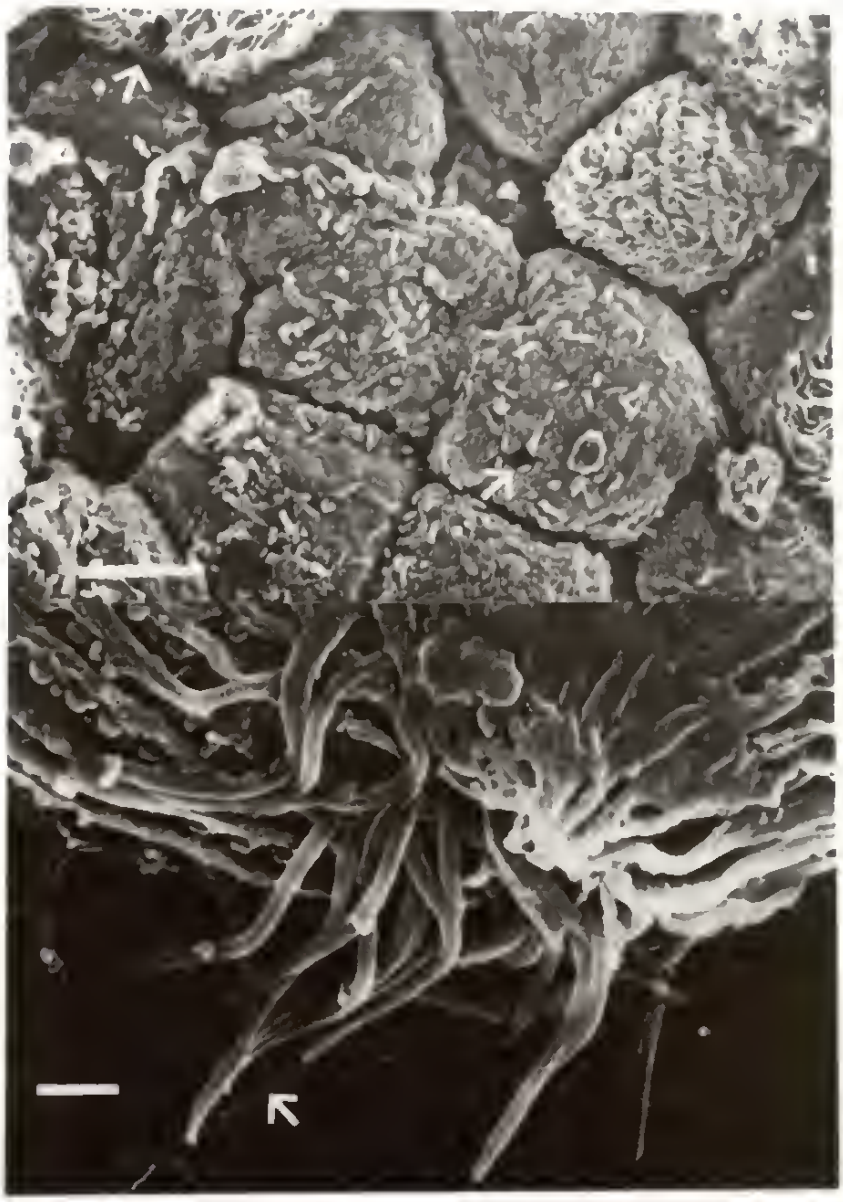


Figure 9. Cyst nearing end of schizogony showing presence of oral apparatus in schizonts. Arrows indicate oral apparatus. Bar = 10μ

Figure 10. Conical depression at apex of partially deciliated tomite with arrow indicating apical filament arising from this depression. Bar = 1μ



The Immune Response of Channel Catfish (*Ictalurus punctatus*) to Sheep Red Blood Cells, *Tetrahymena pyriformis* and *Ichthyophthirius multifiliis*

by

Jerald B. McCartney

INTRODUCTION

The humoral immune response of teleost fishes to numerous antigens has been been extensively studied and excellent reviews are available (Corbel, 1975; Cuahing, 1970; Ingram, 1980). Lewis, et al. (1979) reported the presence of B and T cell analogs in the peripheral blood of immunized channel catfish (Ictalurus punctatus). These cells were characterized as T cells possessing surface receptora for injected sheep red blood cells and forming roaettes. The B cells were found positive for bound antibody on the cell membrane, and the B lymphocytes in the peripheral circulation were considerably larger than the T lymphocytes.

A hapten-carrier effect indicating B and T lymphocyte cooperation was present in the sea robin (Prionotus evolans). Using this same hapten-carrier effect no increase in antibody affinity (Ko) has been found, (Yocum et al., 1975) but a decrease in affinity over time has been (Everhart, 1972). This is opposite to the mammalian system.

The antibodies (immunoglobulins) of fishes have been cited to react indiscriminately with cloaely related heterologous proteins. Studies with bovine serum albumin injected goldfish (Carasius auratua) found these fish produced antibody that reacted with bovine serum albumin but did not cross react with sheep serum albumin as did the rabbit controls. Using the standard quantitative precipitin test goldfish sera had three equivalence points with bovine serum albumin whereas the rabbit serum uaed had one. Other differences were the fish sera contained more low affinity antibody than the rabbit and that fish immunoglobulins migrate slower electrophoretically than rsbbit Ig's (Everhart snd Shefner, 1966).

Characterization of fish antibodies (immunoglobulins) have produced some conflicting reports, but most recent data suggest the presence of only the mu

chain analogous to IgM in bony fishes. This macroglobulin may be conjugated in several forms, from monomeric to tetrameric, causing difficulty in classifying the Ig's by sedimentation coefficients (Marchalonis, 1971; Action et al., 1971). Much of the confusion surrounding the clarification of fish antibodies is due to the heterogeneity of telost immunoglobulins both within and among species (Corbel, 1975).

The complement system is active in telosts using both the alternate and classical pathways (Ingram, 1980; Masura et al., 1981-1). Although most of the proteins of the classical pathway have not been purified C_5 has been and was found to participate in a membrane attack complex similar to the mammalian system (Masura et al., 1981-2).

Since fish are poikilothermic many questions have arisen about the temperature dependence of the humoral immune response of these animals. Many studies have shown the immune response to be entirely dependent on temperature and the antigen (immunogen) used with route of administration and species of telost being of secondary importance (Heartwell, 1975). All stages of the immune response from antigen presentation, B cell maturation, T help and supression to the actual release of antibody are probably temperature dependent biochemical processes (Rijkers et al., 1980).

Fish in their natural environment are exposed to wide fluctuations in body temperature and many antigens, some of which are pathogenic. The ability for fish, as with mammals, to mount an efficient and effective immune response is paramount to their survival.

The purpose of this study was to classify the immunoglobulins of channel catfish to sheep red blood cells and determine serological cross reactivity to I. multifiliis in T. pyriformis immunized fish.

MATERIALS AND METHODS

Immunization Studies

In this study 2-4 kg channel catfish acquired from Leisure Acres Fish Farm, designated w-cats, and from T-bar Catfish, designated t-bar cats, were held at 25°C in a 400 liter fiberglass tank. A two liter per minute flow of dechlorinated water was passed continuously through the tank to remove debris and prevent accumulation of ammonia. The fish were fed weekly a sinking pelleted feed and fathead minnows (*Pimephales promelus*) were continuously present in the tank as supplemental feed.

Eight fish were kept in the tank, three had their right pectoral fin clipped and were injected with 0.5 ml of 2% sheep red blood cells (SRBC) in 0.01 M phosphate buffered saline (PBS). SRBC used in this experiment were purchased in Alsevers solution; washed two times in 0.01 M PBS and diluted before injection. Three other fish had their left pectoral fins clipped and were injected with 1.0 ml of *Tetrahymena pyriformis* at 550 organisms per milliliter. The *T. pyriformis* culture, obtained from Carolina Biology Supply, was washed in 0.01 M PBS by centrifugation and freeze-thawed before injection. Two fish were unclipped and injected with 1.0 ml of 0.01 M PBS. All injections were given intraperitoneally (I.P.) at the base of the left pelvic fin. All fish were bled by cardiac puncture with a 21 gauge, 1 1/2 inch needle and were restrained by rolling them up in a dip net. A pre-inoculation blood sample was drawn and each fish's titer recorded to SRBC, *T. pyriformis* and *Ichthyophthirius multifiliis.* All post inoculation samples were pooled, according to their respective antigens, the serum removed, titered and then frozen. Three milliliters of blood was drawn from each fish on a weekly basis.

The hemagglutination (HA) and hemolysis titers were determined on whole, complement killed (56°C, 30 minutes), and 0.01 M 2-ME alkylated sera. The HA

and hemolysis titers were repeated one year later on frozen samples using whole serum and complement killed serum (45°C, 30 minutes). To stop the hemolysis of SRBC, they were fixed in 2.5% or .1% gluteraldehyde at 4°C for one hour in the Alsevers solution. They were then washed two times in 0.01 M PBS by centrifugation and resuspended in 0.01 M PBS as a 2 percent solution. The sera for titration was diluted in 96 well round bottom micro-titer plates using 25 µl diluting rods and 25 µl of 0.01 M PBS in each well as diluent. Fifty µl of 2 percent SRBC were then added to each well, the plate covered and read in 4 hours. Normal w-cat sera, normal t-bar cat sera and PBS only served as controls. The end point of hemolysis was determined by 100% lysis of the SRBC macroscopically. The end point of HA was determined by an undisturbed button in the well.

The titers of the T. pyriformis injected fish were determined weekly to SRBC but not to T. pyriformis. Frozen serum samples were used to determine the anti-protozoal titers at the end of the experiment. A culture of I. multifiliis was maintained in the laboratory by serial transmission on naive, and non-naive immunosupressed (25 mg/kg hydrocortisone acetate, I.P.) 20-30 cm t-bar channel catfish kept at 22°C in 80 liter aquaria. Trophozoites were collected by rubbing the fish, gently, by hand in a two liter syracuse dish filled with aquaria water. Mobile trophozoites were transferred by Pasteur pipet to distilled deionized water in plastic petri dishes. The trophozoites were then transferred by pipet to aquaria water that had been filtered through a .22 µm millipore filter in glass petri dishes. The trophozoites would then encyst and undergo schizogony. Tomites would be present in the dish by 18-24 hours at 22°C. T. pyriformis and tomites of I. multifiliis were enumerated by placing 100 µl of a thoroughly mixed culture, in triplicate, on glass slides and fixing the protozoa in Noland's solution. The fixed protozoa were tallied with a hand counter at

35 diameters and the three results averaged. To determine the titers of the immunized channel catfish to T. pyriformis and I. multifiliis flat bottom 96 well micro-titer plates were used. The sera were diluted as with the SRBC assay and 50 µl of T. pyriformis at 1080 organisms per milliliter or 50 µl of tomites of I. multifiliis at 400 organisms per milliliter were added to each well. The end point of immobilization titer was 100% cessation of motility after 2 hours of incubation at 22°C. The end point of lysis titer was determined by rupture of all the T. pyriformis organisms in the well at the end of two hours.

All fish were boosted with their original immunogen, same concentration and volume, at week 4 and week 7 of the experiment. All fish, but the controls, were immunosuppressed with 33 mg/kg hydrocortisone acetate I.P. at week 8 and again at week 10.

Chromatography of Immune Catfish Sera

SRBC immune channel catfish sera was chromatographed using Sephadex G-200 gel (Pharmacia Fine Chemicals) and a 25 cm, 50 ml column. The column was loaded with 1.5 ml of sera collected at week 4 of the experiment. Five centimeters of head pressure were maintained and 36, 2 ml fractions were collected by hand. Each fraction was stained with Bio-Rad protein dye and optical density determined at 595 nm using a Bausch and Lomb Spectronic 20. Collected fractions were assayed for hemagglutination and hemolysis of SRBC as previously described.

Sephacryl S-200 (Pharmacia Fine Chemicals) column chromatography was used to separate serum proteins from SRBC immune sera collected at week 8 of the experiment. The column was prepared according to the manufacturer's instructions and 3 ml of sera was placed on the column. The column was 80 by 2.5 cm with an elution volume of 35 ml. The flow rate with 15 ml of head pressure was 20 ml per hour and 70, 2 ml samples were collected with an automatic fraction collector. Samples were assayed for anti-SRBC activity and protein concentration as before.

A 10 cm DEAE column was used to separate I. multifiliis immune serum proteins from T-bar cats. The serum was precipitated with half saturated ammonium sulfate and dialized against 0.01 M PBS, (three changes) for 24 hours at 4°C. The proteins were lyophilized and reconstituted to 10 mg per ml of protein. Thirty mg of protein was applied to the DEAE column and 4 cm of head pressure maintained and a flow rate of 18 ml per hour. Seven 2 ml fractions were collected by hand, assayed for protein and immobilization titers to I. multifiliis. PBS at a pH of 7.2 and 0.01 M concentration was used in all the columns as a buffer.

Immunization of Rabbits

New Zealand white rabbits were used to produce Ig's to channel catfish sera components. The rabbits were immunized according to the schedule in Table 1. All injections were given as 1 ml, divided into three sites, subcutaneously. The antigens were mixed 50:50 v/v with their respective carrier. All rabbits were given a booster 21 days later and these were mixed with PBS in all cases. Blood was collected using Xylene and the marginal ear vein of the rabbit, allowed to clot at room temperature, centrifuged, then the serum removed and frozen. Pre-inoculation serum was collected from each rabbit for controls.

Detection of Rabbit Ig's to Catfish Sera

Double diffusion in gel on glass slides as described by Ouchterlony (1968) was used to detect the production of Ig's by the rabbits. Whole catfish sera was placed in the center well and 1:2 serially diluted rabbit sera was placed in the peripheral wells. Sera producing precipitin lines at a dilution of 1:8 or greater was saved for immunoelectrophoresis (IEP).

Immunoelectrophoresis (IEP) as described by Nowotny (1969) was accomplished using chilled IEP buffer (pH 8.2) and 12 ma of current for two hours. Whole catfish sera, DEAE column fraction 4 and S-200 gel fractions 11, 22 and 43 were

electrophoresed. Whole, immunized or unimmunized, rabbit sera were placed in the center troughs and allowed to incubate 24 hours at 22°C in a moistened chamber. Slides were washed, dried and stained with amido black according to Uriel (1971).

Table 1. Immunization schedule for rabbits using catfish sera components.

Rabbit	Inoculant	Day of Sera Collection	Carrier
4-1	SAS/2 ppt Whole Catfish Sera	0, 7, 14, 28	PBS
71-1	DEAE - fraction 4	0, 7, 14, 28	Freunds Inc. Adjv.
75-2	S-200 fraction 11	0, 14, 21, 28	Freunds Inc. Adjv.
74-2	S-200 fraction 22	0, 14, 28	Freunds Inc. Adjv.

