STUDIES ON *ICHTHYOPHTHIRIUS MULTIFILIIS* FREEZING APPARATUS, CRYOPRESERVATION, LABORATORY CULTURE, AND INTERSPECIFIC TRANSFER

by

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B.S., University of California, Davis, 1978

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Parasitology

Interdepartmental Program

Division of Biology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1981

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INTRODUCTION

The ciliate fish parasite, Ichthyophthirius multifiliis Foquet, 1876, is a potentially lethal parasite on most, if not all, fresh water fishes throughout the world (49). The expansion of fish farming as a promising means of providing high quality protein diets in many countries, including the United States, has been faced with the problem of I. multifiliis infections depleting or destroying entire pond populations (57). Research to further understand the pathogenic course of the infection (21-25), transmission from pond to pond (48, 56), and the host's capability in overcoming or resisting infection continues to be crucial in the search for an effective means to control the parasite.

In vitro culture of I. multifiliis has been unsuccessful to date (26, 36). Maintaining significant quantities of laboratory stock by host to host passaging is unpredictable at best, with variation in pathogenicity of organisms collected from different sources and in host susceptibility. Frequently, fish become immune to the parasite after recovering from sublethal infections (25). Cryopreservation would solve most supply problems, with collection and storage of organisms from the original host until they are needed for experimental purposes. The immunological defenses against I. multifiliis in fish have not been clearly defined. Both serum antibodies specific for I. multifiliis, and a change in epithelial mucus may be means of resisting infection.

The research presented here includes several aspects of the problem outlined above. Laboratory maintenance and interspecific transfer of I. multifiliis are discussed. Serum and epithelial mucus may have immune components and were studied in immune fish. Finally, a

controlled freezing system and a method for cryopreserving *I. multifiliis* have been developed.

LITERATURE REVIEW

Ecology and Distribution

I. multifiliis is endemic in fresh water bodies throughout the temperate and tropical regions of the world. While mostly lethal among fingerlings and fry (10, 27), its appearance and severity mainly depend on ambient temperature and the health of the fish.

Ambient temperatures above 27°C or below 0°C stop the life cycle. Above 27°C, all but the cyst stage are destroyed. Optimal temperatures for development are between 21 and 24°C (48).

It has been suggested that the trophozoite may be the overwintering life stage (32). Rather than remaining statically in that form, the life cycle probably continues with the trophozoite stage predominating. Bauer, et. al. (6) observed very slow reproduction between 2 and 4°C. Ychehckar and Uspenkaja (68) found that low temperatures slowed development and prolonged the trophozoite stage. The trophozoite remained on the fish until sufficient reserve materials (glycogen, protein, DNA, RNA, and enzymes) accumulated for encysting and replication.

Meyer (48) found *I. multifiliis* incidence in farm ponds in 10 states of the lower Mississippi Valley region to be greatest in winter and spring months, with peak outbreaks in April and October. At those times, the ambient temperatures were within the range for *I. multifiliis* growth while temperature fluctuations reduced fish resistance to infection.

Hatchery fish are particularly prone to *I. multifiliis*. Crowding stresses the fry and fingerlings in particular, reducing resistance mechanisms and increasing susceptibility. Preventing introduction of *I. multifiliis* and reducing stress both are effective control measures to

minimize outbreaks (10).

I. multifiliis is present in low numbers in most fish populations (53). Fish in this study which had no history of the disease living in optimal temperatures for parasite development became heavily infected when stressed by one or a combination of conditions such as food deprivation, oxygen stress, handling, temperature fluctuation, and bacterial or fungal disease.

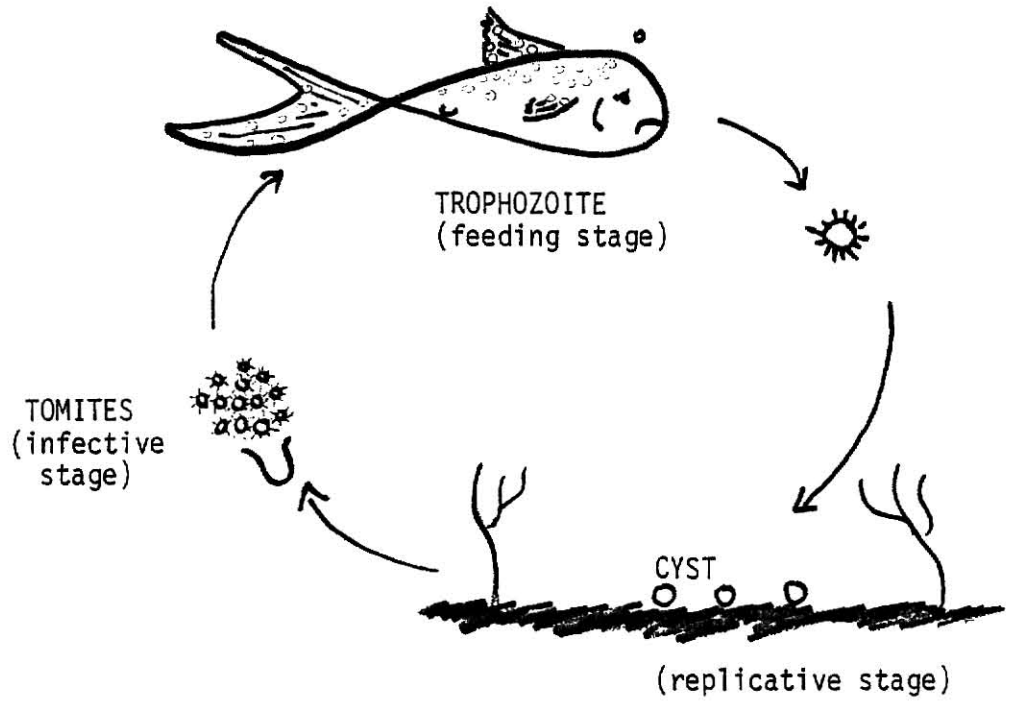
Life Cycle

The life cycle of I. multifiliis is a simple one and well documented (7, 10, 15, 21, 26, 27, 38, 57). However, the details of each stage are still being elucidated. The life cycle is diagrammed in Figure 1.

The tomite, or motile infective stage, is 30 to 40 microns in diameter. Upon contact with a host, the tomite secretes hyaluronidase (66) to rapidly break through the fish epithelium, the connective tissue of which is primarily hyaluronic acid. It burrows down to or near the basement membrane of the epithelium. There it begins to feed on a variety of host tissues, including blood, epithelium, and mucus (23, 36). Crilley and Bucklew (12) have identified structures in the pellicular region of the trophozoite, or feeding stage, which may be involved in extracellular digestion. The host cells around the trophozoite divide and flatten to form a capsule around it, creating the white pustule characteristic of the disease. The trophozoites mature, reaching a maximum size of 1 mm in diameter (53) before leaving the host. The trophozoite secretes a proteinaceous cyst wall and settles onto the pond substrate to divide into several hundred to a thousand tomites (57). Division may begin while the trophozoite is still on the fish.

FIGURE 1

Life Cycle of Ichthyophthirius multifiliis



Some trophozoite forms do not make a cyst wall, but divide up to eight times and stop. Possibly, the latter cells lack sufficient quantities of one or more reserve materials (68) and were forced to leave the dead host prematurely. The division in the cyst in optimum temperatures is completed within 24 hours. The cyst bursts, releasing the tomites which are infective for 24 hours and live up to 48 hours. Tomites generally do not feed.

The mechanism by which the tomite locates the host and selectively colonizes certain areas of the body (e.g. gills) more than others is not understood. Kozel (35) has identified an "apical filament" on the tomite which may be involved in the orientation of the tomite and facilitate infection processes. Tomites have been observed to be attracted to fish scales and such attraction may be along some kind of chemical gradient.

The time required to complete one life cycle is directly related to the environmental temperature. This is most directly observed in the time required for a trophozoite to mature. At 2 to 4°C, it takes four or more weeks (6, 36). At 15 to 16°C, it takes 11 to 13 days (10) while at the optimum temperature range of 21 to 24°C, it takes only 3 to 4 days (48). Beckert (7) observed the entire cycle to take 4 to 5 days at 24°C.

Nearly all cultured fresh water fish are susceptible, to varying degrees, to I. multifiliis. Members of the catfish, sunfish and trout families (10, 15, 21, 53) are very vulnerable to infection of all parts of the body. Others, such as some goldfish, are primarily affected in the gills, and not on other body surfaces (53). In general, the gills and fins are the first and most heavily parasitized areas. The dorsal

body surface is invaded second and the lateral and ventral surfaces are the least parasitized. (21)

Pathology

The course of infection in ichthyophthiriasis, or "white spot disease," has been well documented by Hines and Spira (21-25). If the fish carries a sufficient parasite load, it will die. The symptoms of I. multifiliis infections are nonspecific and common to infection by most external parasites. The course of infection was described in six stages for the mirror carp. The same pattern was observed in this study in the channel catfish.