RESULTS

The w-cats used in this study possessed a pre-immunization hemolysis titer of 1:8, no HA titer and immobilization titer to *T. pyriformis* and *I. multifiliis* of 1:4. T-bar cats possessed a pre-immunization hemolysis titer to SRBC of 1:64 and were not used except as sera controls in the hemolysis assay. The control and *T. pyriformis* inoculated fish developed a hemolysis titer of 1:16 on week three of the experiment. This titer remained constant throughout the rest of the experiment.

The catfish seemed to develop an anamnestic response to the SRBC with the primary response peaking at week three (Fig. 1). The secondary response was not as well indicated by the sera assayed at the time of collection, but was apparent when all the sera was assayed together. The extent of the secondary response may have been masked by the corticosteroid treatment. The hemolysis

titers of all sera dropped to 1:8 after alkylation of the Ig's by 0.1 M 2-ME. The titers also dropped to 1:8 after heating the sera at 56°C for 30 minutes. Heating the sera at 45°C for 30 min stopped hemolysis past a 1:8 dilution and allowed hemagglutination to take place. The HA titer after heating was often higher than the hemolysis titer (Fig. 1). Fixing the SRBC in 2.5% or .1% gluteraldehyde also stopped hemolysis but caused the hemagglutination titers to become erratic. HA was not performed on sera after steroid injection.

Fish immunized with T. pyriformis also showed a secondary response, but of shorter duration. The antibody cross reactivity with I. multifiliis shows an increasing titer to this organism after immunization with T. pyriformis (Fig. 2). Titers of control fish (immohilization) remained constant to T. pyriformis and I. multifiliis throughout the experiment at 1:2.

Intraperitoneal injection of hydrocortisone acetate of 33 mg/kg on week 8 caused a drop in titer no matter which assay was used. Two weeks after immunosupression the antibody titers were rising again except in the I. multifiliis cross reactivity study. Titers after the second steroid injection (week 10) could not be determined as all experimental fish died three days after this injection. The fish were necropsied but the etiological cause of desth was not determined. Numerous hemorrhages throughout the serosal surfaces of internal organs and the fins was suggestive of hemorrhagic septicemia (Aeromonas hydrophilla). The titers of control fish did not rise against I. multifiliis or T. pyriformis as they did for SRBC.

Column Chromatography

Sera from t-bar catfish immune to I. multifiliis were chromatographed on DEAE. The graph of the fractions collected from the DEAE column show a peak in absorbance at fraction 4 (Fig. 3). This fraction possessed immobilization activity from the t-bar cats to I. mulfifiliis and was used in the immunization of rabbit 71-1.

The Sephadex G-200 column resolved the sera of SRBC injected catfish from week 4 of the experiment into 2 fractions. Tubes 6-13 and tubes 27-32 had HA activity against SRBC (Fig. 4). Although the sera had hemolyzing activity before being chromatographed only HA activity was present afterwards.

The Sephacryl S-200 column gave good resolution of the week 8 SRBC injected catfish serum, with three protein peaks being eluted. Activity to SRBC was present in all three peaks with agglutinating properties in peaks 1 and 3. The hemolyzing property of immune catfish sera was present in peak 2 (Fig. 5).

DISCUSSION

In a study by Chiller et al. (1969) of rainbow trout (Salmo gairdneri) immunized with SRBC it was found that larger trout (greater than 200 grams) were able to develop rosette forming cells in the anterior kidney and the spleen. Fish smaller than 200 grams did not develop either a humoral or cellular response to SRBC given repeated injections. Phase contrast and electron microscopy revealed five morphological classes of cells able to form rosettes. These cells were lymphocytes, plasma cells, blast like cells, macrophages and cells resembling eosinophils. Those fish with rosette forming cells developed HA titers to SRBC of 1:64 or greater.

In a later study by Chiller, et al. (1969) plaque forming cells (PFC) were also found in the anterior kidney and the spleen of rainbow trout immunized with SRBC. PFC were found as early as day 3 post injection and peaked at day 14. The number of PFC had dropped considerably by day 30 of the experiment. Trout not exposed to SRBC had very few PFC but the control fish in the tank with the immunized fish developed an increase in the number of PFC's although lower in number than the immunized fish. SRBC immune trout sera was chromatographed using Sephadex G-200 and three protein peaks were eluted from the

column. Peak one had agglutinating activity against SRBC, peak two contained some antibody plus the complement components, and peak three was stated to contain natural hemolysins for SRBC.

Heartwell (1975) found the sera of channel catfish immunized with Flexibacter columnaris to be resolved into three peaks on Sephadex G-200. Antibody activity was in the first peak or excluded fraction of the column. DEAE chromatography indicated two groups of Ig's of differing charges. Ultracentrifugation showed the Ig's to be 800,000 to 900,000 molecular weight and all antibodies from the primary and secondary responae to be sensitive to to alkylation by 2-ME. IEP of immune catfish sera against F. columnaris antigen indicated that as the immune response proceeded the Ig's increased in their cathodal (gamma) migration. A second Ig arc became apparent during the anamnestic response to F. columnaris.

Marchalonis (1971) also found the sera of gold fish (Carassius auratus) and carp (Cyprinus carpio) to be fractioned into three peaks on a Sephadex G-200 column. The antibody activity to injected human IgM was found in the first peak eluted from the column.

The results of these previous studies closely agree with the results from this study. Large channel catfish were used in this experiment for several reasons. One, more mature teleosts have been shown to produce a better immune response than smaller fish (Chiller et al., 1969). Two, large volume blood samples are easily drawn with lesa risk of internal injury or development of anemia. Three, large fish are more reaiatant to disease and, with the exception of low oxygen tensions, are more difficult to kill.

The catfish were kept at 25°C as this is the temperature at which they reproduce and are most active. Since antibody formation is temperature dependent in poikilotherms 25°C was presumed to be the temperature to which channel

catfish are physiologically adapted (Tait, 19691; Rijkers et al., 1980).

Before the beginning of the experiment, fish of both sexes were in the tank but it was the breeding season for the fish and fighting began in the tank (Clemens and Sneed, 1957). Therefore, the male fish were replaced with females, and the spawning activity ceased. Besides the damage the fish were doing to each other it has been shown that fish low on the social scale in an artificial environment have a reduced or absent immune response (Barrow, 1955). The increased stress of the fighting and release of endogenous glucocorticosteroids may be forwarded as an explanation for this phenomenon.

The cross reactivity in a rabbit serological system and cross protection of channel catfish from I. multifiliis by injection of T. pyriformis antigen has been studied by Goven et al. (1981). In this study cross reactivity was shown in a catfish serological system. It was found that immobilization titers to T. pyriformis and I. multifiliis could be varied widely by changing the time of incubation with immune sera or altering the number of organisms per well. For this reason immobilization titers were done at the end of the experiment as a group. It has been stated that the cross reacting antigens between I. multifiliis and T. pyriformes are on the cilia (Goven et al., 1981). It would be interesting to speculate the reason the I. multifiliis did not lyse in the T. pyriformis immune catfish sera was that there were no Ig's to the pellicle or cell membrane of the tomites and complement could not form a membrane attack complex in close enough proximity to the pellicle to lyse the cell. Serum from catfish immune to I. multifiliis from an infection had been found to be lytic to tomites of I. multifiliis in this study. Beckert (1975) found agglutination and immobilization of tomites and trophozoites in immune catfish serum to be a repeatable phenomenon hut that lysis was not. The antibody mediated immune

response of teleosts to I. multifiliis and T. pyriformis has been studied and several references are available on this topic (Areerat, 1974; Beckert, 1975; Goven et al., 1980; Hines and Spira, 1974; Joshi, 1966). Very little information on the cellular response of fish to these protozoans is available except some on the pathology (Hines and Spira, 1974).

The immunosuppressive effects of some drugs have been tested on fish. The immune supressing effect of oxytetracycline was noted in the carp with delay or deletion of scale graft rejection. This effect was noted only if the drug was administered intra-muscularly (Rijkers et al., 1980). The immunosuppressive effect of hydrocortisone acetate has not been studied on teleosts but of its many known effects in the mammalian system, one of these is suppression of protein synthesis. Yokote (1970) found that 25 mg/kg doses of hydrocortisone acetate would cause degranulation of the beta cells in the pancreas of the carp leading to diabetes. In a similar study Robertson et al. (1963) found that rainbow trout implanted with hydrocortisone repositories developed the degeneration noted in spawning steelhead trout and salmon. The thymus underwent atrophy and the lymphocyte counts of the fish were lowered. In both studies the mucus of the fish was visibly altered and they became extremely susceptible to infection with I. multifiliis.

In this study channel catfish injected with 33 mg/kg hydrocortisone acetate entered a catabolic state even though feeding well. The fish became extremely susceptible to infection with I. multifiliis plus other pathogens as well. A second I.P. injection of 33 mg/kg hydrocortisone would sometimes cause the mucus and epithelium to fall off the fish in sheets. Decreases in humoral immunity may be a reason for the increased susceptibility to disease as maybe the suppression of the inflammatory process and general cachexia of the fish. Steroids not only affect the fish but the protozoa as well. Growth of T. pyriformis is

inhibited by several steroids of mammalian origin at concentrations of 10^{-3} to 10^{-4} M (Buetow, 1964).

From this study and supported by the study of Heartwell (1975) the following can be stated about channel catfish humoral immunity. One, they develop an anamnestic response to particulate antigen. Two, their Ig's are macroglobulins of greater than 200,000 molecular weight. Three, these Ig's have a gamma electrophoretic mobility and are of two antigenic types and charges.

The heterogeneity and complexity of the immune response of teleosts to different types of antigens makes the interpretation of results difficult in the fish humoral system. A multitude of naturally occurring protective proteins and the fact that fish Ig's will autodissociate upon storage indicates caution when reporting results (Ingram, 1980).

The fact that fish held together in a tank seem to be able to transfer immunity or antigens to each other so that unvaccinated fish can develop immunity seems to warrant further study.

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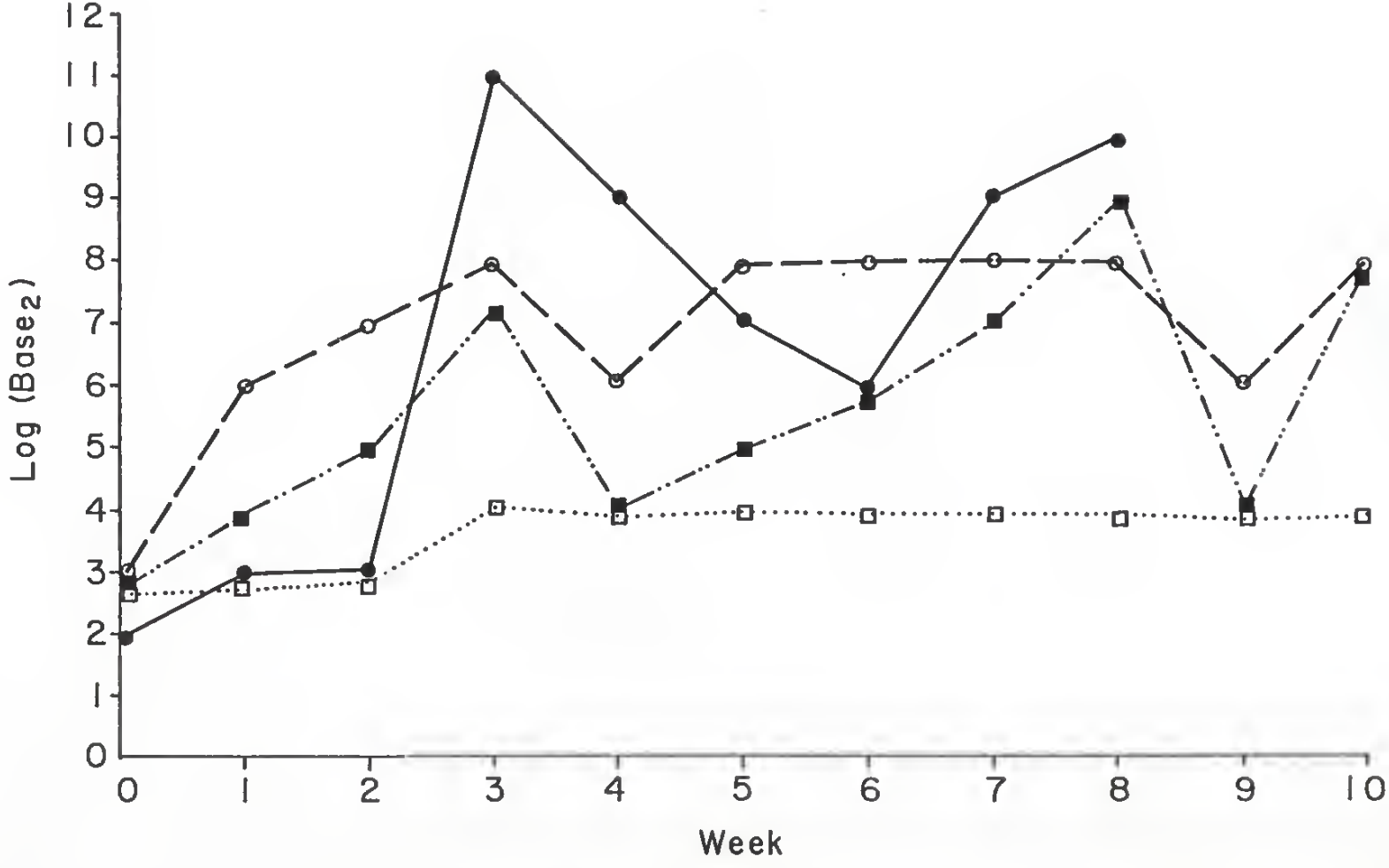
Figure 1. Weekly titers of channel catfish immunized with sheep red blood cells. Closed circles equals hemagglutination after 45°C for 30 min. Open circles are hemolysis at time of collection and closed squares are hemolysis after freezing the sera for one year. Open squares are the unimmunized control fish kept in the same tank. Immunized fish were boosted at weeks 4 and 7 and immunosuppressed at weeks 8 and 10.