1. Fish appear normal but parasites have already penetrated the skin, gill, and fin epithelium.
2. Fish often jump and surface. Superficial vessels of the integument become visible. Skin epithelium and mucus thicken. Parasites are walled off, except in the gills.
3. Superficial vessels become engorged. Parasites become visible all over the body. Neutrophils are numerous in the spongiose dermis.
4. Fish are no longer hyperactive, but swim very slowly and congregate at the bottom of the tank. Fins begin to fray. The epithelium is no longer in ordered layers.
5. Fish are noticeably weaker. Fins are very frayed. Epithelial and mucal coats are thick and often clumped or patchy. Once fish reach this stage, they do not recover. The epidermis returns to normal thickness and mucus cell proliferation ceases.
6. The fish are moribund. Mucus is gone from the body and no

mucus vacuoles are found in the remaining epithelium. The eyes are sunken, the cornea is opaque, and the gills are pale and very necrotic. Epidermis and corium are easily detached and the epidermis is lost down to the basal layer. Most parasites are gone from the body.

Hines and Spira (21) also described pathologic effects of the disease. The loss of skin and exposure of the vascular network of the dermis in the later stages of infection upset the mechanics of osmoregulation. The gill infection caused gill lamellae to thicken. The fish then became very vulnerable to even the slightest drop in dissolved oxygen levels because gas diffusion was less efficient. Changes in liver tissue were caused by fat reserve mobilization in response to increased energy requirements during infection, especially in the last two stages when the fish stopped feeding. The haemopoetic areas of the kidney and spleen increased and were exhausted from massive leukocyte production (neutrophils, lymphocytes, fine reticular phagocytes, and later predominance of undifferentiated blast cells). The leukocytes were named according to morphology, not in terms of observed functional equivalents to mammalian systems.

Mortality resulted from several dysfunctions. The severe hyperplasia of gill filaments coupled with proliferative and degenerative skin pathology both reduced oxygen absorption and waste (ammonia and urea) removal. The nitrogenous wastes were found to accumulate in mirror carp (24). The loss of skin and mucus also reduced the ability of the fish to osmoregulate. Secondary fungal and bacterial infections commonly accompanied I. multifiliis and sometimes killed the host before the parasitic disease had run its course. Compounds produced by the parasite and the cell decomposition products may also be toxic to the fish (6).

Laboratory Culture

Maintaining laboratory supplies of I. multifiliis is done through serial passaging in susceptible fish hosts. In vitro systems are being investigated, with limited success to date. In order to maintain an in vivo supply, a fairly specific set of conditions must be met.

Hlond (26) developed an in vitro medium based on fish slime, on which the tomite stage was maintained, without completion of the life cycle. Kozel (36) has developed an effective growth medium based on tissues the trophozoite consumes. The trophozoites develop up to the tomite stage but do not complete the life cycle.

Beckert (7) and LeValley (38) maintained in vivo supplies of I. multifiliis by exposing naive fish to infected moribund fish and removing the latter within 24 hours of death. Flow-through troughs housed the fish, and no gravel substrate was placed on the bottom. Ambient temperature in LeValley's study was 18°C, slightly below optimum for both I. multifiliis growth and efficient fish immune defenses.

CONTROLLING COOLING RATES WITH A VARIABLE VACUUM IN A DEWAR FLASK

Introduction

The rate of cooling is an important variable in optimizing cell survival in freezing proceedures (17, 37, 41, 47, 50, 51, 54, 63, 64). Many cell types require slow cooling rates, from 0.4°C/min for schistosomules (63) to 1.8°C/min for lymphocytes. Many cell types require a cooling rate of 1°/min (2, 19, 47, 51). Cryoinjury from cooling too rapidly seems to result mainly from intracellular crystallization (37, 41, 46). Cooling too slowly may decrease survival through dehydration and denaturation (17). By varying cooling rates, the best rate for a specific cell type can be determined.

There are many systems on the market for regulated cooling. Some are limited in cooling rate control with low reproducibility of results. Others, such as those with computerized controls (5, 44), can be expensive though highly accurate. The system described combines the benefits of low cost (under $130 for the Dewar flasks and accessories), easy assembly, and high reliability. It was first described by Leibo and Mazur (37) and altered by Zeilmaker and Verhamme (69) to control cooling rates by varying the pressure in the Dewar flask. Drawing a vacuum from the Dewar flask slows the cooling rate as much as 2°C/min below that achieved at atmospheric pressure. This modified system is sufficiently pliable to enable the operator to select from a spectrum of cooling rates, thus making it useful for many of the cell lines in work cited above. The modified system can also be adjusted to increase heat flux during the liquid/solid phase transition encountered when cell samples are not seeded to initiate extracellular freezing (37).

This paper presents cooling rate data within a narrow range achieveable with this system, makes suggestions as to broader applications, and cites preliminary freezing results using this system.

Materials and Methods

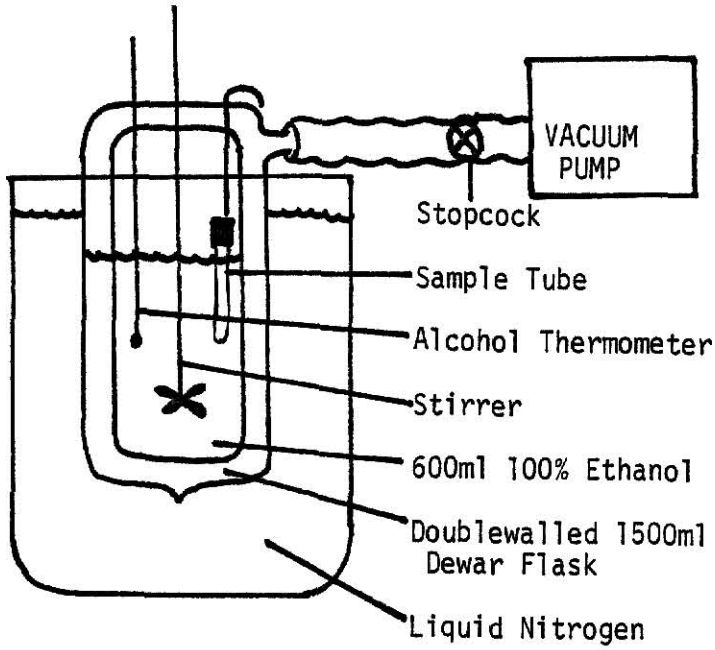
Figure 2 illustrates the modified system. An alcohol thermometer was substituted for the sample tube thermocouple. At low cooling rates for large cells, such as I. multifiliis, the difference between the sample and the alcohol bath will be less than 1°C, introducing no significant error (37). The bottom of a Dewar flask was sealed and a sidearm created in the outer wall which connected to a vacuum/pressure pump (Millipore Corp.). Cell culture tubes containing I. multifiliis or polypropylene ampules containing myeloma cells were suspended around the perimeter of the flask.

Two variables were used to alter the cooling rate. Changing the ethanol volume was effective for coarse adjustment. The Dewar flask used for the volume tests was evacuated and sealed with no sidearm added. The vacuum could not be measured by the glassblower. Extrapolation of the results below suggested an estimate of 29" Hg (737 mm Hg). A constant volume of 600 ml ethanol was used for the vacuum tests since the rate of -1°C/min was included in its range. The vacuum was drawn before each test and the flask sealed off by a stopcock.

Temperature readings were taken every five minutes, beginning when liquid nitrogen was added to the outer container and ending at -45°C. The endpoint of -45°C was selected because water desorption terminates at about -40°C (41), and other successful methods of protozoan cryopreservation terminate between -40 and -50°C (47). The samples were

FIGURE 2

SCHEMATIC DRAWING OF THE COOLING APPARATUS



then plunged into liquid nitrogen and stored. Liquid nitrogen was added after each reading to maintain a constant volume. Five vacuum levels, 5" Hg (127mm Hg) through 25" Hg (635mm Hg) at five-inch (127 mm) intervals, were tested five times each. Cooling rates and the overall relationship between vacuum (V) and cooling rate (CR) were determined by regression analysis. Standard deviations were calculated at each volume.

Results and Discussion

The graph and equation defining the relationship between vacuum levels and cooling rates in this system are illustrated in Figure 3. Although cooling is known to be a curvilinear function (37), linear regression amply defined the rates, with an average correlation coefficient (r^2) for individual cooling rate determinations of 0.996 $\pm$0.002. The overall correlation coefficient for the relationship between V and CR was 0.91. Cooling rate data are presented in Table 1.

At a constant vacuum, the cooling rate was altered significantly by 100-ml changes in ethanol volume (Table 2). The relationship between volume and cooling rate is shown in Figure 4. Table 3 presents data on the effects of varying cooling medium volume on cooling rates. At volumes other than 600 ml ethanol, the resulting cooling rate/vacuum lines should parallel the one shown in Figure 3. From these lines, one could predict the volume and vacuum level for a specific cooling rate. Larger volumes, in larger Dewar flasks, may be desirable for projects requiring that more samples be frozen. Because of the smaller surface area/volume ratio of larger flasks, the cooling rate/vacuum lines would have shallower slopes.

Preliminary results of freezing *I. multifiliis* in this system

FIGURE 3

Relationship between cooling rates and vacuum in the modified Dewar flask

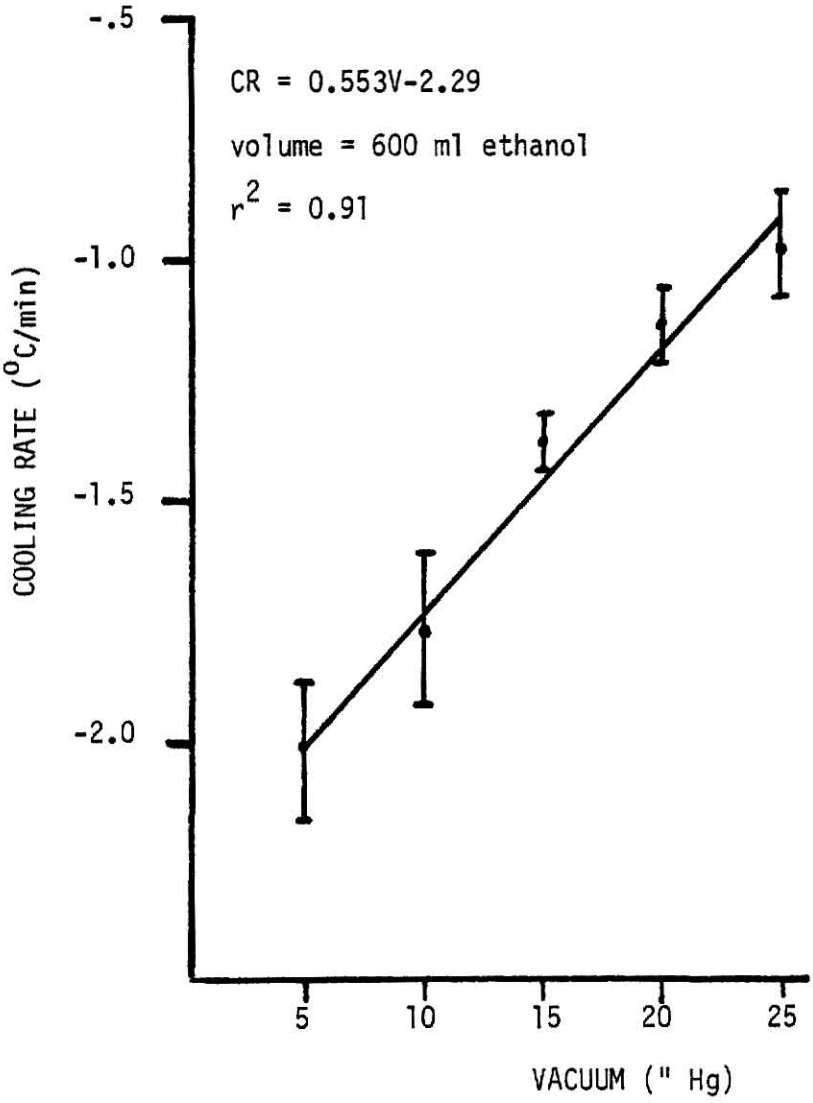


TABLE 1

Cooling Rates (^{o}C/min) in 600 ml Ethanol Cooling Medium

Varying Dewar Flask Vacuum

Vacuum (in. Hg)		1	2	3	4	5	Average Rate ± St. Dev.
5"	rate	-2.169	-2.042	-2.919	-1.925	-1.830	-2.032 ± .138
	r^2	.9994	.9974	.9980	.9974	.9966	
10"	rate	-2.049	-1.588	-1.701	-1.745	-1.80	-1.778 ± .153
	r^2	.9982	.9968	.9958	.9956	.9966	
15"	rate	-1.380	-1.393	-1.49	-1.39	-1.315	-1.394 ± .055
	r^2	.9934	.9944	.9950	.9948	.9972	
20"	rate	-1.277	-1.183	-1.111	-1.094	-1.072	-1.146 ± .077
	r^2	.9950	.9946	.9930	.9934	.9966	
25"	rate	-0.910	-0.962	-1.19	-0.853	-0.922	-0.976 ± .11
	r^2	.9952	.9926	.9980	.9908	.9942	

Average r^2 = 0.996 ± .002

TABLE 2

Effect of Volume on Cooling Rate[a]

Ethanol volume (ml)	Cooling rate (°C/min)
400	-0.91 ± .05[b]
500	-0.82 ± .03
600	-0.73 ± .01

[a] vacuum in Dewar flask approximately 29" Hg

[b] mean ± 1 S.D., where n=5

FIGURE 4

Cooling Rates in the Sealed Dewar Flask

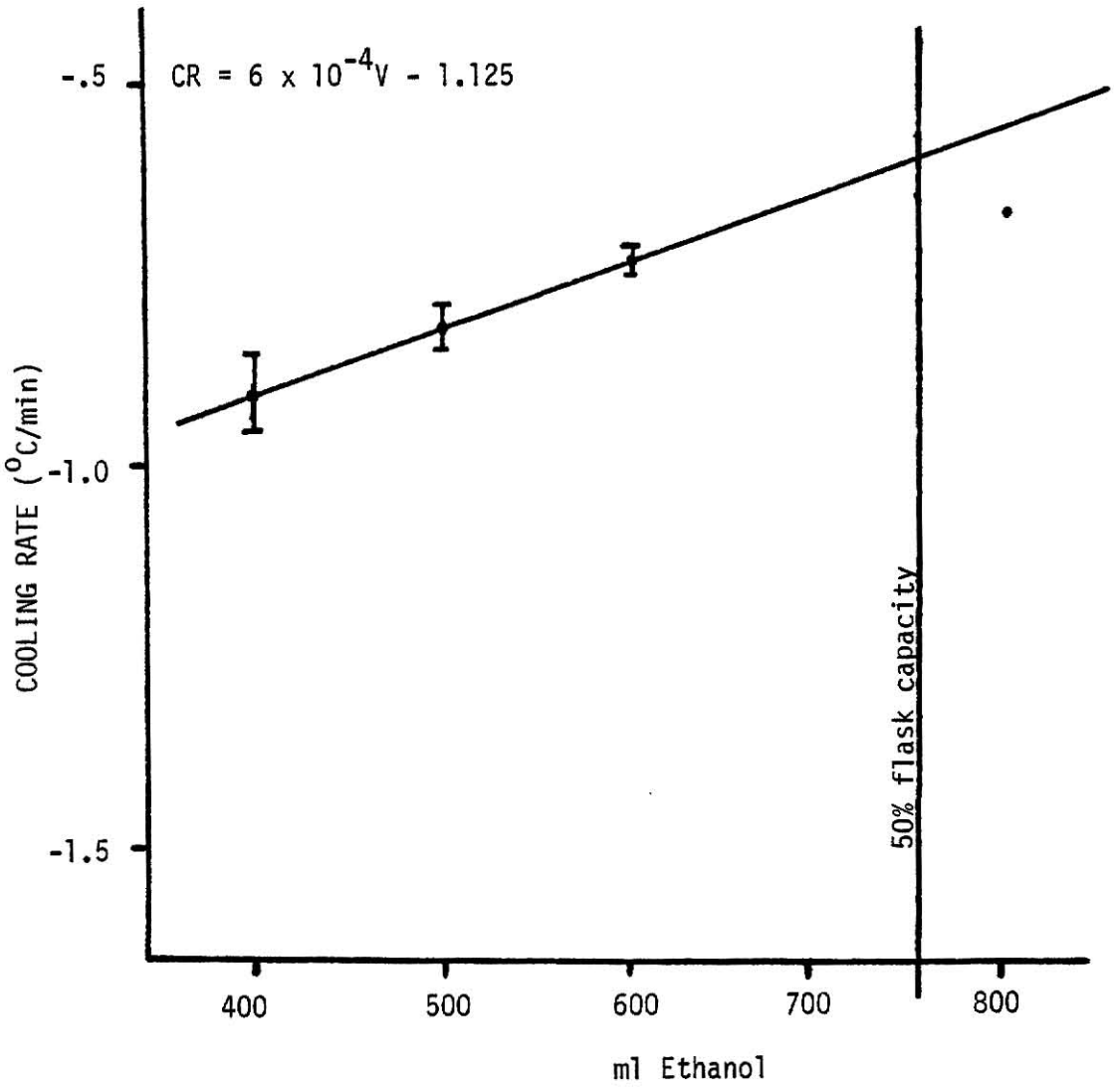


TABLE 3

Cooling Rates of Sealed Dewar Flask

Varying Cooling Medium Volume

Volume		1	2	3	4	5	Average ± 1σ
400 ml	rate	-0.923	-0.822	-0.916	-0.949	-0.942	-0.910±.05
	r^2	.9994	.9950	.9936	.9964	.9974	
500 ml	rate	-0.871	-0.814	-0.810	-0.816	-0.791	-0.820±.027
	r^2	.9958	.9920	.9964	.9966	.9968	
600 ml	rate	-0.721	-0.714	-0.738	-0.723	-0.733	-0.726±.009
	r^2	.9966	.9964	.9968	.9948	.9954	
800 ml	rate	-0.668					
	r^2	.9966					

indicated about 60-65% survival of the trophozoites and cysts, using a rate of -1°C/min. Cells frozen at -0.8°C/min survived, but in quantities too low to count. Cells frozen in a Linde Biological Freezer (BF-5) did not survive. All samples were seeded to initiate extracellular ice formation prior to controlled cooling. Foreman and Pegg (17) cited embryo preservation as one of the few systems requiring tightly controlled cooling rates. I suggest that large unicellular organisms may also require tight control because of similar, low surface area/volume ratios. The lower ratio indicates more time is required for sufficient dehydration throughout the cell. This would be accomplished with a cooling rate slow enough to allow dehydration and equilibration of water concentration within the cell prior to freezing, yet high enough so as to prevent denaturation.