Log (Base2)
12
11
10
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7
6
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4
3
2
1
0
0
1
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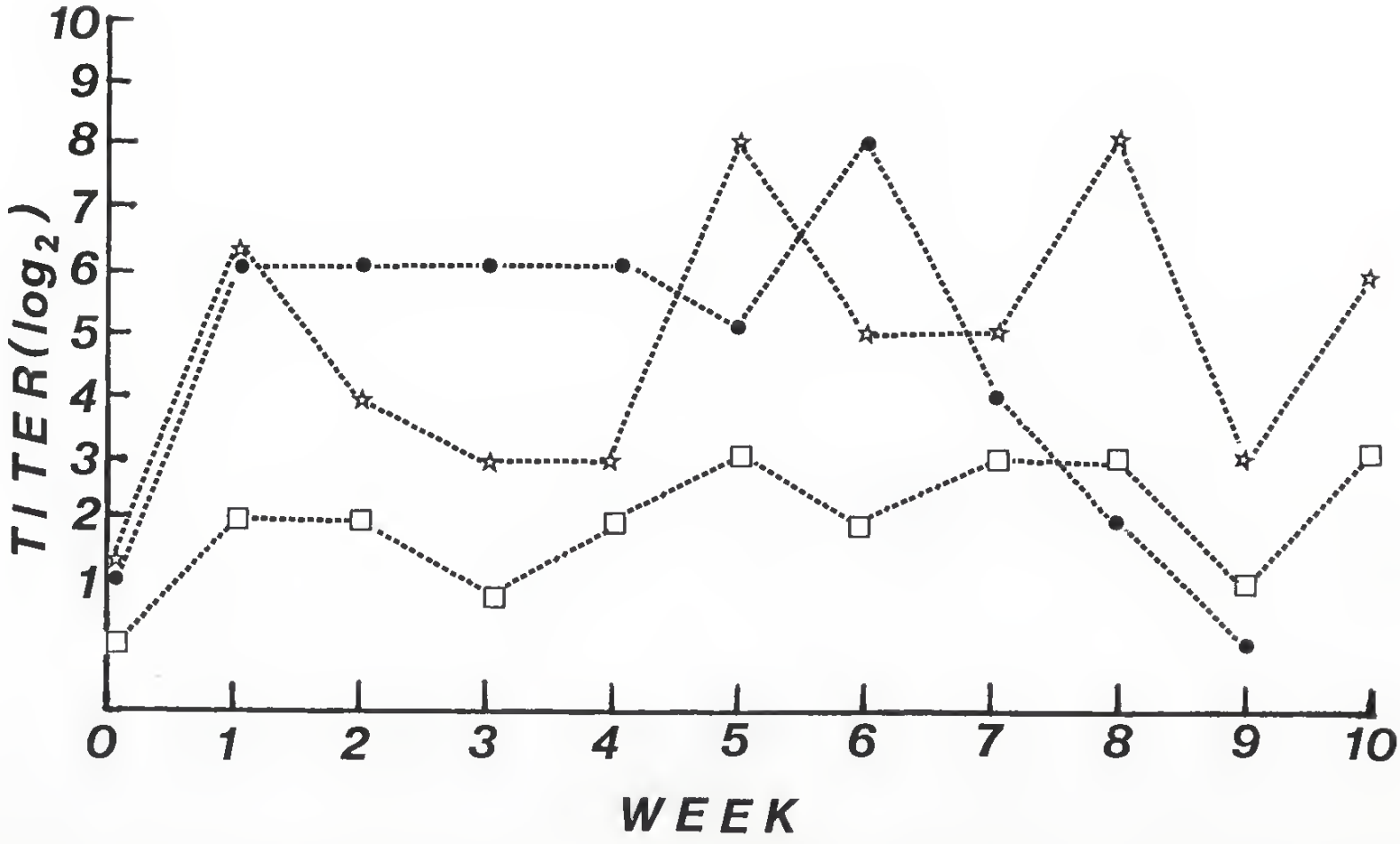
4
5
6
7
8
9
10
Week

Figure 2. Weekly immobilization and lysis titers of T. pyriformis injected fish and cross reactive immobilization of I. multifiliis. Closed circles equals immobilization of I. multifiliis. Open stars are the immobilization titers and open squares the lysis titers using T. pyriformis organisms.


TITER(log2)
10
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6
5
4
3
2
1
0
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10
WEEK

Figure 3. Fractions eluted from DEAE column of I. multifiliis immune channel catfish sera.

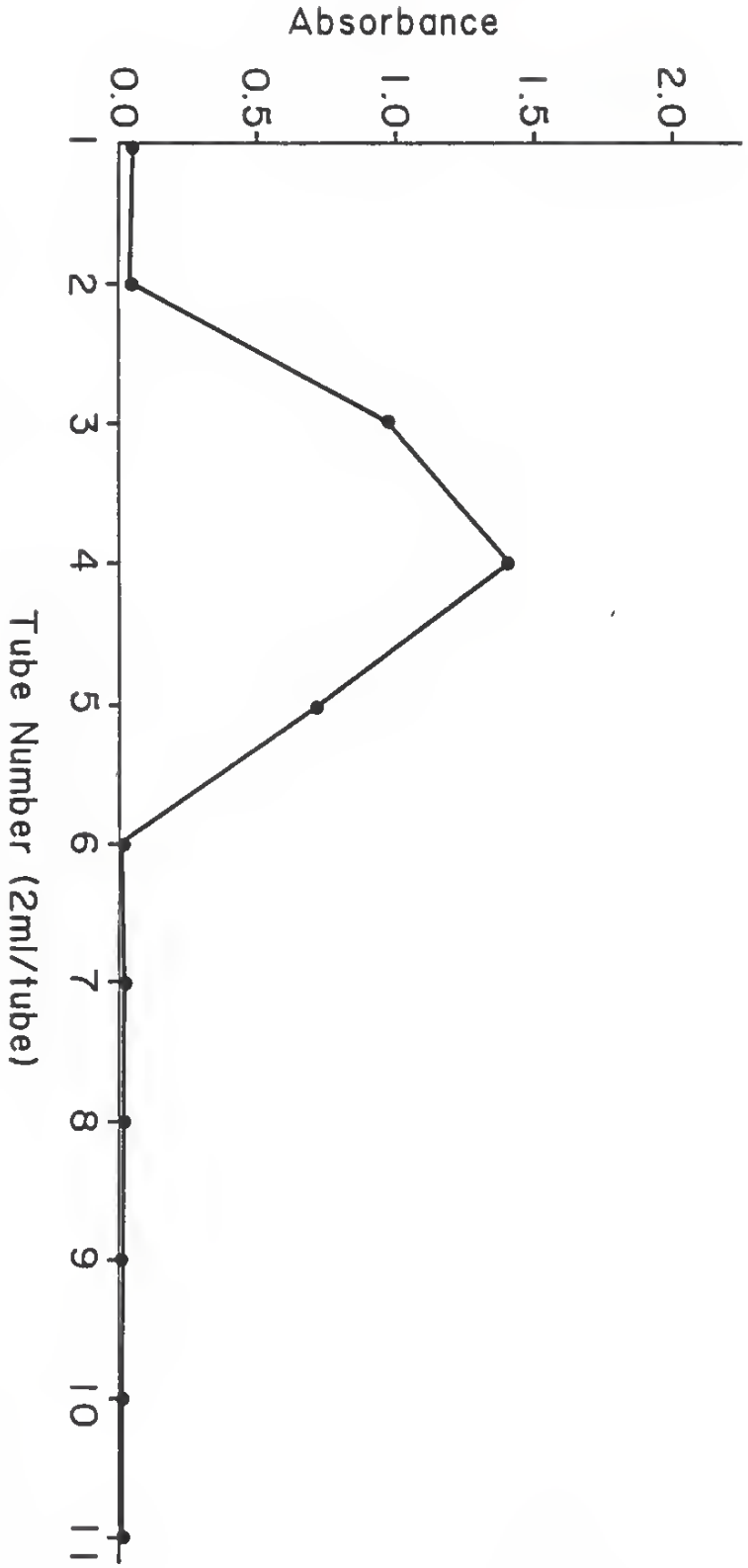

Absorbance
0.0
0.5
1.0
1.5
2.0
1
2
3
4
5
6
7
8
9
10
11
Tube Number (2ml/tube)

Figure 4. Elution curve of Sephadex G-200 column. Bars indicate part of the curve where hemagglutination was present from SRBC immunized channel catfish.

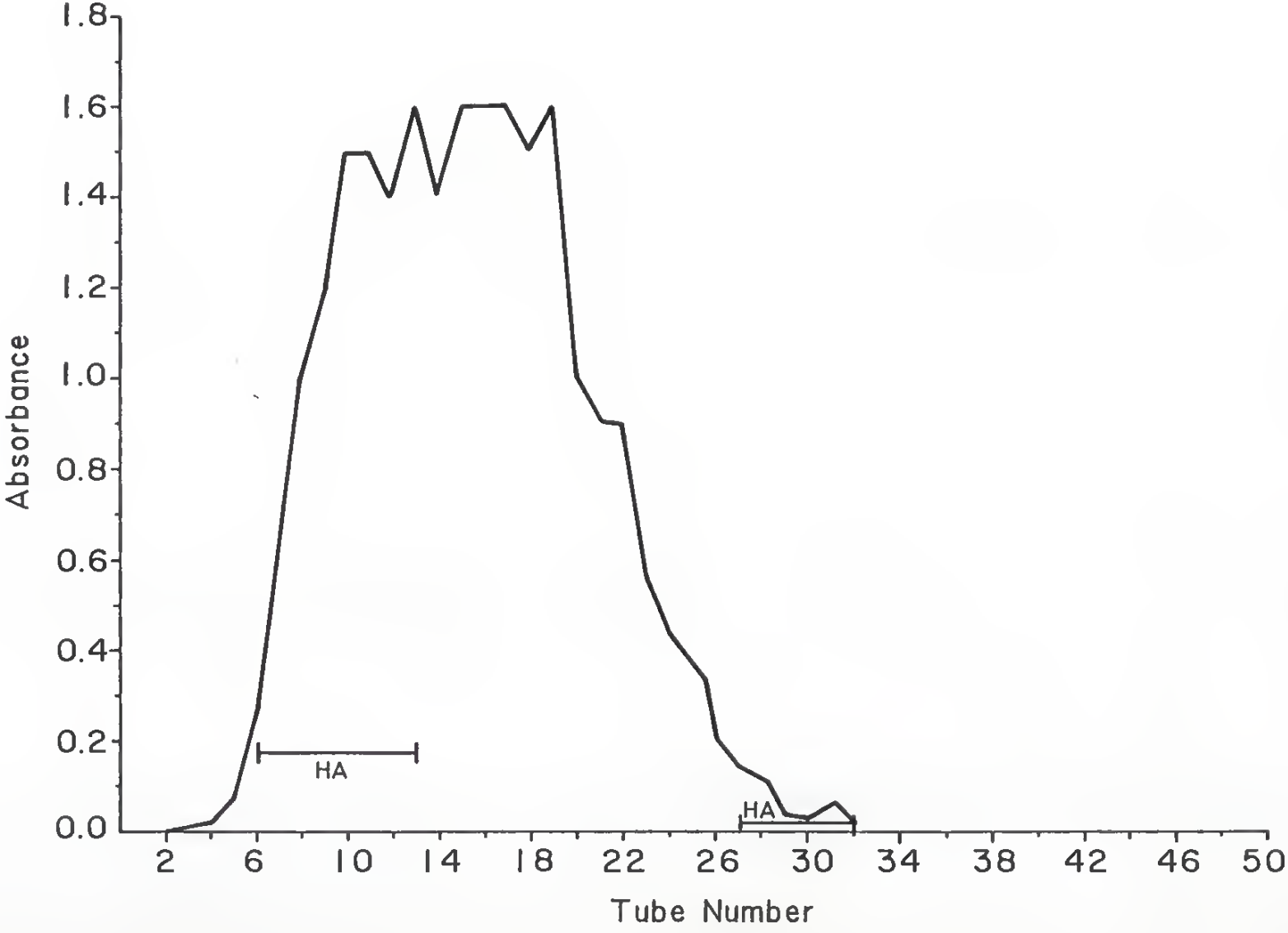

Absorbance
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
HA
HA
2
6
10
14
18
22
26
30
34
38
42
46
50
Tube Number

Figure 5. Chromatography of SRBC immune catfish sera. Bars indicate where activity was present with hemagglutinating activity (HA) present in peaks 1 and 3.

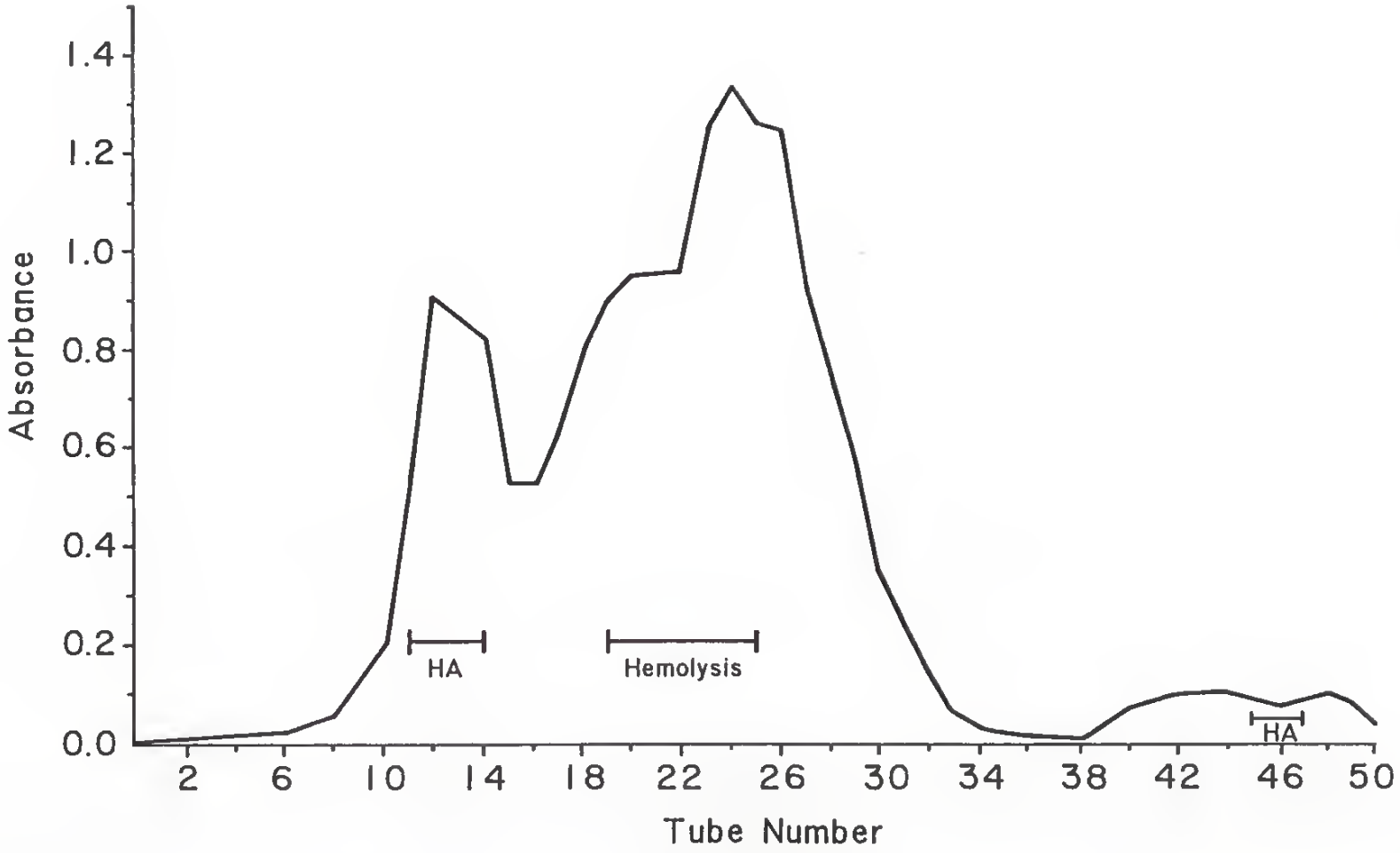

Absorbance
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
HA
Hemolysis
HA
2
6
10
14
18
22
26
30
34
38
42
46
50
Tube Number

Figure 6. Immunoelectrophoresis of channel catfish sera. Top well, fractions 11, 12 and 13 from Sephacryl S-200 column. Bottom well whole catfish sera. Trough is rabbit 71-1 sera immunized with fraction 4 of DEAE column.

Figure 7. IEP of catfish sera immunized with SRBC. Top well is fractions 21, 22, 23 from Sephacryl S-200 column. Bottom well fractions 45, 46, 47 and trough is rabbit 71-1 sera.

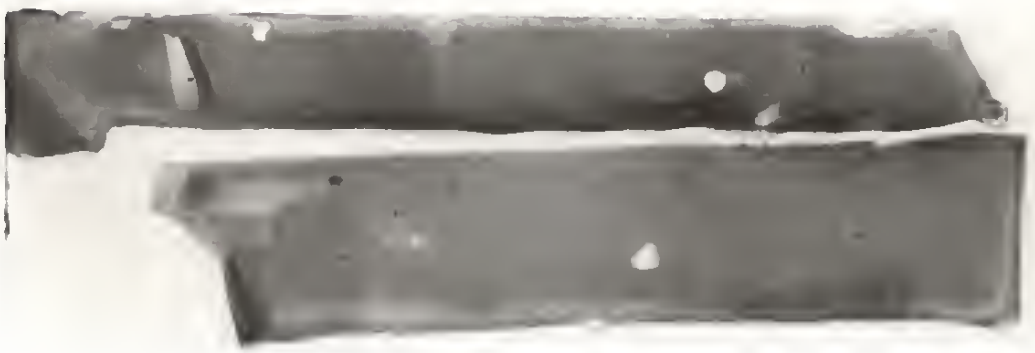
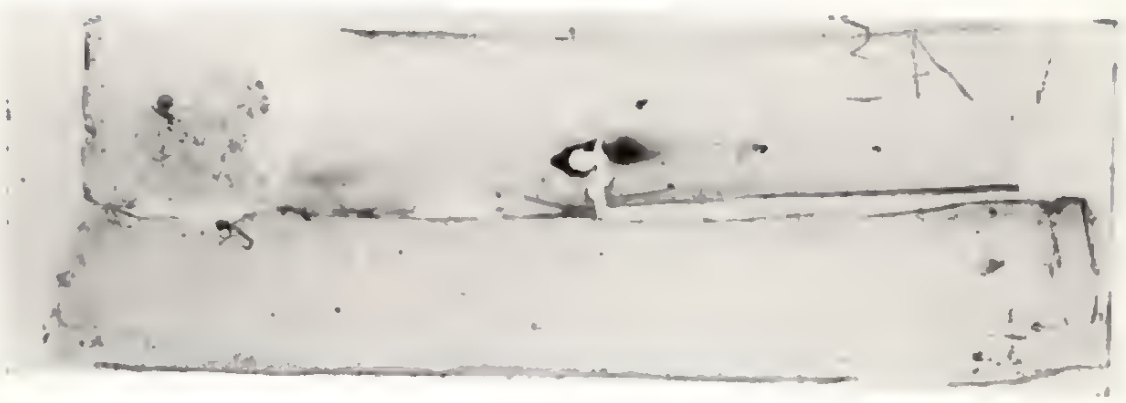
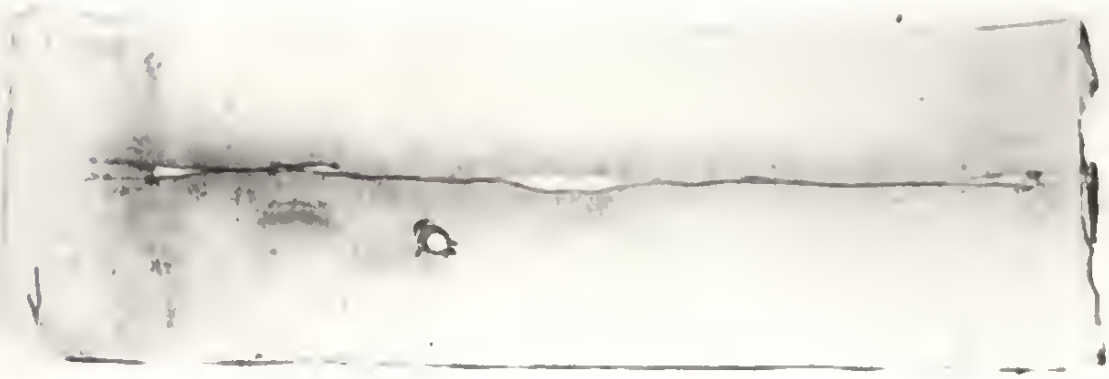


Figure 8. IEP of catfish sera. Top well is whole catfish sera, bottom well is fractions 11 and 12. Trough filled with rabbit 75-2 sera immunized with fraction 11 of Sephacryl S-200 column.

Figure 9. IEP of catfish sera. Top well is fraction 22 from Sephacryl S-200 column, bottom well is whole catfish sera. Trough filled with rabbit 74-2 sera were immunized with fraction 22.





Early Detection of Ichthyophthiriasis Using the Enzyme-Linked Immunosorbance Assay

by

Jerald B. McCartney

INTRODUCTION

The enzyme-linked immunosorbance assay or ELISA has become a powerful tool for the detection of disease and disease agents. The versatility of this assay is given by the fact that any agent that is, by definition, an immunogen can be detected by an ELISA and the sensitivity is such that nanogram quantities of proteins can be measured (Mills, 1982).

A review of the literature would be a monumental undertaking as over 80 articles were published on ELISAs of parasitic protozoans alone from 1977-1981. O'Beirne and Cooper (1979) gave an accounting of the history of the assay and a partial review with 232 references cited. The use of ELISA in respect to parasitic infections and the problems encountered in that system are treated by Ruitenber and Knapen (1977). The main difficulty with the parasite serological system is the false positives created by cross reactivity of antiparasitic Igs between and among genera of taxonomically related organisms. This problem is not restricted to the parasites, (van Dueson and Whetstone, 1981) but is being overcome by the use of monoclonal antibodies (Gamble and Graham, 1984). A major use of the ELISA, besides immunology, is in the field of epidemiology (Saunders et al., 1977). Also, many commercial ELISAs are available for use in diagnostic labs or for the clinician, such as, the ELISA used to detect occult heartworm infections (Dirofilarius immitis) in canids.

In this study an ELISA was developed to test for the presence of Ichthyophthirius multifiliis in aquaria and pond waters. The ability to determine the numbers of tomites present without the time consuming task of filtering, staining, identifying, and manually enumerating this organism would be advantageous for several reasons. The dynamics of an outbreak of Ichthyophthiriasis could be monitored and could determine the efficacy of chemotherapeutics or

vaccines after two or three generations of the parasite had cycled through the fish (Dickensen, 1983). An aquarium could be checked for the presence of an occult infection when adding naive fish to an existing population and lastly, large numbers of fish held for disease free certification could be checked for the presence of I. multifiliis without carefully inspecting each fish.

To reduce the incidence of misdiagnosis of Ichthyophthiriasis a monoclonal antibody against this parasite was produced to circumvent cross reactivity of mammalian and teleost immune sera to other ciliates present in water shown by Goven et al. (1981).

MATERIALS AND METHODS

Production Of Immune Rabbit Sera and Conjugates

Trophozoites of I. multifiliis were collected from moribund fish and transferred by Pasteur pipet through 3 washes of distilled deionized water (DDW) in glass Petri dishes. Eighty-five trophozoites from fathead minnows (Pimephales promelus) and 200 trophozoites from channel catfish (Ictalurus punctatus) were then placed in 1 and 2 ml of 0.01 M phosphate buffered saline respectively, after the DDW from the transfer had been drawn off, and frozen. The antigen was thawed and diluted 1:1 v/v with Freunds complete adjuvant and 1 ml injected into rabbits 2-1, 3-1, and 4-1 divided into three cites. Rabbit 2-1 received trophozoites from fathead minnows, rabbits 3-1 and 4-1 received trophozoites from channel catfish. Blood was collected prior to inoculation and 14 days post inoculation. The rabbits were boosted with the same antigen at day 14 and blood was collected once weekly for a month from the marginal ear vein of each rabbit. Normal sera and sera with immobilization titers greater than 1:512 against tomites of I. multifiliis were frozen. Normal and immune sera from the rabbits was precipitated with half saturated $NH_4SO_4^-$, centrifuged and the precipitate dialyzed exhaustively against 0.01 M PBS at 4°C. The concen-

trated Igs were then lyophilized and kept at 4°C. The normal and immune Igs were then conjugated to horse radish peroxidase (HRP) according to the method of Nakane and Kawaoi (1974).

The Filter Membrane ELISA

Tomites hatched from trophozoites, cultured in glass Petri dishes in biologically conditioned water (BCW),* were washed by centrifugation in 0.01 M PBS and fixed for 1 hour (4°C) in 2.5% glutaraldehyde, recentrifuged to remove the fixative and diluted with 0.01 M PBS to 400 tomites ml. This antigen was stored in sterile plastic serum vials at 4°C for use in the ELISA. Tetrahymena pyriformis was treated the same as tomites except it was reconstituted to 1780 organisms/ml.

To determine the rate of reaction 50 µl of thoroughly mixed tomite or T. pyriformis antigen was applied to a 5 µ pore size, polycarbonate filter in a 25 mm polycarbonate filter holder (Nuclepore Corp.). Fifty ml of aquaria water in which a light infection of I. multifiliis was present on the fish was filtered in an identical apparatus and 50 ml of BCW was also filtered to determine the background of the system. The filter holders were then filled (approximately 0.5 ml) with a 1:500 dilution of rabbit 2-1 immune sera and incubated for 30 minutes at 22°C. The antisera was flushed from the filter with 10 ml of PBS in a 20 ml syringe and the filter holder filled with a 1:1000 dilution of HRP-labeled goat anti-rabbit IGs (Cappel Labs) and incubated as before. The substrate for the peroxidase, 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonate) or (ABTS) was prepared according to Saunders (1979) and applied to the filter holder with a 3 ml syringe. The filter holder had a covered 25 gauge needle in place to keep the reagents from dripping from the filter holder (Fig. 1). Color reactions were read at 5, 10 and 15 minutes by aspirating the substrate

*BCW = aged aquaria water with fish and I. multifiliis present, filtered through a 0.22 µ milipore filter.

from the top hole of the holder with a Gilford spectrophotometer set at 480 nm. When 15 minutes had elapsed the substrate was forced through the filter with an air filled syringe into a plastic serum tube and read again at 20 and 25 minutes (Fig. 2).

To test the lower limits of detection, incremental volumes from a thoroughly mixed 400 organisms/ml solution, were transferred with an Eppendorf variable pipet and applied to filters. Fifty ml of aquaria water was filtered as was 50 ml of BCW to use as a negative control. Igs, enzyme and substrate was added as before and the substrate was expelled from the filter holder after 15 minutes of incubation at 22°C and read (Fig. 3).

ELISA Using the Microtiter System

Plates for the microtiter assay were prepared by coating the plates by immersion in (0.1 M) poly-l-lysine for 0.5 hour at 22°C. The plates were then washed by immersion in DDW and air dried. Tomites, trophozoites or T. pyriformis were added to the plates and allowed to interact with the lysine polymer for 1 hour and then fixed with 2.5% glutaraldehyde for 0.5 hour at 4°C. The plates were then washed by immersion in DDW and stored upside down at 4°C. Six to 10 trophozoites were placed in each well and 50 to 400 tomites or T. pyriformis organisms were used per well.

Sera was diluted in empty microtiter plates with 25 µl diluting tulips in 50 µl of PBS. A transfer plate was used to transfer the diluted sera or Igs to an antigen coated plate containing 50 µl of PBS. All seras, Igs and anti-seras were incubated 30 minutes at 22°C. Unbound antibody was removed with 7 washes of PBS using a plate washer and HRP-conjugated goat anti-mouse Igs were diluted 1:1000 before use (Fig. 4). Substrate was prepared as before with 100 µl added to each well and other color reaction stopped at 30 minutes. The stopping reagent was 0.1 M HF adjusted to pH 4 with NaOH in 0.001 M edetic acid (Saunders et al., 1978).

Igs tested by this procedure included HRP conjugated rabbit 2-1 normal and I. multifiliis immune sera, sera from I. multifiliis injected mice, T. pyriformis injected channel catfish and the media from mouse hybridomas producing Igs against I. multifiliis.

Production of Monoclonal Antibody

Three, BALB/c mice were immunized subcutaneously with sonicated trophozoites that had been washed 3 times in DDW and concentrated to 50 ml in 0.01 M PBS. The mice were injected with 0.5 ml of antigen on days 0, 30 and 60. One week after the last injection the spleens were removed from the mice for fusion of the B cells present and sera was collected at this time. The fusion and cloning was performed in the immunology laboratory by Dr. G. W. Fortner. Production of antibody was screened in the previously described microtiter ELISA using straight media from the hybridomas with positive wells being titered. Positive wells were also cross checked for specificity using an immunofluorescence assay (IFA) adapted from Markiw and Wolf (1978).

The Immunofluorescence Assay

Trophozoites and tomites of I. multifiliis and T. pyriformis were taken from culture with a Pasteur pipet and placed within circles drawn on glass microscope slides with a latex pen. Two circles approximately 2 cm in diameter were drawn on each slide, one for the test and one for the control. The organisms were air dried to the slide then placed in acetone and stored at -20°C. Test seras or culture media from the ELISA positive hybridomas were used to flood the circle containing the protozoans and incubated at 37°C for 0.5 hours in a moistened chamber. The slides were then washed in 3 different baths of 0.01 M PBS, 5 minutes in each bath. Fluorescein isothiocyanate (FITC) labeled goat Igs, (anti-mouse, heavy and light chains, Cappel Labs) were diluted 1:25 in PBS with 1% Evans blue dye added to turn the solution

from yellow to light blue. This dye will reduce non-specific fluorescence. The FITC-labeled Igs were applied to the circles and incubated and washed as before. The slides were then air dried, the circles flooded with glycerin, a cover slip applied and read with a Leitz fluorescent microscope. Normal mouse sera and HAT medium were used as negative controls. Immune mouse sera diluted 1:50 was used as the positive controls. Slides were photographed for reference.