Myeloma cell lines were frozen without being seeded. During the phase transition interval, the vacuum was decreased 3 to 5" Hg (76 to 127mm Hg) which increased heat flux and permitted consistent cooling. The vacuum was then increased to the original level for the remainder of the cooling process. About 65 to 70% of the cells survived. In selecting volume and vacuum settings for cooling unseeded samples, the desired rate should be in the middle of the range covered by a specific volume to allow vacuum changes in either direction. When a large number of unseeded samples are cooled, or when total sample volume exceeds 20 ml, a larger drop in vacuum may be required to absorb the latent heat of fusion. A small sample volume (under 5 ml) may require no change at all. Large volumes of seeded samples may require a vacuum decrease of 0.5 to 1" Hg (12.7 to 25mm Hg) throughout the freezing process.

CRYOPRESERVATION OF *I. MULTIFILIIS*

Introduction

Cryopreservation is a method by which cellular material can be maintained in a viable state while stored in liquid nitrogen or other low temperature environments. It is useful for preserving genetic integrity of organisms altered over time in normal culture practices, and provides a predictable supply of those cells for experimental purposes. The American Type Culture Collection (1) uses cryopreservation techniques routinely and encourages research into new methods to preserve new cell lines and improve viability of cells already being frozen.

Many protozoan species have been cryopreserved, including several ciliates (13, 14, 28, 67). *I. multifiliis* has not been successfully preserved to date (13). To do so would greatly enhance research efforts with this parasite. The maintenance of an *in vivo* serial passaging system is a costly, time consuming task with highly variable yields of cells.

A multitude of variables must be considered in freezing cells. The primary variable is that of freezing rate (13, 44, 45). Other important variables are: the cryoprotectant used, additional freezing solution components, thawing rate, components in post-thaw dilutions, and the type of vessel in which the cells are frozen (63). Variables tested in this study were components of the freezing and thawing solutions, thaw rate, and cryoprotectant concentration. The original technique came from S.P. Leibo (pers. comm. to L.S. Rodkey) for preserving lymphocytes. *I. multifiliis* survived a modification of that freezing technique.

Materials and Methods

Cells: I. multifiliis was maintained in vivo as described later in this thesis. The hosts were pithed and placed in 125 ml of fish tank water, or biologically conditioned water (BCW), which had been passed through a 0.22 micron filter (Millipore Corp.). The host was removed when most of the trophozoites had left it, which took from 3 to 6 hours. The more mature the trophozoites, the more rapidly they left the host. The cells were then concentrated by centrifugation at 2000 rpm for 10 min at 4°C. Volume was reduced to less than 10 ml. The cells were held on ice until the cryoprotectant was added.

Several methods to concentrate the cells were attempted. Centrifugation (speeds up to 5000 rpm) and a separating funnel were both used to concentrate the cells. The separating funnel was pretreated with Desicote (Beckman Instruments, Inc.) to reduce parasite adherance and promote collection at the bottom of the funnel. Methyl cellulose and cooling on ice prior to centrifugation were tried to slow the movement of the trophozoites so they could be concentrated. Methyl cellulose was added in concentrations varying from 0.5 to 20% methyl cellulose. Success was measured in terms of the concentration that kept the cells pelleted the longest without killing them. Filters, mesh sizes from 350 to 710 microns, were used to both concentrate the cells and separate them from sloughed fish skin and mucus.

Cryoprotectant: DMSO (dimethyl sulfoxide) was selected as the cryoprotectant based on the technique from S.P. Leibo and the recommendation of P.-M. Daggett (13) that DMSO was more successful with ciliates than glycerol. Hwang, et al. (28) used 10% DMSO to freeze the ciliate Tetrahymena pyriformis. A final concentration of 5% DMSO was

used in most of this study. Concentrations of 6% and 7.5% were also tried. DMSO is toxic to cells. Trophozoites and tomites were exposed to several DMSO concentrations at room temperature (21°C) and on ice (4°C). The former would be encountered after dilution and the latter prior to the controlled freezing process.

Osmolarity: The 5% DMSO freezing solution and various dilution solutions (below) were tested for osmolarity. The effects of the presence of fetal bovine serum (FBS) and glucose, and the concentration of glucose in the dilution and freezing solutions were measured.

Freezing: The freezing was done as follows. All components were held on ice until they were added to the seeding bath. One tenth ml cells were placed in a 20-ml screw-cap tube to which 0.75 ml FBS was added. DMSO was diluted to 40% with a 20% glucose-BCW solution. The DMSO mixture (0.25 ml) was added to the tube for a final dilution of 5%. In some tests, the FBS was replaced with an equal volume of BCW or the glucose was omitted from the DMSO mixture.

The tube was suspended in a -5°C bath for 5 min and seeded (37, 51). Seeding is the initiation of extracellular ice crystal formation by touching the solution with an ice crystal on the end of a Pasteur pipette. Five to 30 min later, the tube was placed in the controlled cooling system described earlier (9), and cooled from -5 to -45°C at a rate of 1°C/min (vacuum set at 23.75" Hg for less than 5 tubes, 23" Hg for 6-10 tubes). At -45°C, the tubes were plunged in LN_2 and stored in a liquid nitrogen vapor freezer.

Thawing: The cells were thawed at one of three rates. Slow rates were in a 2°C ice slush in which the ice cap on the end of the tube was left in place for 10 min, then cleared away. Medium rates were in a

room temperature bath (21-24°C) and fast rates were in a 37°C bath. The tubes of cells were agitated in the bath until the ice crystals dissolved. They were then put on ice and diluted with 2 ml cold 20% glucose-BCW, then 2 ml cold FBS, and then 4 ml 20% glucose-BCW. The solutions were added dropwise and vortexed after each drop. The tubes were held on ice until the contents were washed by centrifugation and resuspension two or three times with BCW. The cells then warmed to room temperature and were tested for viability.

Viability: Two tests were used to assess survival of the cells. The fluorescein diacetate assay (FDA) is based on the Rotman and Papermaster (59) study which demonstrated intact cells take up diacetyl fluorescein and enzymatically hydrolyze it into fluorescein which fluoresces at 450-500 nm. The dye leaks through the membrane of damaged cells so they do not fluoresce (46). Ten µl of a 0.01 M FDA solution was added to 2 ml BCW and this mixture was then added to a 2 ml cell suspension. After 20 min incubation in the dark, the number of cells fluorescing and the total number of cells were counted. The assay was first tested on unfrozen cells. They fluoresced bright green while formalin-killed cells did not fluoresce.

The second assay was the presence of tomites 24 hours after thawing. Live cysts and trophozoites would undergo replication and the density of tomites would be related to the proportion of surviving cysts and trophozoites. A high density of tomites was scored ++, medium density +, low density +/-, and absence was -.

Results

Concentration: In the separating funnel, trophozoites would not

settle as cysts for up to 24 hours, while some would settle immediately. Sloughed skin also settled to the bottom along with the cysts. The cysts still managed to adhere to the sides of the funnel. Washing them off increased the volume, diluting the parasites.

Centrifugation effectively concentrated the parasites. At 2000 rpm, $4^{o}C$, the trophozoites would still swim up from the pellet within one minute of centrifugation. It was not until late in the study that it was found that the trophozoites would stay down if spun at 5000 rpm, and they would live and reproduce normally. The sloughed skin and mucus also pelleted and were frozen along with the cells.

Slowing the trophozoites down long enough to concentrate then was accomplished using methyl cellulose. Cooling them on ice before centrifugation did not help. The results of the methyl cellulose effects on the trophozoites are in Table 4. The most effective concentration was 2.5% methyl cellulose, with a long holding time and no parasite mortality.

Filtration using a range of mesh sizes was variably successful in separating the cysts and trophozoites from other items in the water. The wide variation in parasite size, from 250 µm to 1000 µm in diameter made it impossible to catch a majority of the cells in one mesh. The degree of development changed from one culture to the next, so one mesh size would not work consistently. Table 5 lists the mesh sizes and what passed through them.

DMSO Toxicity: Figure 5 shows the results of trophozoites and tomites exposed to a range of DMSO concentrations at $4^{o}C$ and $21^{o}C$. The concentration to which they would be exposed in freezing is 5%, or 1:20. At room temperature and 4^{o}, the trophozoites survive much longer than the 5 min preseeding incubation period they would experience during freezing.

TABLE 4

Effect of Methyl Cellulose on Trophozoite Motility and Survival

% Methyl Cellulose in water	Time (min) to recovery		% Survival
0.5	2 min	100% recovered	100
1.0	4 min 5 min	50% recovered 100% recovered	100
2.5	1 min 6 min	10% recovered 80%	100
5.0			70
7.5			75
10.0	1 min	50%	100
12.5	3 min	20%	20
15.0	4 min	0%	0
17.5	6 min	10%	20 or more
20.0	6 min	10%	10