RESULTS

The results of Figure 2 show that stopping reagent is not needed for the filter system since the substrate is removed from the enzyme. There was a cross reactivity shown between T. pyriformis and I. multifiliis although there were over 4 times as many T. pyriformis organisms applied to the filter. The aquarium filtrate with an infection of I. multifiliis present also shows a positive reaction.

Figure 3 indicates that the ELISA on the filter papers would not begin to show the presence of tomites before at least 10 were present on the filter and the curve plateaued when more than 40 organisms were present.

Figure 4 was a test of the conjugated rabbit Igs against I. multifiliis. This test indicates the conjugated Igs could be used at a dilution of 1:1000 in the microtiter system to give a reliable color reaction with 0.5 hour of incubation. The use of deciliated tomites as antigen shows most of the rabbit Igs are binding to the cilia of the plated tomites.

Figure 5 shows that Igs against I. multifiliis are being produced by the hybridomas B3F and A152. The mouse used for fusion had a titer considerably higher than normal.

Figure 6 shows that immunofluorescence is present on many parts of the trophozoite indicating the mouse produced Igs against several different antigenic sites. The fluorescence is nearly uniform.

Figure 7 and 8 are of the monoclonal A152 indicating by the halo effect the Igs are directed against the cilia (Fig. 7). Figure 8 shows this antibody is not reacting with the cilia of T. pyriformis.

Figure 9 is of the specificity of Igs produced by clone B3F. This antibody(s) seems to be reacting with the internal organelles of the trophozoite.

Figure 10 is the negative control for the mouse sera. There appears to be antibody present against nuclear material that is fluorescing. This was found to be present in the goat anti-mouse FITC labeled Igs and is possibly the chromatin binding the goat Igs or the FITC.

DISCUSSION

The cross reactivity between I. multifiliis and other ciliates present in aquaria water indicated that if rabbits were immunized with whole tomites a problem with false negatives would be encountered (Goven et al., 1981). Tests using Paramecium caudatum indicated that the rabbit sera against tomites would not react with this organism as it did with T. pyriformis.

The monoclonal A152 did not cross react with T. pyriformis in the IFA and this antibody could be used to purify antigen from sonicated or digested tomites for use in a catfish serological system (Goven et al., 1981), much in the manner used for the nematode Trichinella spiralis (Gamble and Graham, 1984). The purifed antigen could also be used to immunize mammals for the production of reagents to use in the filter ELISA. The HAT media associated with the culture of the hybridomas significantly reduced the background of the assay and could possibly increase the sensitivity of the filter assay to indicate the presence of just 1 or 2 tomites. The tomites are thought to be randomly distributed in aquaria or pond water and are non-chemotactic (Beckert, 1975). The filter assay, by taking water samples in different places, could prove or disprove

this. Many of the assays attempted were complicated by the presence of a prozone in the extremely sensitive ELISA, especially in the rabbit Ig system. The further the sera was diluted, serially 1:2, the higher the absorbance of the color reaction would become (de Savigny, 1980).

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Figure 1. Illustration of the equipment used in the membrane filter ELISA to detect *I. multifiliis*.

Figure 2. ELISA using membrane filters. I = I. multifiliis antigen, A = Aquaria filtrate, T = Tetrahymena pyriformis antigen, and B = background color reactions. Note color reaction stops after flushing substrate from filter. Antisera was from rabbit 2-1 immunized with I. multifiliis.

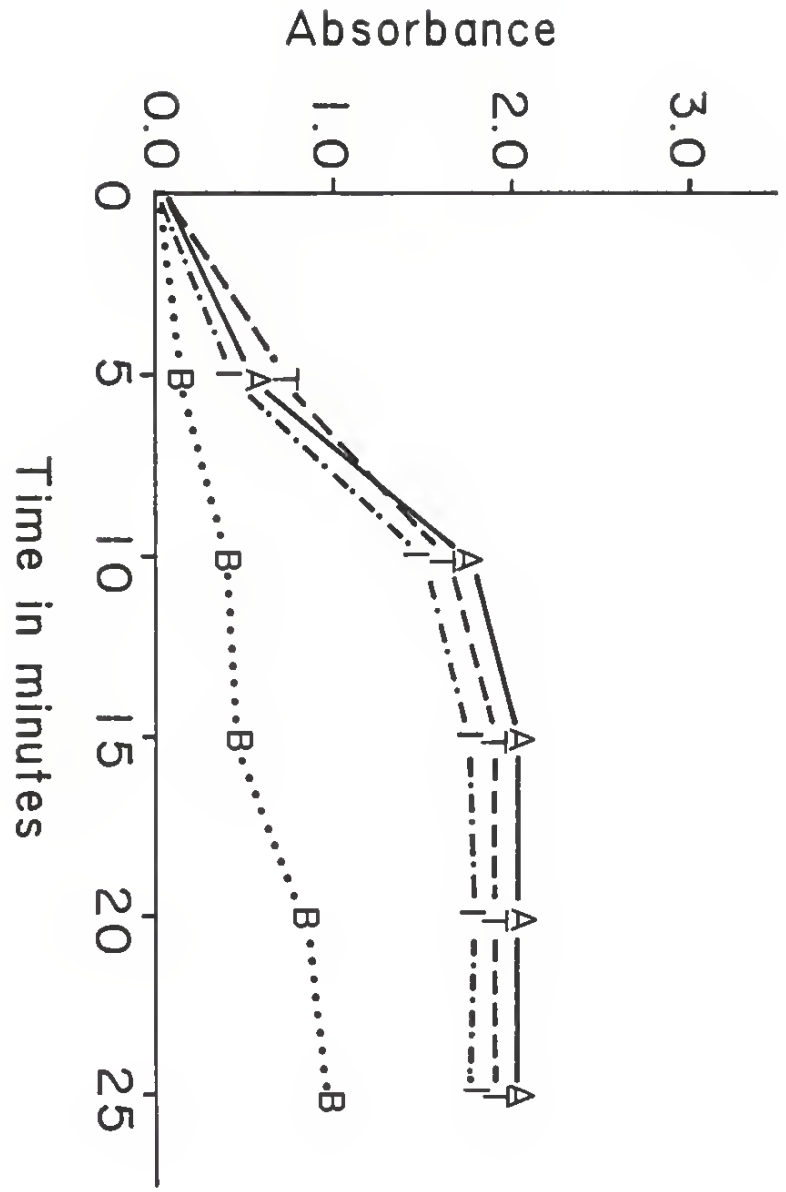

Absorbance
0.0
1.0
2.0
3.0
0
5
10
15
20
25
Time in minutes
A
T
B

Figure 3. Sensitivity of filter ELISA for detecting tomites. The color reaction does not rise above background until greater than 10 tomites were present on the filter.

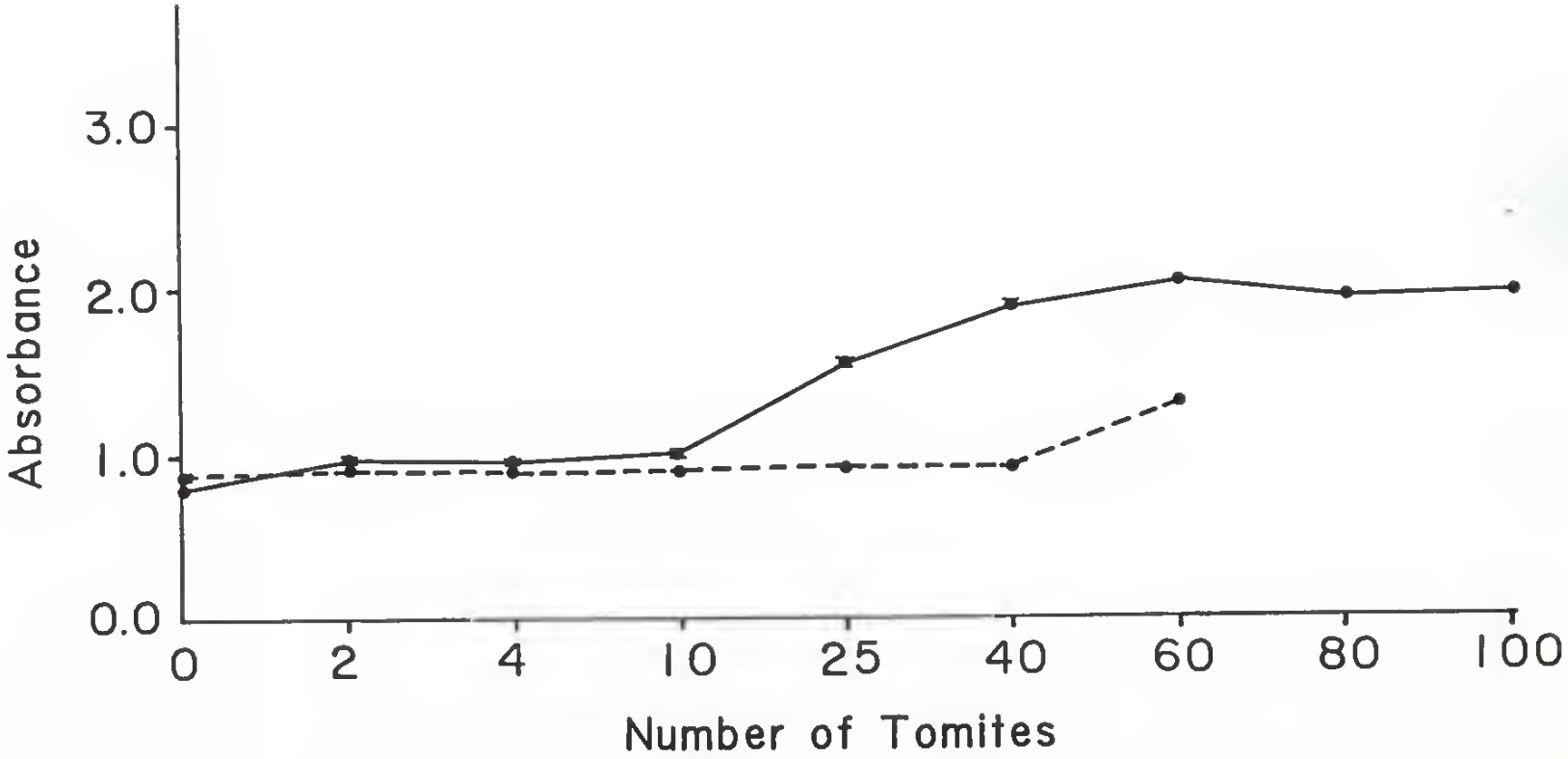

Absorbance
3.0
2.0
1.0
0.0
0
2
4
10
25
40
60
80
100
Number of Tomites

Figure 4. ELISA using HRP-conjugated rabbit anti I. multifiliis, diluted 1:100 before serial dilution in microtiter plates. Open circles indicate color reaction using whole tomite antigen, squares are deciliated tomites and triangles equals background. True dilution at 2^3 would be 1:800.

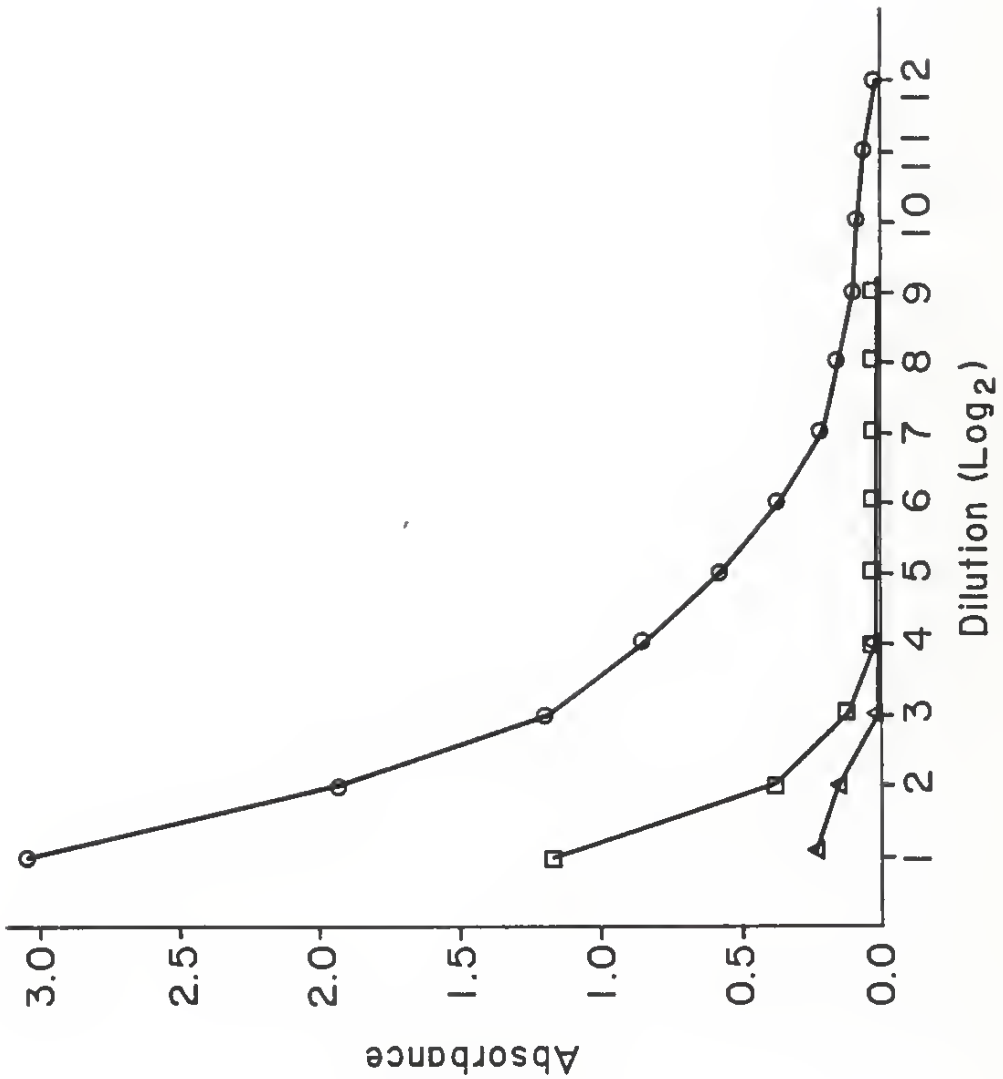

Absorbance
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12
Dilution (Log2)

Figure 5. ELISA in microtiter plates from antibody producing hybridomas and the mouse used for fusion, mouse sera was diluted 1:100 before serial dilution. *-----* = clone B3F, 0-----0 = clone A1S2, □-----□ = normal mouse sera, ✪-----✪ = background, ●-----● = immune mouse sera.