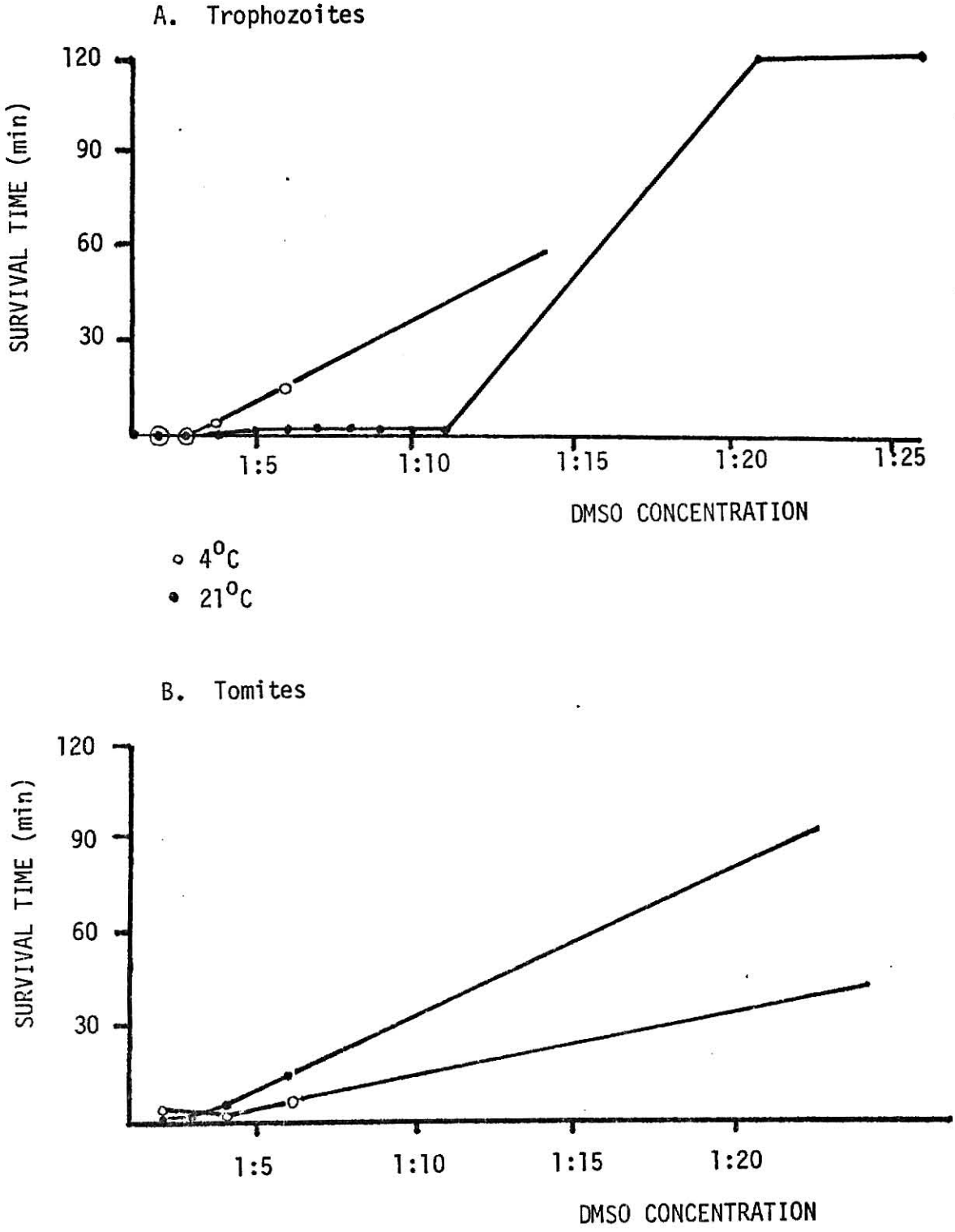
TABLE 5

Filtration of Cysts and Trophozoites

Mesh Size (μ)	Caught	Passed Through
710	clumps of sloughed skin and mucus	all cysts and trophozoites smaller pieces of skin and mucus
508	some cysts and trophozoites, skin and mucus	some cysts and trophozoites skin and mucus
437	large cysts and trophozoites, skin and mucus	debris and smaller trophozoites
350	cysts, trophozoites, sloughed skin and mucual debris	very small trophozoites

FIGURE 5

I. multifiliis survival in DMSO



Tomites have not been frozen, but the 1% DMSO solution they were exposed to after thawing was not lethal when they were not washed after thawing.

Both trophozoites and tomites exposed to DMSO were tested for infectivity. While the low concentrations of DMSO were not toxic to the parasites, the fish died within 24 hours when exposed to the DMSO in the solution with the parasite in liter bowls. When placed in 8-l aquaria, DMSO-treated tomites produced infections 60% as heavy as untreated tomites. The DMSO has low toxicity and can be washed out of solution and permits the parasite to complete its life cycle.

Osmolarity tests: The results of the osmolarity tests are in Table 6 and Figure 6. The tests were done in three groups. BCW and dechlorinated tap water were present in each group to standardize the readings.

Bovine serum albumin substituted for FBS reduced osmolarity only slightly in freezing and dilution solutions. The presence of glucose made the greatest difference in all solutions. The osmolarity decreased linearly as glucose concentrations were decreased. When fully diluted, the solutions were still much higher in concentration than the BCW in which the parasite lives.

Freezing: The results of the freezing experiments are shown in Table 7. Successful conditions are compiled in Table 8. The duration of storage in liquid nitrogen ranged from 1 to 87 days. Storage time did not correlate with parasite survival. Stirewalt, et. al. (63), also found no change in viability over storage periods up to one month.

All DMSO levels required glucose and FBS. Some survival was obtained without FBS at the 5% DMSO level, but the presence of FBS yielded a greater proportion of successful freezing trials, since it has cryoprotective functions (64). Post-seeding incubation time varied

TABLE 6

Osmolarity of freezing and dilution solutions

	Solution	mOsm
BCW		0
dechlorinated tap water		0
1	Freezing solution 1: 1 ml BCW, 0.75ml FBS, 0.25 ml 5% DMSO & 12% glucose in BCW	1082
2	bovine serum albumin subst. for FBS	1045
3	12% glucose omitted	943
4	BCW subst. for FBS	897
5	BCW subst. for FBS, 12% glucose omitted	792
6	Dilution 1: #1 with 2 ml FBS and 6 ml 20% glucose in BCW	982
7	bovine serum albumin subst. for FBS	744
8	BCW subst. for FBS	828
9	20% glucose omitted	249
10	BCW subst. for FBS, 20% glucose omitted	177
11	Dilution 2: #3 with 2 ml FBS and 6 ml 20% glucose in BCW	840
12	bovine serum albumin subst. for FBS	786
13	BCW subst. for FBS	756
14	20% glucose omitted	184
15	BCW subst. for FBS, 20% glucose omitted	125
16	Glucose gradient: #6 with 3 ml BCW & 3 ml BCW-glucose	539
17	4.5ml BCW & 1.5 ml BCW-glucose	384
18	#11 with 1.5 ml BCW & 4.5 ml BCW-glucose	698
19	3 ml BCW & 3 ml BCW-glucose	515
20	4.5 ml BCW & 1.5 ml BCW-glucose	351

standardized with 100 and 500 mOsm salts

FIGURE 6

Osmolarity of Freezing and Dilution Solutions

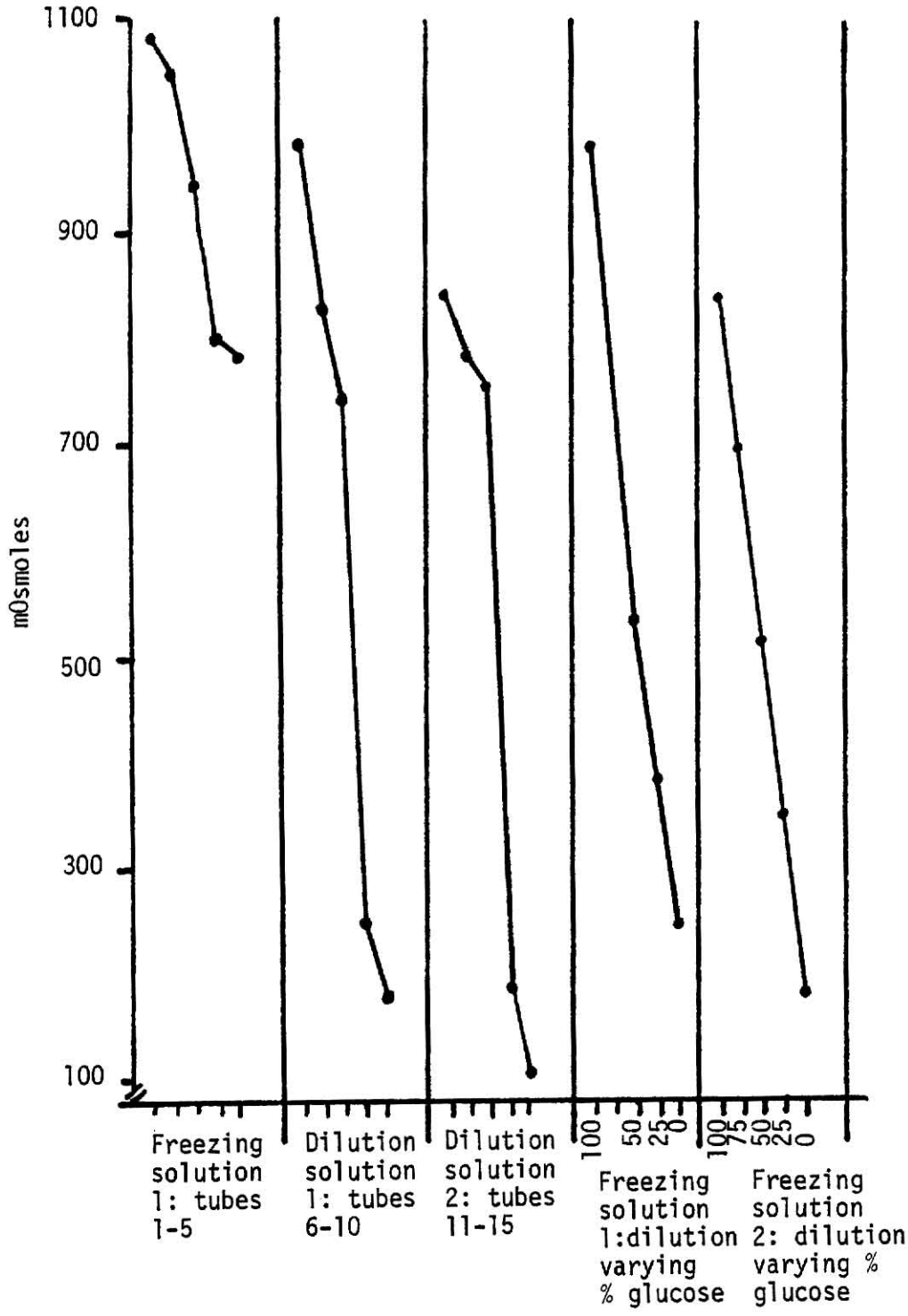


TABLE 7

Freezing Results

Days Frozen	Freezing Variables					Thaw Variable		Survival	
	Glucose	FBS	postseeding incubation (min)	%DMSO	Methyl cellulose	Thaw rate	post-thaw incub. (min)	FDA	Replic.
* 40	+	+		5	-	F[a]		+	
* 7	+	+		5	-	M	30	63	
* 13	+	+	30	5	-	S	79		++[b]
	+	+	30	5	-	M	70		+/-
	+	+	30	5	-	S	40		+/-
*	+	+	30	5	-	M	27		++
	+	+	30	5	-	M	20		-
* 12	+	-	5	5	-	S	130		+
*	+	-	5	5	-	S	108		+
	+	-	5	5	-	S	86		-
	-	+	5	5	-	M	70		+/-
71	-	+	5	5	-	S	274		-
	-	+	5	5	-	S	265		-
	-	+	5	5	-	S	260		-
* 87	+	+	7.5	5	-	S	72	13	
	+	+	7.5	5	-	M	82	0	
*	+	+	7.5	5	-	S	64	42	
	+	+	7.5	5	-	M	79	0	
	+	+	7.5	5	-	S	68	0	
28	+	+	5	5	-	M	56	8.5	-
1	+	+	19	7.5	-	S	60		-
	+	+	19	7.5	-	S	60		-
	+	+	19	7.5	-	S	60		-
	+	+	19	7.5	-	S	60		-
* 9	+	+	25	6	+	F	60	32.7	+
	+	-	25	6	+	F	60	18.9	+/-
14	+	+	25	6	+	M	64	0	+/-
	+	+	25	6	+	M	64	0	+/-
	+	+	25	6	+	M	64	0	-
	+	+	25	6	+	M	64	0	-
17	+	+	25	6	+	S	148	0	+/-
*	+	+	25	6	+	S	126	24.9	+
*	+	+	25	6	+	S	129	20.2	+
26	+	+		5	+	S		0	
	+	+		7.5	+	S		0	
*	+	+		5	-	S		57.7	
*	+	+		7.5	-	S		75	
	+	+		6	+	S		0	
* 27	+	+		7.5	+	F		17	
	+	+		5	+	F		0	
*	+	+		5	-	F		11	
	+	+		7.5	-	F		3	
	+	+		6	+	F		0	

a. S= 5 to 33°C/min; M= 39 to 85°C/min; F= 86 to 112°C/min
b. ++ = very dense tomites; + = dense; +/- = rare; - = absense
* = successful freezing conditions

from 5 to 30 min and did not correlate with survival.

Components which varied with DMSO concentration were the presence of methyl cellulose as a concentrating agent, the thaw rate, and the post-thaw incubation time. At 5% DMSO, methyl cellulose could not be used to concentrate the cells, and a slow thaw rate with 1 hour of incubation was used. An average of 37% of the cells survived. At 6% DMSO, methyl cellulose enhanced survival and either a fast thaw rate with 1 hour incubation or a slow thaw rate with 2 hours incubation could be used. Twenty-six percent of the cells survived. The data at 7.5% DMSO were less abundant, and the need for methyl cellulose and the most effective post-thaw incubation times were not clearly defined. These results are tabulated in Table 8.

Discussion

The success of a freezing system depends on overcoming the osmotic changes encountered during cooling and warming. One must provide components in the cell suspension to minimize the impact of water loss and increased solute concentration without overcompensating, which would result in dehydration of the cells.

The cooling rate determines the degree of osmotic stress to which the cells may be exposed, and how much time the cryoprotectant has to remove intracellular water. If cooled too rapidly, enough water may not leave the cell before it freezes and the membrane will be disrupted (46, 52, 54). Controlled cooling rates introduced the alternative problem of cooling too slowly. The cell may be severely dehydrated before freezing. Or, solutes may build up in the cell from water loss and kill the cell before it would die of dehydration (45, 46). Less controlled cooling systems

TABLE 8

Successful Freezing Conditions

% DMSO	Days Frozen	Glucose (1.5%)	Fetal Bovine Serum (37.5%)	Methyl Cellulose (1.25%)	Thaw Rate	Post-thaw Incubation (min)	% Survival
5	7-87	+	+	-	Slow	73	37
6	9-17	+	+	+	Slow	127	26*
6	9-17	+	+	+	Fast	60	
7.5	26-27	+	+	-	Slow		46**
7.5	26-27	+	+	+	Fast		

* survival for all 6% DMSO

** N=2, survival for all 7.5% DMSO

than the one presented here have been successful with ciliates (28), cooling from 20 to -20°C in one step and -20 to -196°C (liquid nitrogen) in a second step. This was not tried here. I. multifiliis is a large cell and requires more time to adjust water and solute concentrations than do smaller cells. Stirewalt, et. al. (63) found the same conditions to be necessary in freezing schistosomules of Schistosoma mansoni.

The cryoprotectant is the primary defense against freezing damage. The purpose is to aid the cell in water loss while protecting it from possible harmful effects of the process. There are both penetrating and nonpenetrating cryoprotectants. DMSO is a penetrating cryoprotectant and works by reducing electrolyte concentration and allowing osmotic removal of water at low enough temperatures so as to minimize damaging effects from concentrated solutes (45). Nonpenetrating cryoprotectants include glucose, sucrose, and glycerol (which penetrates mammalian cells). Dalgleish, et. al. (14), selected DMSO over glucose and sucrose for the haemoprotozoan Babesia rodhaini because the penetrating cryoprotectant significantly increased survival.

The concentration of cryoprotectant must be balanced between the toxic and protective levels. The DMSO concentrations recommended for ciliates were between 6 and 10% (13). Hwang, et.al. (28) used 10% DMSO for ciliate Tetrahymena pyriformis. The cells need only survive 5 to 10 min in DMSO concentrations used in the freezing solution, which was the criterion Morris, et. al.(50) used to select DMSO concentrations. The cells need to survive several hours in the post-thaw dilution concentration of DMSO, which is what Daggett (13) suggested for selecting DMSO concentrations to use. Dalgleish, et. al.(14), found DMSO to be more toxic at room temperature than at 0 or -5°C, which was also

observed in this study. The DMSO levels at 5 to 7.5% in this study were not immediately toxic to cells at 0°C. Post-thaw levels of 1% DMSO also were not toxic over several hours.

The thawing rate was not frequently discussed in cryopreservation literature as an important variable in other freezing protocols. Many used 35-40°C water baths and thawed samples in 1-2 min (14, 28, 60, 63). The method by Leibo and Mazur (37) used a room temperature bath (20°C) to thaw cells more slowly. O'Dell and Crowe (54) suggested a connection between cooling and thawing rates. Rapid cooling followed by warming too slowly would aggravate the damage done by intracellular ice and reduce survival. Conversely, the methyl cellulose in this study may have reduced dehydration in higher DMSO concentrations (6% and 7.5%) so a slow warming rate with prolonged post-thaw incubation could handle the dehydration stress.

The post-thaw incubation in the presence of DMSO is not a common variable in other methods. Most methods dilute the DMSO to less than 0.1% very rapidly and/or wash it out directly after thawing (e.g. 50, 64). Leaving the cells in hypertonic solution may assist in slow recovery of solute and water balance control before the solution is reduced to the osmolarity of the usual growth medium (37). Stirewalt, et. al.(63), incubated cells 30 min before washing out the freezing solution. In this study, the period of post-thaw incubation improved survival, the duration of incubation depending on the DMSO concentration and the thaw rate. The incubation at osmolarities higher than the usual BCW environment may have provided a stepwise rehydration. By varying glucose concentrations in the dilutions, the osmolarity could be changed to improve rehydration conditions and, therefore, cell survival.

The freezing and thawing conditions in which the parasite survival was the highest favored cells within a specific size range. The parasite size ranged from 250 to 1000 µm and the cells at either extreme were cooled too slowly and too quickly, respectively. The average-sized parasites survived best under conditions used because water loss occurred slowly enough so the cells were frozen before they became dehydrated. Using the mesh filters mentioned earlier to not only concentrate the parasite but also reduce size variation would probably increase survival of those cells that are frozen (63).

To improve this freezing system, I. multifiliis should be separated from the fish debris and a buffer should be added to the freezing solution. By separating the cells, the concentration of cells could be more closely controlled and the best concentration used. Too many cells decrease the cryoprotectant's ability to interact with each cell and facilitate water loss, resulting in increased proportions of cells with intracellular ice. Too few cells may allow too much exposure to concentrated salts released into solution and kill a higher percentage of cells (46). Kosinski's (33) method of ciliate sterilization may work for cysts, and perhaps for trophozoites if they can be slowed down long enough. Leibo and Mazur (37) and Daggett (13) used balanced salt solution to buffer their freezing solutions. Stirewalt, et. al. (63), buffered the medium for schistosomules with lactalbumin hydrolysate medium with Earle's salts.