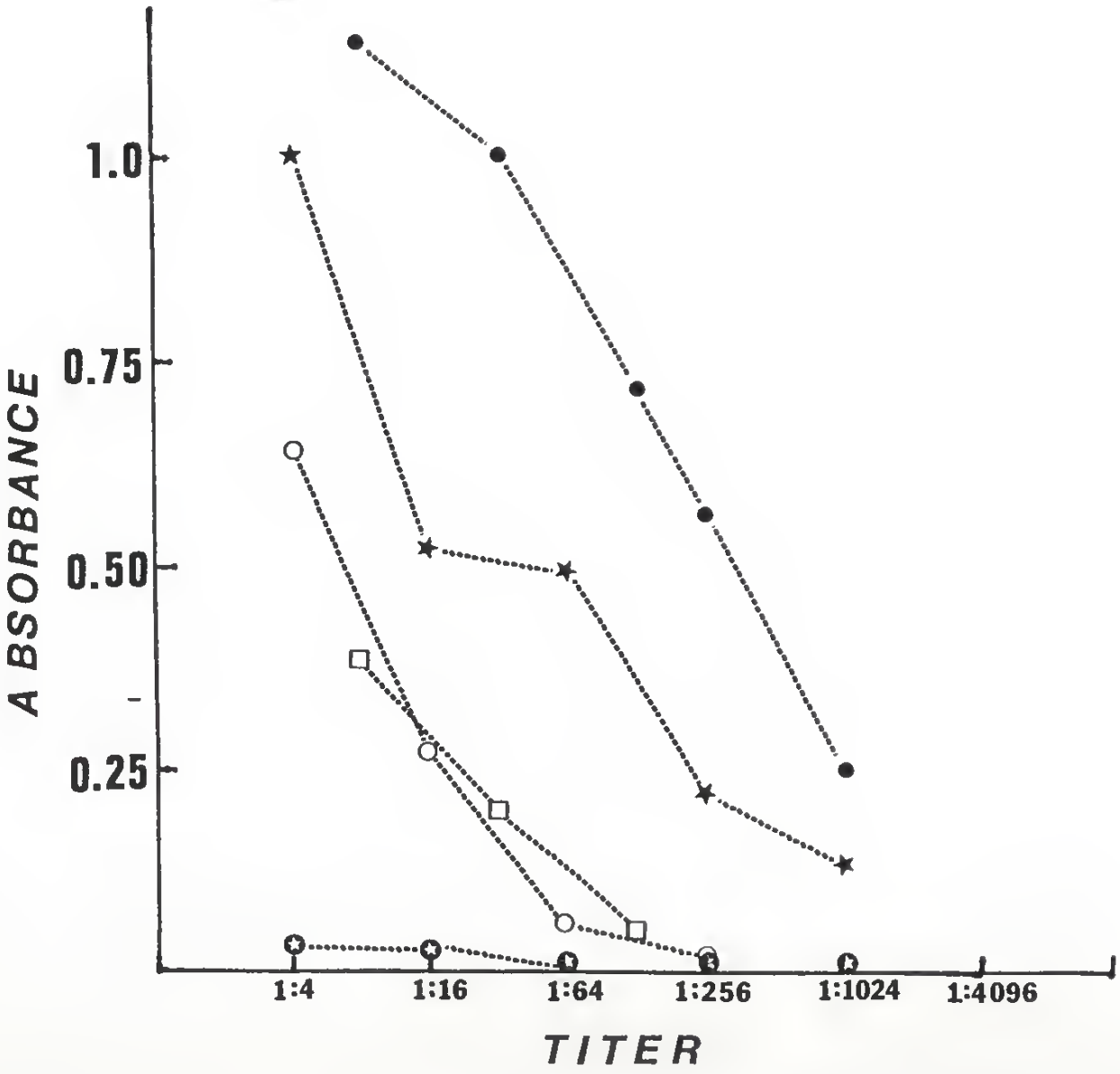

1.0
0.75
0.50
0.25
A BSORBANCE
1:4
1:16
1:64
1:256
1:1024
1:4096
TITER

Figure 6. IFA test with sera of I. multifiliis immunized mouse used in fusion experiments. The antibodies present are binding to the whole trophozoite. 100X.

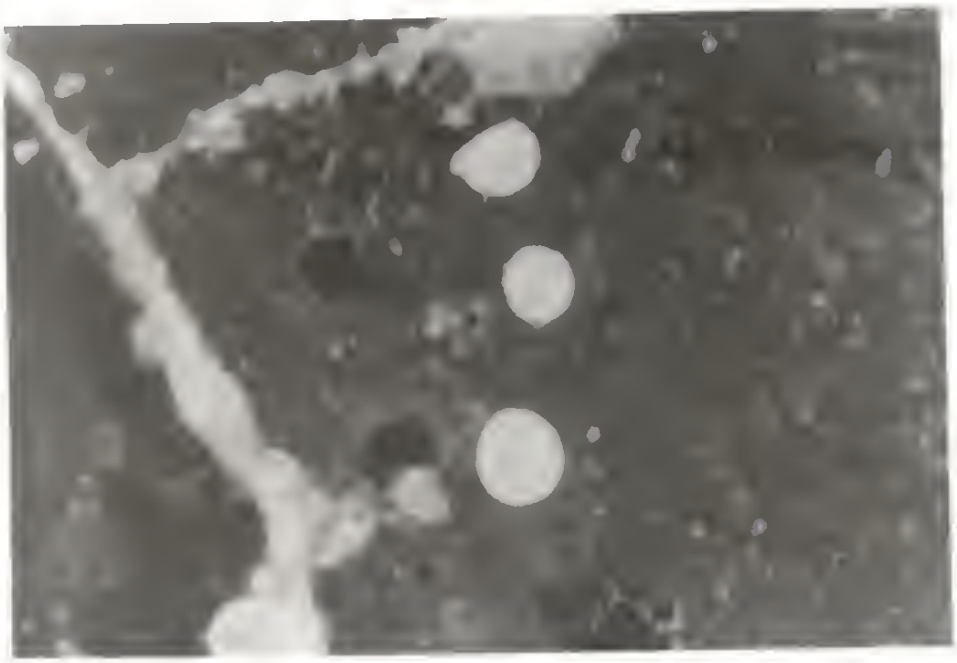
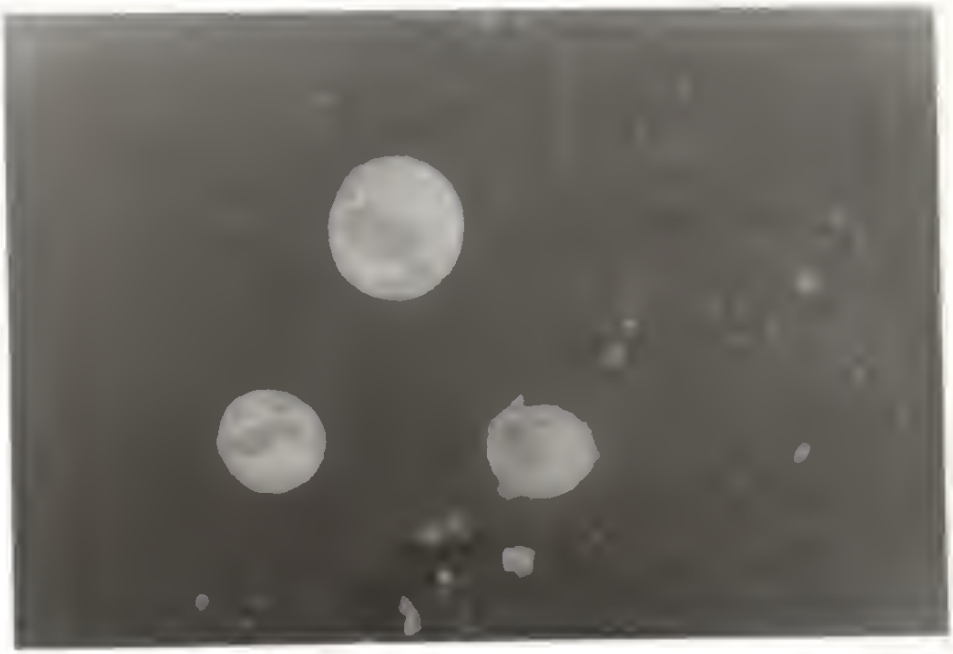


Figure 7. Immunofluorescence of schizonts of I. multifiliis produced by Igs from hybridoma A1S2. The antibody is directed at the surface of the schizont where the cilia are present.

Figure 8. Test using IFA to check for cross reactivity of hybridoma A1S2 to cilia of Tetrahymena pyriformis. Only non-specific fluorescence is present.

Figure 9. Specificity of Igs produced by hybridoma B3F. The binding appears strongest on some of the internal organelles of the trophozoite. 100 X.

Figure 10. A negative control using normal mouse sera. There is some specific fluorescence in the nucleus. The orange is non-specific fluorescence. 250X.



The Toxicity and Prophylactic Use Of Dinitrochlorobenzene Against *Ichthyophthiriasis* in Channel Catfish

by

Jerald B. McCartney

INTRODUCTION

The immune system of fishes, the teleosts in particular, have in recent years been studied extensively with most of the information available pertaining to the humoral immune system of fish (Ridgeway, et al., 1966; Corbel, 1975; Cushing, 1970; Ingram, 1980).

The channel catfish (Ictalurus punctatus), as a representative of the teleosts, has most of the cells and organs needed to have a complete, functional cellular immune system. The only organs not present when compared to mammals are the lymph nodes and these are functionally replaced in the kidneys. There is also a lack of gut associated lymphoid tissue and IgA does not appear phylogenetically until the amphibians (Good et al., 1966). The cells missing are the mast cells and basophils with the subsequent lack or unreported presence of IgE.

The thymus is present in the gill chamber between the dermis and epidermis and does not atrophy during life but remains the same size from larva to adult. Functional T lymphocytes are present after the larval stage and are able to participate in graft rejection. Much of the classical work on graft rejection was performed by Goodrich and Nichols (1933) who found that autografts of goldfish scales (Carrasius auratus) were accepted and that homografts were rejected with varying degrees of tissue destruction. Hildeman and Cooper (1963) proved the immunological basis of first and second set scale rejection. Kallman (1961), using inbred strains of fish, found the genetics of histocompatability to be the same as those in mammals. The hapten-carrier effect has been studied in fishes (Yocum et al., 1975) and dinitrophenol bound proteins are being used to contact immunize trout (Anderson, et al., 1982). The T cell helper and supressor functions have also been studied in carp

(Wishkovsky and Avtalion, 1982). Delayed hypersensitivity reactions have been demonstrated in fiahes phylogenetically from the elasmobranchs to the teleoats with the hag fish (Agnatha) being the lowest fish lacking this sequence of immune responaes. Finstad and Good (1966) prepared a list of criteria defining adaptive immunity and the lamprey was the lowest fish to fulfill all the criteria including delayed hypersensitivity. Dreyer and King (1948) gave immunizing intraperitoneal injections of horse serum to aeveral species of teleosts held at 18°C. Ten seconds after injection with a large second dose anaphylaxis was indicated by fanning of the dorsal fin, increased excursions of the operculum then ainking to the bottom of the aquaria with the dorsal fin contracted againat the body. The reaction was only specific for horse serum and could be diminished with epinephrine. ln mammals immediate hypersensitivity is mediated by IgE primarily, but the mediator in fish has not been defined.

The purpose of this study was to produce a period of prophylaxis against the parasite Ichthyophthirius multifiliis by using the contact irritant 1-chloro 2,4-dinitrobenzene (DNCB) to make the skin of the channel catfish an unsuitable habitat for thia protozoan. No information on DNCB with reapect to fish is available in the literature and the only known use of this compound in animals was as a stimulator of T lymphocytea when applied topically and used to diagnose combined immuno-deficiency in horses (Hodgkins et al., 1978).

MATERIALS AND METHODS

Five, 80 liter aquaria were filled with dechlorinated tap water and left without fish in them for 24 hours. Aeration with a bubble-up filter provided 7 ppm dissolved oxygen and no activated charcoal was used in the filter, only glass wool. Ten, 15 cm channel catfish were placed in each aquarium with 1.0,

0.5, 0.25, 0.01 and 0.0 ppm DNCB added from a 10 g/l, in 95% ethanol stock solution. Ethanol (95%) was added to the control aquarium to equal the ethanol concentration in the 1 ppm test aquaria (8 ml) equivalent to a 0.01% ethanol solution. Beckert (1975) reported a 0.05% ethanol solution not to be detrimental to channel catfish or I. multifiliis. Fish were observed every 24 hours for 10 days and the percent surviving recorded.

To test the toxicity, DNCB was directly applied on the fish at 10 g/l, DNCB in 95% ehtanol, solution was applied by spraying 20 cm channel catfish with a filled syringe in a dip net and by painting the DNCB solution on the fish with a brush. Thirty six, 20 cm catfish were divided into 3 equal groups with one group painted and one group sprayed. The third group was painted with 95% ethanol only for a control. The fish were held in 150 l stainless steel tanks at 18°C for 25 days. A small stream of dechlorinated water maintained the temperature and removed debris from daily feeding of sinking catfish food.

To determine the histopathology of applying DNCB directly to the skin 30, 10 cm channel catfish were divided into two groups. These fish were placed in 80 liter aquaria filled with ground water and bubble-up filters filled with activated charcoal provided aeration. The fish were acclimated to the aquaria for 3 days and when no signs of disease were apparent one group was removed in a net and painted with the 10 g/l solution of DNCB on the dorsal surface. The other group was painted with 95% ethanol on the dorsal surface and both groups returned to their respective aquaria. The fish were fed daily with Tetra-min tropical fish food. One fish was removed every other day for 16 days, pithed, and a 0.5 cm section removed for histopathology. The section was made transversely at the level of the anus and placed in 10% buffered neutral formalin.

After collection and fixation the vertebral column was trimmed from each specimen and replaced in the fixative. The specimens were then imbedded in parafin, sectioned with a microtone and stained with hematoxylin and eosin. Untreated fish from the same lot were also sectioned and stained for use as a normal reference. The histopathology slides were photographed and the prints arranged in sequence for comparison. The cells were compared to normals found in Grizzel and Rogers (1976) and the same terminology used. All the photographs were taken at 250 power within 3 fields of the lateral line. After 14 days the 8 remaining fish in the aquaria were exposed to a moribund fish with ichthyophthiriasis and the sequence of infection recorded.

To test the prophylactic effect of DNCB against ichthyophthiriasis 125, 10 cm channel catfish were divided into 5 groups and sprayed, in a dip net, with either 10 g/l DNCB in 95% ethanol, 95% ethanol alone or the above solutions diluted 50:50 v/v with aquaria water before application. One group of 25 fish were sprayed with aquaria water only as a control. Activated charcoal impregnated with a resin to absorb ammonia was used in the bubble-up filters and fish were fed tropical fish food daily as before and observed for mortalities. On day 4 of the experiment an ichthyophthiriasis moribund 15 cm channel catfish was placed in each aquaria and left there until it died. The aquaria were observed daily for mortalities and the dead fish removed. The percent surviving was recorded until all fish had expired. Water temperature was not controlled and varied from 21°C to 26°C through the course of the experiment.