The thawed I. multifiliis should be tested, eventually, for cell surface antigenic determinants. Uspenskaya and Lozina-Lozinsky (67) found surface antigens to be rearranged after thawing as opposed to unfrozen controls. Two antigens were the same between the frozen and control cysts. One antigen on the control was not on the frozen cysts

while two antigens on the frozen cysts were absent from controls. They did not test the possibility of the two antigens on frozen cells being subunits of the third antigen on the unfrozen cells. The degree of change in antigenic structure would be instrumental in determining usefulness of frozen cells in immunological studies.

The freezing system described here for I. multifiliis is a start for developing a high yield cell culture system. The constants in this study including freezing rate, using glass screw-cap tubes as the freezing vessel, and the dilution components may need modification to improve survival, with accompanying adjustments in DMSO concentration, glucose concentration, and the number of cells per tube frozen. These components are highly interdependent and alterations in one precipitates the need to change the other components.

OBSERVATIONS AND EXPLORATORY STUDIES

Laboratory Culture

Channel catfish served as parasite hosts in this study. Stocks came from the Tuttle Creek Reservoir Fisheries Research Laboratory, creeks around Tuttle Creek Reservoir, and the Culver Fish Farm (McPhearson, Kansas). The first two supplies were primarily fingerlings. The hatchery fish were six to eight inches (15-21 cm) long. The pond and creek fish were assumed to have not been previously exposed to I. multifiliis, although this was not borne out during parasite culturing attempts. The third group was known to be naive, and were fairly consistently susceptible to the parasite. Optimal size was 4 to 6 inches (10-15 cm). The fingerlings frequently died from stress, bacteria, and/or fungus before exposure to the parasite, or before the parasite could complete development. Mature trophozoite numbers were also low on those fish which carried the infection to term, and many had to be sacrificed to obtain sufficient cells for freezing and other experiments. Fish over eight inches (21 cm) had to be exposed to a large number of tomites or they would recover and resist carrying large parasite loads.

To reduce mortality from other diseases, such as Aeromonas hydrophila and Costia, fish from the Fisheries Laboratory and local creeks had to be treated as soon as they were brought into the laboratory. Treatments of salt (dip, 1.5ppt, 120 min) and formalin (dip, 1:4000 v/v, 60 min) were tried. Both reduced other sources of mortality. Results of the treatments are in Table 9. When the salt dip was followed by the formalin dip 24 hours later, the fish not only survived to carry an infection, they also carried it longer, allowing the trophozoites to grow larger. The formalin dip and

TABLE 9

Time Channel Catfish are able to Support Parasite Given Different Preliminary Treatments

	No Treatment	Salt Dip	Formalin Dip	Both
	3 days	2 days	5 days	5 days
	3	3	6	5
	4	3	7	4
	4	4	7	3
	7	7	7	8
	7	8	3	6
	5	9	3	5
	4	10	3	5
	7	14	8	6
	4	8	10	6
	5	11	11	7
	6	8		6
	6			13
	5			13
	5			11
	5			14
	5			6
	5			8
	5			9
	4			
	5			
	5			
No. dying from other causes	21	12	2	4
Average duration ± standard deviation*	5 ± 1.1	7.25 ± 3.5	6.4 ± 2.5	7.4 ± 3.1
% non-*I. multifiliis* mortality	50%	50%	15%	17%
Rank order of preference	4	3	2	1

*none of the duration values are significantly different from the rest.

combined treatment were not significantly different in terms of duration of infection, but the combination appeared to yield more large trophozoites. All fish were allowed to recover for at least 48 hours after the last treatment before exposure to the tomites.

Several foods were used to maintain the fish. The hatchery fish did well on a pelleted diet (components in Appendix I). The Fisheries Laboratory and creek fingerlings refused the pellets and became severely emaciated. Brine shrimp was successful for a few months, but supplies did not keep well in the freezer and water frequently became fouled, with accompanying fungal and bacterial problems. The fingerlings did well on Tetramin flaked food (Carolina Biological Supply).

Both open and closed tank systems were used. The closed system was composed of 45-l glass aquaria with undergravel filters set at slow filtration rates. They were limited in efficacy because of the ability of fish that recovered from sublethal infections to become resistant and protect naive fish put in with them. Hines and Spira (25) also encountered this difficulty. The open system was composed of 150-l stainless steel troughs with a very slow water flow rate and aeration through airstones. In addition to reducing resistance problems, the incidence of disease decreased in both hatchery fish and those from other sources.

The most successful in vivo culture system, then, was similar to that used by Beckert (7) and LeValley (38). New hosts were added to the infected tanks as soon as the old hosts became moribund. The fish were from a known source or "cleaned up" with formalin and salt. They were kept in large, flow-through tanks with a light water flow rate and additional aeration. They were fed at or just below maintenance level so they would be under some stress and be more susceptible when exposed

to tomites. Old hosts were removed no more than 12 hours after losing their trophozoites.

Observed Life Cycle

Fish were maintained in conditions outlined above. The life cycle was measured in time from exposure to tomites to the natural death or sacrifice (usually at stage 5 or 6 of the disease) of the host fish, or loss of the trophozoites (recovery). The fish's health and size, and the number of tomites to which it was exposed all varied the severity of the infection and the maturity of the trophozoite when it left the host. Trophozoite maturation refers to the trophozoite reaching sufficient size and energy reserves to produce tomites. These variables were controlled by using fish within the size range 7 to 16 mm in length. All fish were actively feeding and showed no visible signs of stress or disease condition.

Data are summarized in Table 10. The average maturation time was 5.5 days $\pm$ 2 days. The average was higher than that reported in the literature (4 days), though not significantly. Variation was due to failure to detect some trophozoite loss as a second generation was produced to increase the infection on larger fish. These fish took longer to reach terminal stages of the disease and the recorded parasite carrying time is longer. For the majority of the fish, one generation of the parasite was sufficient to kill, and some died before the trophozoites were fully mature.

Interspecific Transfer of *I. multifiliis*

The existance of strains or subspecies within *I. multifiliis* is an issue with evidence for both sides. Morphological characteristics and the ability to transfer the parasite from one host species to another are

TABLE 10

Time in Trophozoite Maturation

Tank 1	Tank 2	Tank 3
4	6	4
7	7	8
9		2
6		9
7		5
5		4
6		4
3		6
7		4
4		5
9		7
3		
2		
7		

Average life cycle = 5.56 days $\pm$ 2 days

Range: 2 to 9 days

criteria descriptively measuring genetic variation in this species. The more variation, the more likely it is that subgroups do indeed exist.

Transfer of I. multifiliis from tropical fish to channel catfish was accomplished several times in this study. This suggests that there may be only minor differences between groups of the parasite. Hypotheses to explain the divergent observations of this and other studies are discussed.

Materials and Methods: Tropical fish with I. multifiliis were obtained from local aquarium shops. These were added to naive fingerling channel catfish in 45-l tanks with undergravel filters. The fish were on a Tetramin diet. Infected channel catfish were introduced to naive fish in the same conditions. The time from exposure to the tomites to the time the fish either died or lost the trophozoites was considered one generation. The number of generations between the original parasite population to the establishment of the parasite on the fingerling channel catfish was a measure of the adaptation needed in the parasite population to efficiently infect catfish. An established infection was one which was able to create a lethal condition on more than 90% of the host fish.

Results: The results are shown in Table 11 and detailed in Table 12. The transfer from a channel catfish source generally took one generation, two if the original parasite numbers were low. The transfer from tropical species took three generations. Virulence was light in Xiphophorus to extremely virulent in Symphysodon parasite populations. The tropical fish and channel catfish sources were not significantly different in adaptation time or virulence.

Discussion: The three generations needed to establish tropical fish I. multifiliis in channel catfish was more related to building up the population from the very low number of parasites on the small tropical fish

TABLE 11

Generations Needed to Transfer I. multifiliis to Channel Catfish

Donor Host	No. Generations	Virulence
Channel catfish (*Ictalurus punctatus*)	1 or 2	light to heavy
Platys (*Xiphophorus variatus*)	3	light to moderate
Brown Discus (*Symphysodon aequi fasciata*)	3	moderate to heavy
Plecostomus punctatus	3	light
black molly and guppy	3	very heavy

TABLE 12

Adaptation of *I. multifiliis* to Channel Catfish from Tropical Fish Hosts

Xiphophorus variatus (Meek) "Platy"

	Day	
first generation	1	naive catfish exposed to infected platys
	2-3	trophozoites released from platys
	4	first signs of infection
	7	some catfish died of infection, others lost trophozoites
second generation	10	more catfish added; show no signs of infection
	16-18	fish died from infection
third generation	24	fish from nest group of fish died from infection
fourth generation	28	next group died

Symphysodon aequi faciata "brown Discus"

	Day	
first generation	1	trophozoites left Discus
	5	catfish infected
	9	trophozoites left catfish
second generation	11	trophozoites visible on catfish
	14	trophozoites dropped off some catfish
	16	fish died
third generation	17	fish exposed on day 14 all infected
	18-19	fish died

continued in 4 to 5 day cycles with moderately lethal infections

Mollies and guppies

	Day	
first generation	1	mollies and guppies added to catfish
	2-8	trophozoites dropped off tropical fish and returned without visibly infecting catfish
second generation	8	more catfish exposed
	15-16	first and second groups of catfish died
third generation	17	new catfish exposed
	23	fish died of infection
fourth generation	31	next group died

than to poor infectivity on the new host. There was variation in the tropical fish parasite virulence on channel catfish, but intraspecific populations exhibited a similar range in virulence.