RESULTS

The use of DNCB in the laboratory gave the empirical impression that it would stop the infection of I. multifiliis on channel catfish and this was indicated several times when an infection would begin to manifest itself in an aquarium where it wasn't wanted, especially in fish being kept for serology. These fish were removed and sprayed with DNCB in ethanol and returned to the same aquaria and the infection would usually abate. These fish were mostly 25 cm or more in length. The sloughing of the mucus was also noted in these fish and one could easily tell if a fish had been missed by the spray.

In preliminary bioassays some catfish were kept in DNCB in the 0.1 to 0.05 ppm range for months and some unusual chronic pathologies manifested themselves. Most obvious was the fish would develop a light yellow color analogous to fish in very turbid water. The most notable were the clubbing of the gill lamellae, visible in wet mounts, and rejection of intramuscular parasites never before observed to happen. Many of the channel catfish, especially wild strains, were infected with Clinostomun marginata a trematode infesting herons. These metacercaria are motile when removed from the fish and are commonly termed yellow bass grubs. After 20 to 30 days in DNCB many of these muscular stages would either migrate out of the catfish or be rejected. This would leave a deep lesion on the side of the fish and sometimes the peritoneum would be exposed leading to the demise of the fish. C. marginata has not been reported to leave a host and must have been rejected due to the DNCB treatment and was not observed in fish untreated with DNCB. Due to these chronic pathologies it is not recommended to leave fish in DNCB contaminated waters for long periods of time.

Fish painted or sprayed with DNCB in 95% ethanol or 95% ethanol alone sloughed their mucus coat and some epithelium. The trophozoites present on

on the fish would also be sloughed and the tissue shreds on the bottom of the aquaria would contain trophozoites. These were observed microscopically and due to the lack of cytoplasmic streaming were assumed to be dead. Since no mortalities were observed with 95% ethanol application even in very small fish this could be used in conjunction with a treatment that destroys the tomite stage of the parasite only by placing the fish, after dipping, into uninfected water. Flammability of the solvent and cost could be a drawback to this protocol.

Figure 1 shows the 96 hour LD50 of DNCB to be 0.3 ppm. Fish kept in lower concentrations for long periods of time developed pathologies previously discussed.

Figures 2 thru 9 show the effects on the skin of DNCB. Initially there is a sloughing of mucus and some cells and this is visible grossly when the fish are placed back in the aquaria after being sprayed or painted. The mucus regenerates rapidly and the discolored areas disappear in 6-12 hours. The discoloration was present with 95% ethanol but not in the diluted solutions. On day 2 (Fig. 2) the alarm substance cells are beginning to vacuolate and the nuclei are missing or pyknotic. The alarm substance cells continue to degenerate until by day 12 (Fig. 5) nearly all the alarm substance cells have disappeared and only the goblet and epithelial cells are left. The epithelial cells did not appear to degenerate but the thickness of the epithelium was reduced considerably. The alarm cells began to regenerate on day 13 (Fig. 6) and by day 16 (Fig. 8) the regeneration was nearly complete. Fish painted with DNCB in ethanol (10 g/l in 95%) would change colors and become darker (steel grey) on days 4-10 post treatment. Histopathologically this was due to an increase and redistribution of melanophores in the dermis and epithelium.

Figures 9 and 10 show the effects of 95% ethanol alone. Except for the removal of mucus there was very little cellular change during treatment and no

mortality. Figure 11 is a trophozoite under the epithelium showing the damage caused by the parasite and the fact that it does not penetrate the dermal layer, although it damages it. Figure 12 is normal epithelium from a healthy fish.

Table 1 is a record of the mortalities associated with ichthyophthiriasis in aquaria. There was initial mortality from application of the undiluted DNCB in this size of channel catfish. Trophozoites began to appear on the fish before they were purposely infected and apparently had contact with I. multifiliis before the experiment began. There did not appear to be an advantage to the treatments in this situation as all fish were dead by day 10 of the experiment. The fish in the 95% ethanol treatment group died prematurely due to a plugged oxygen source and subsequent anoxia.

DISCUSSION

Further study on the uses of this drug (DNCB) is warranted since histologically the epithelium is not maximally altered until day 8-12 post treatment and fish should be challenged at this time and the results noted. Also from a more practical viewpoint fish from a pond known to contain I. multifiliis should be brought into holding tanks in the high incidence seasons of spring and fall and dipped in DNCB and ethanol before placing into tanks and the efficiency of the drugs recorded.

Pfeiffer (1963, 1977) discussed the fright substance (alarm) cells present in the skin of many fishes. These cells were found to release, upon disturbance of the epithelium, chemicals that alert other fish of the same species and sometimes other species of danger in the area. The fish then illicit evasive action behavior specific to that group or family of fishes. Channel catfish evasive behavior consists of going to the bottom and hiding. The release of alarm substance at frequent intervals would assumably stress the

fish and penetration of I. multifiliis into the epithelium would release alarm substances and add further to the stress, besides the physical damage. Destroying mucus and epithelium with DNCB and ethanol did stress the fish as noted by their behavior patterns and they did hide in the corners of the aquaria remaining immobile for a period of time post treatment.

There is some question as to whether enhancing cellular immunity would aid in reducing the incidence of ichthyophthiriasis. Since this study did not reveal the presence of lymphocytes or macrophages in the epidermis of the channel catfish and since I. multifiliis does not penetrate into the dermis there may be no contact between the immune cells and the parasite. There may be cytotoxic substance elaborated by these cells inhibiting the parasite but this has not been investigated.

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Figure 1. 96 hour LD50 for designated Dinitrochlorobenzene concentrations.

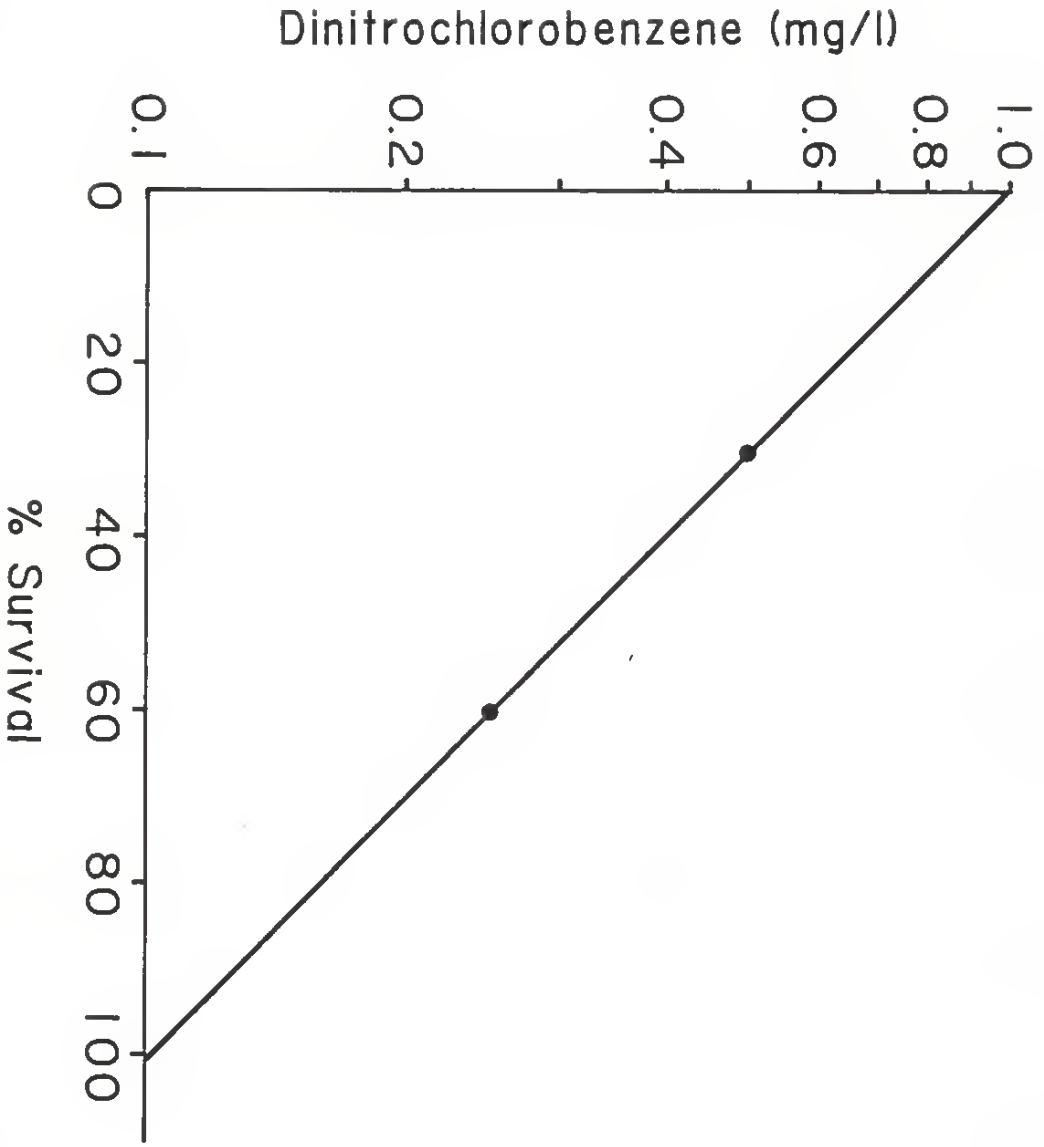
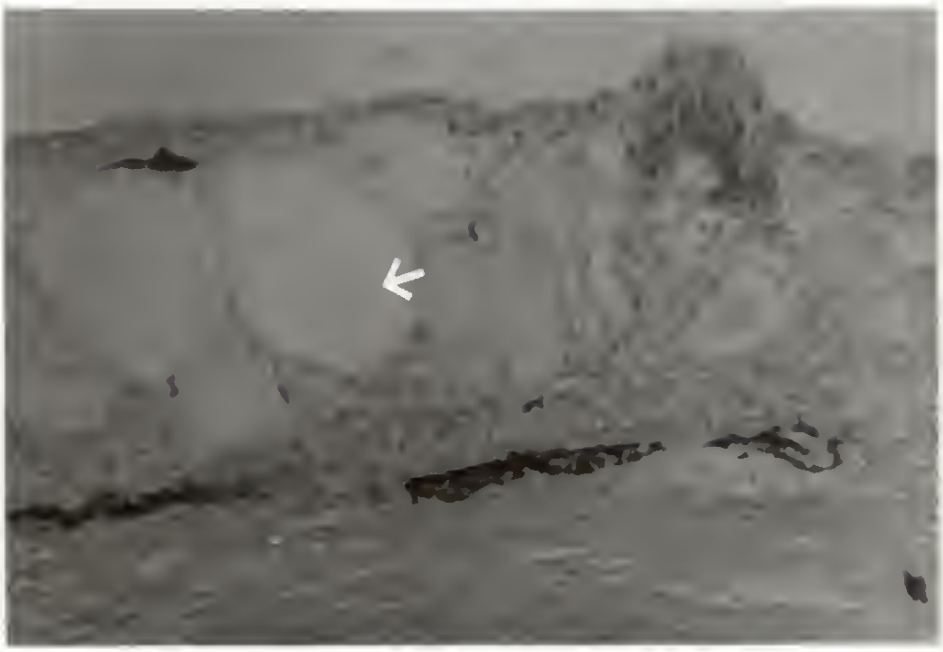

Dinitrochlorobenzene (mg/l)
0.1
0.2
0.4
0.6
0.8
1.0
% Survival
0
20
40
60
80
100

Figure 2. Two days post treatment with DNCB. Note vacuolization of alarm cells. Arrow indicates an alarm substance cell.

Figure 3. Four days post treatment with DNCB. Alarm substance cells are becoming more eosinophillic and melanophores are distributing from basal layer into the epithelium (Arrows).



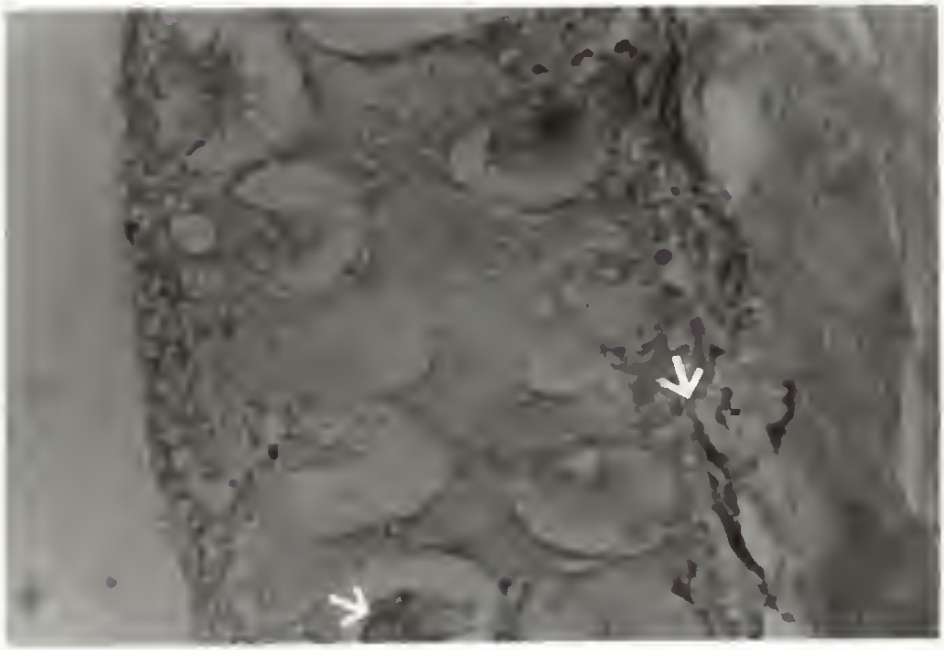


Figure 4. Eight days post treatment with DNCB. Alarm substance cells are nearly absent and islands of melanophores are present in the thickened epithelium.

Figure 5. Twelve days post treatment with DNCB. Any alarm substance cells left are degenerate and the thickness of the epithelium is greatly reduced.

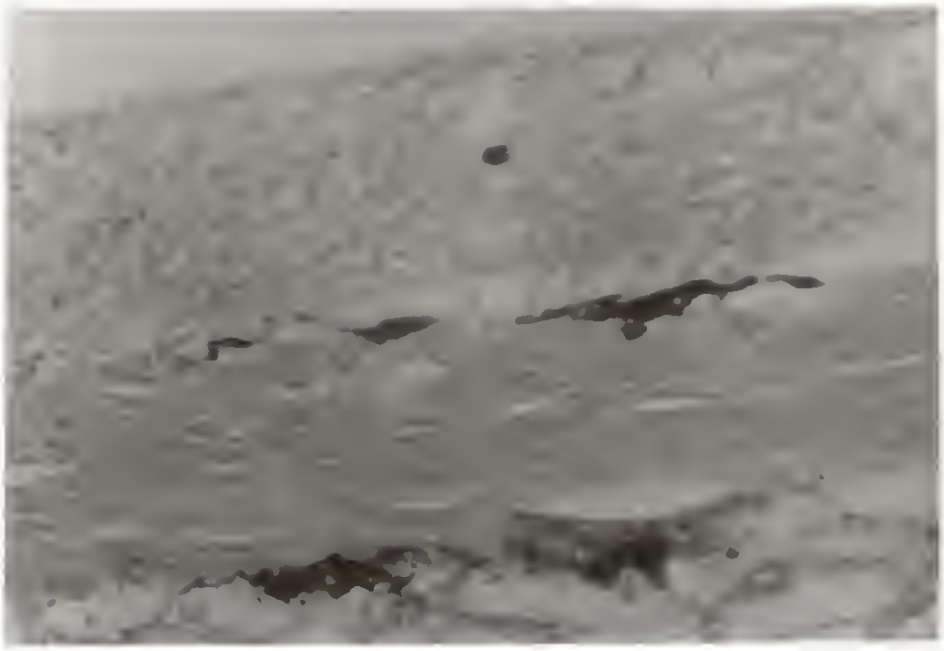


Figure 6. Thirteen days post treatment (DNCB). The alarm substance cells are beginning to regenerate but islands of melanophores, indicated by arrows, are still present in the outer epithelial layers.

Figure 7. Fourteen days post treatment with DNCB. The epithelium has returned to near normal. Arrow indicates the lateral line.

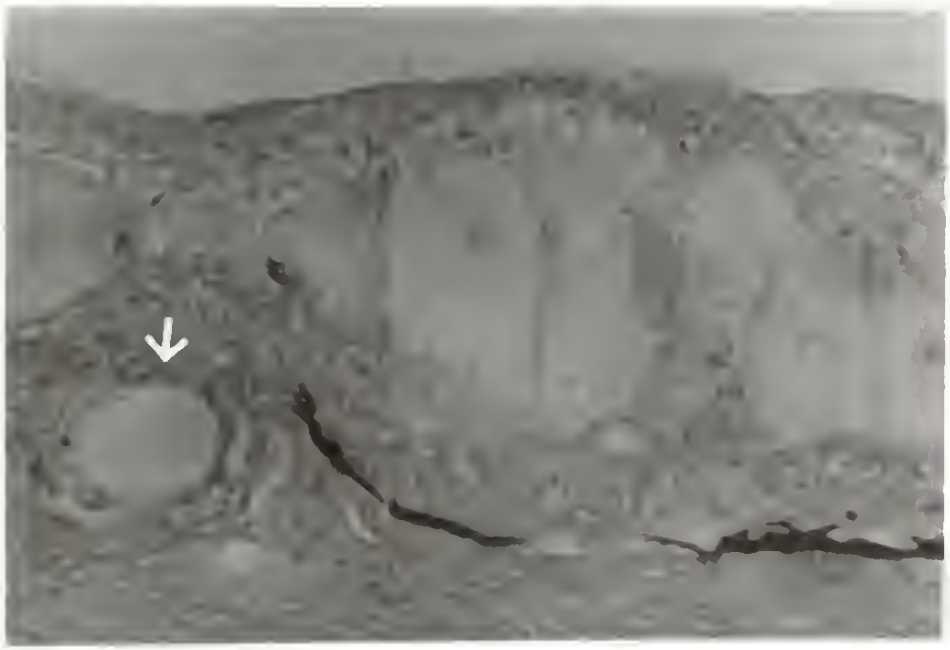


Figure 8. Sixteen days post treatment with DNCB. There is revacuolization of the alarm substance cells in fish exposed to tomites of I. multifiliis.

Figure 9. Two days post treatment with 95% ethanol alone. The epithelium is normal but the epidermis has pulled away from the dermis and is an artifact.

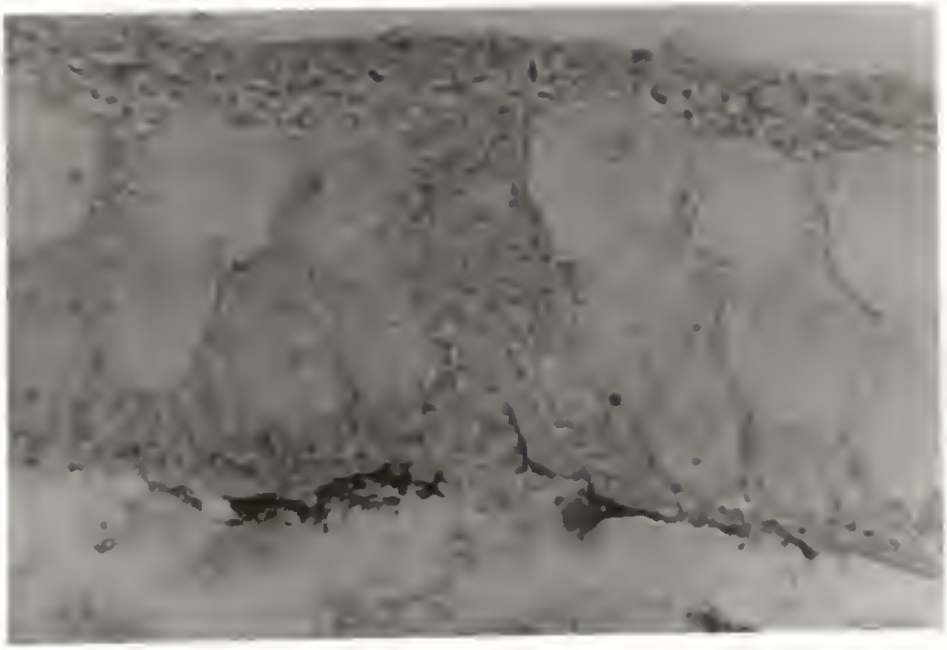


Figure 10. Thirteen days post treatment with 95% ethanol alone. The epithelium is still normal and the arrow indicates the mucus layer.

Figure 11. Sixteen days post treatment with DNCB. This is a trophozoite of I. multifiliis, the arrow indicates the saggital section through the nucleus of the protozoan. Note the damage to the alarm substance cells and the erosion of the dermis.

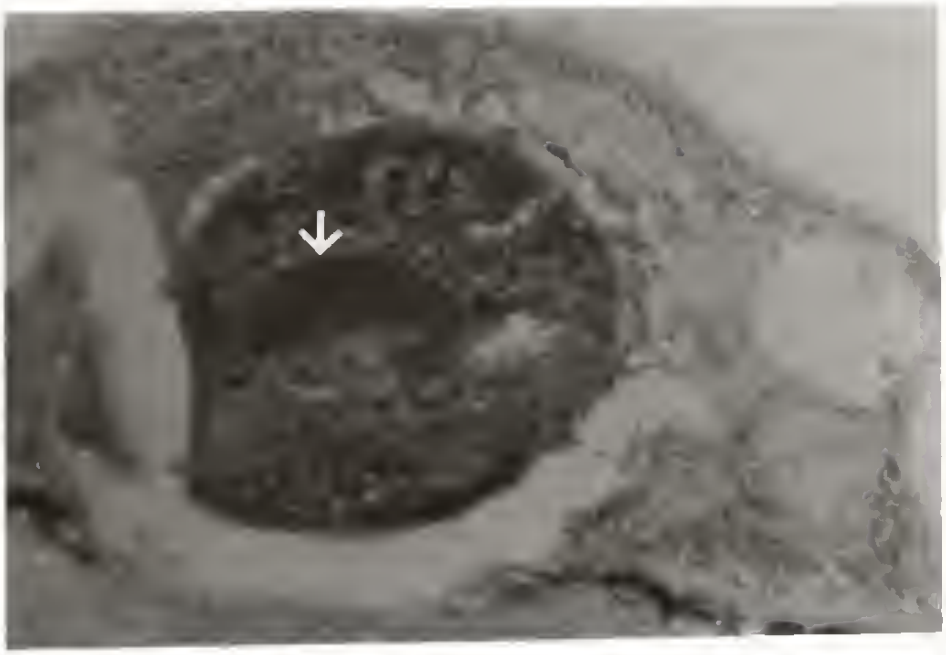


Figure 12. Normal catfish epithelium with arrow indicating goblet (mucus producing cell. The epithelium thins over the lateral line.

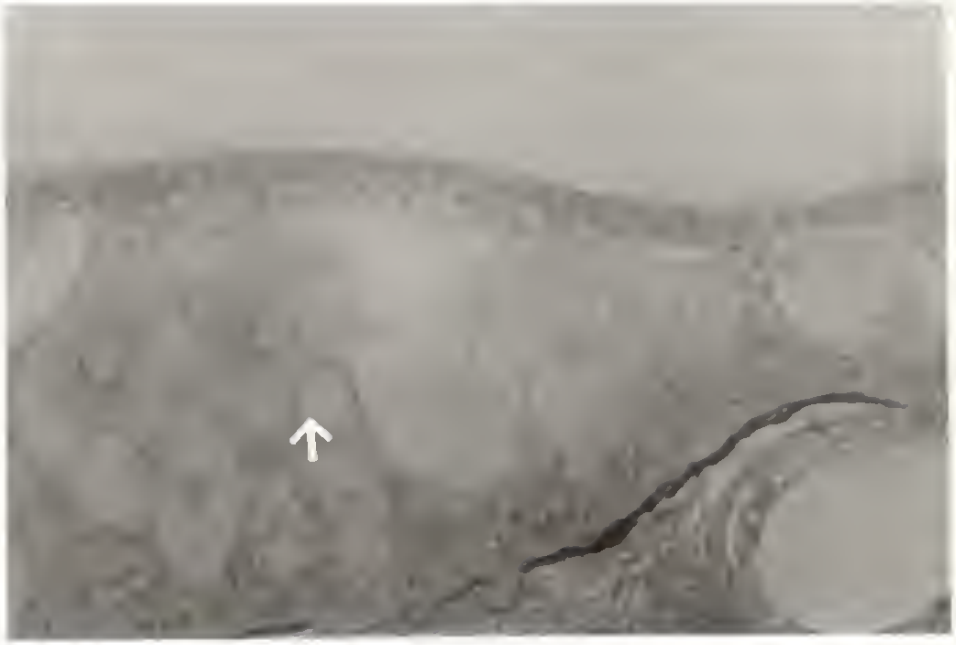


Table 1. Percentage survival of DNCB/ethanol treated fish challenged with *I. multifiliis*.

	Treatment				
DAY	95% ethanol	DNCB in ethanol	50% ethanol	50% DNCB in ethanol	water only
1	100	64	100	100	100
2	100	64	100	100	100
3	100	64	100	100	100
4	0	44	96	96	88
5	0	32	96	96	84
6	0	32	96	96	84
7	0	32	96	96	84
8	0	32	84	58	68
9	0	32	44	36	32
10	0	0	0	0	0

STUDIES ON *ICHTHYOPHTHIRIUS MULTIFILIIS* AND THE IMMUNE SYSTEM OF *ICTALURUS PUNCTATUS* WITH EMPHASIS ON EARLY DETECTION OF DISEASE, CHEMOTHERAPEUTIC AGENTS AND PRODUCTION OF BIOLOGICAL REAGENTS

by

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B.S., Kansas State University, Manhattan, KS 1980

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Division of Biology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1985

All stages in the life cycle of the ciliated parasite Ichthyophthirius multifiliis were examined using the scanning electron microscope. Different procedures were used to prepare the tomite, trophozoite and cyst stages. Photomicrographs of freeze dried and critical point dried organisms fixed with glutaraldehyde are presented along with views of trophozoites and tomites deciliated by dibucaine hydrochloride to reveal the pellicle and buccal cavity normally covered by cilia. Theories involving the exocyst and endocyst production of the cyst stage were introduced relative to the physiology of the organism.

The kinetics of the immune response to sheep red blood cells (SRBC) and serological cross reactivity between Tetrahymena pyriformis and I. multifiliis were studied. Partial characterization of the SRBC binding immunoglobulins found them to be sensitive to 2-ME alkylation and able to cause complement associated lysis of SRBC. Temperatures of 45°C for 30 minutes inactivated the complement proteins and freezing sera for a year reduced complement activity. Column chromatography of Igs on Sephacryl S-200 gel showed the SRBC active sera components to be divisible into three fractions. Titration of the column elution indicated two fractions with hemagglutinating activity against SRBC, one a high molecular weight fraction and the other with lower molecular weight than the complement fractions eluting in the middle portion of the curve. Immunoelectrophoresis (IEP) of these proteins with rabbits immunized with different column fractions indicated there to be two antigenically different proteins in the large molecular weight hemagglutingating portion of the elution curve. The proteina migrated towards the cathode. The IEP of the SRBC lysing portion of the curve indicated several proteins present and the continued presence of these gamma migrating Igs. Serological cross reactivity was found between I. multifilliis and T. pyriformis in channel catfish immunized with the

latter. The sera had both lysing and immobilizing activity against T. pyriformis and immobilizing activity alone against I. multifiliis. The lysis and immobilization assays were found to produce inherent variability depending on the viability of the microorganisms.

The enzyme-linked immunosorbance assay (ELISA) was applied to the catfish serological system and for the detection of tomites of I. multifiliis. Cross reactivity between I. multifiliis and other ciliates found in the environment of the channel catfish produced difficulties with the assay that were reduced by using mouse monoclonal antibodies against I. multifiliis. Fluorescent antibody techniques confirmed the results of the ELISA. The microtiter system was used in screening seras of rabbits, mice and catfish and a membrane filter system was developed that could be used in the field to detect presence of I. multifiliis in water. The filter system's sensitivity was 10 tomites before the curve blended into background color. Most of the immunoreagents requried for the IFA and ELISA are not commercially available and must be produced as needed.

The use of Dinitrochlorobenzene (DNCB) was investigated as a prophylactic and chemotherapeutic agent in channel catfish for ichthyophthiriasis. The toxicity, by bioassay, was found to be dose dependent with a 96 hour LD_{50} of 0.3 parts per million with the drug being more toxic to smaller fish. Dinitrochlorobenzene is a contact irritant and illicits a cellular immune response in mammals and the effects of this chemical applied to skin of channel catfish was followed histopathologically. The principle effect was destruction of the alarm substance cells in the epidermis of the fish exposed to DNCB with regeneration of these cells by day 16 post treatment. Controlled trials on DNCB treated fish challenged with I. multifiliis indicated the drug did not significantly alter the course of infection although previous clinical trials indicated the drug would aid in the control of ichthyophthiriasis.