Failure to transfer I. multifiliis from one host species to the next has been reported. A FAO report (56) cites a study in which a "strain" obtained from goldfish was highly lethal to goldfish and most other species, while a channel catfish "strain" was only virulent for catfish. This suggested a wide variation in virulence indicating possibly genetically different groups of I. multifiliis. Nigrelli (53) noted I. multifiliis occurs over a wide geographical range and in many environmental conditions and concluded this was evidence of great genetic variation in the species. He also noted that, while reproduction and development rates vary with ambient temperature, optimal temperatures vary for different populations in accordance with local environmental temperature ranges. He suggests this indicated the existance of physiological races. The shape of the macronucleus is generally described as U-shaped, but Nigrelli found it to vary from crescents to spirals in samples collected from temperate to tropical regions, further supporting his hypothesis. LeValley (38) reported a failure to transfer I. multifiliis to channel catfish from tropical fish hosts, indicating a host-range specificity.

Evidence refuting the existance of subgroups also has been reported. Hines and Spira (21) observed that morphology and development were identical in two cultures isolated in their laboratory and in a culture isolated 30 years previously on a different continent. They concluded the parasite was very stable in virulence and morphology. H.E. Klaassen (pers. comm.) has observed six species simultaneously infected with what was most likely a single innoculum. The species were channel catfish, bluegill, white

crappie, black crappie, green sunfish, and a hybrid sunfish. This suggests the parasite has a broad host range.

The data here do not support the idea of species-specific strains of I. multifiliis. Fish in temperate regions may carry a temperate form of the parasite which can infect most temperate fish. Tropical fish, then, would have temperate I. multifiliis when they are kept in temperate areas. Likewise, fish in tropical regions may be hosts for a tropical form. The parasite may be specific for environmental parameters rather than host species. Future studies on the genetic and biochemical nature of the I. multifiliis found in different parts of the world may shed more light on the degree of diversity within the species.

Immunity of I. multifiliis

Fish are capable of specific and nonspecific immune defenses against bacterial (20), viral (20, 55), and parasitic (42) infections. Corbel (11) and Ingram (30) have reviewed specific and nonspecific mechanisms in the fish immune system. The epithelial mucal layer is both specific and nonspecific in function and has been implicated in immunity to I. multifiliis (25). Serum antibody against I. multifiliis has also been demonstrated (4, 7, 8). In order to culture this parasite, the hosts must not be able to resist infection, or must have defense mechanisms depressed. In order to protect fish populations, a means by which these mechanisms may be enhanced must be developed.

This study examined sera from infected and normal fish for their effects on trophozoites and tomites. Mucus samples processing for further protein characterization was done.

Materials and Methods: Six channel catfish were made resistant to

I. multifiliis using the method of Hines and Spira (25). Two of the fish died in the process (see Table 13). A fifth fish was added to the group after it resisted two consecutive challenges. The fish were maintained on a Tetramin diet in a 45-l glass aquarium with undergravel filter.

Mucus was collected weekly. Fish were placed one at a time in 125 ml of dechlorinated tap water. The samples were kept frozen until all were collected. They were concentrated to 10 ml and assayed for protein concentration with the Bio-Rad Standard Protein assay (see Table 14). Naive fish mucus was collected in the same way.

Blood was collected via cardiac puncture from two 0.45 kg channel catfish heavily infected with I. multifiliis. Normal blood was collected from 1.1 kg unexposed catfish. The blood clotted and was centrifuged to obtain serum. Parasites from the infected fish were used in the experiments. Serum was diluted 1:2 or 1:3, or not diluted at all. Three to five trophozoites were added to each dilution and observed until they died.

Results: The mucus was shown to contain sufficient protein to be studied further on electrophoretic gels (Table 15 and Figure 7). The serum results are in Figure 8. The immune sera contained a substance that slowed, stopped, and killed the trophozoites. Normal sera lacked this substance.

Discussion: The lethal substance in the infected fishs' sera seemed to have appeared during the disease process, since it was absent in unexposed fish. Fish are known to have serum IgM, which was named according to its tetramer structure and immunological function (30), not as a counterpart of the mammalian IgM. If the lethal substance were IgM, the serum may be the source of protective properties of the

TABLE 13

Immune Fish History

Fish were treated with formalin (1:4000) and Tetracycline (50 mg/ml; 96 hr) for Aeromonas hydrophilia. Other fish with the same treatment used in other aquaria were able to carry full-term infections

Day	Observation
1	six channel catfish fingerlings exposed to I. multifiliis from channel catfish and Plecostomus.
2	more I. multifiliis added from another tank where infection progressing strongly
8	a few trophozoites had appeared on fins and dorsal portion of all six fish and had fallen off.
11	second generation on all six fish
13	three had lost the trophozoites, three still developing infection
15	trophozoites fully mature on second group of 3.
18	One of the three recovered, the other two still infected
23	Trophozoites left the other two infected fish
28	The two that just lost the infection developed a strong infection and died.
30	Some I. multifiliis appeared on the remaining four fish.
35	Trophozoites gone from all fish.
36	Challenge with I. multifiliis from a virulent culture
38	No signs of infection
52	No signs ever appeared
85	First mucus collected
6 months	Challenged fish with virulent I. multifiliis No infection appeared.

TABLE 14

Bio-Rad Protein Assay

Standard:

Dilutions of Bovine Gamma Globulin from 0.2 to 1.4 mg/ml.

Proceedure:

0.1 ml standard or unknown in tube

5 ml dye reagent, diluted 1:5 in distilled water

vortex very gently

let sit 5 to 60 min at room temperature

measure Optical Density at 595 nm vs a reagent blank

Plot $O.D._{595}$ vs standard protein concentrations. Read unknowns from this curve.

epithelial mucus, also known to have IgM (25, 30). The serum has yet to be proven the source of mucal components (42).

The mucus layer of the fish covers the entire exterior surface of the body. The mucopolysaccharide components are important chemical barriers to parasitic, fungal, and bacterial colonization. Skin mucus has been implicated in specifically resisting or killing certain ecto-parasites (25, 30). The mucus of catfish immune to I. multifiliis probably contains protective properties. Fish previously unexposed to I. multifiliis placed in the aquarium with immune fish did not become infected upon exposure to the parasite. The immune mucus secretions into the aquarium water may be sufficiently concentrated to protect all the fish. Or, these secretions may elicit a response in the unexposed fish providing temporary resistance. It is possible the lethal substance found in serum is also in mucus and released into the aquarium.

Immunization and chemical treatments have been investigated for treating I. multifiliis infections. Immunization by injection of the antigen has been shown to be effective in fish (4, 31, 55) but very costly if considered for a hatchery or farm pond. Chemical treatments have been effective and are being improved (some listed in 49 and 56). A promising, inexpensive chemical treatment was malachite green (0.1 ppm w/ or w/o 15 ppm formalin) but it is no longer permitted for use on food fish since it has been demonstrated to be carcinogenic. Recently, Farley and Heckman (16) found silver nitrate to be effective against tomites while harmless to fish.

Promoting production of the lethal substance in mucus would be an effective and safe means of controlling I. multifiliis infections. This would be especially beneficial in the spring and summer months when the

Figure 7

Bio-Rad Protein Standard Curve

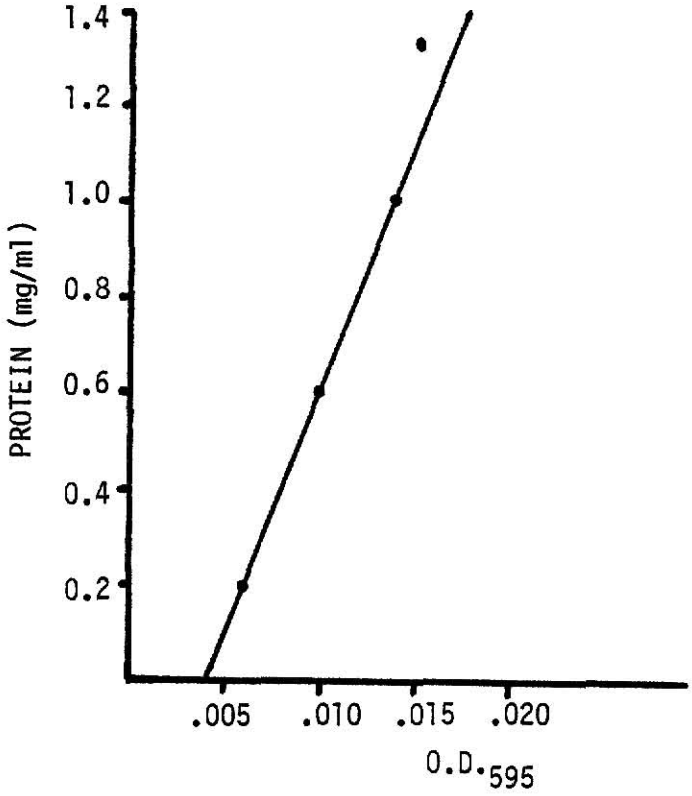


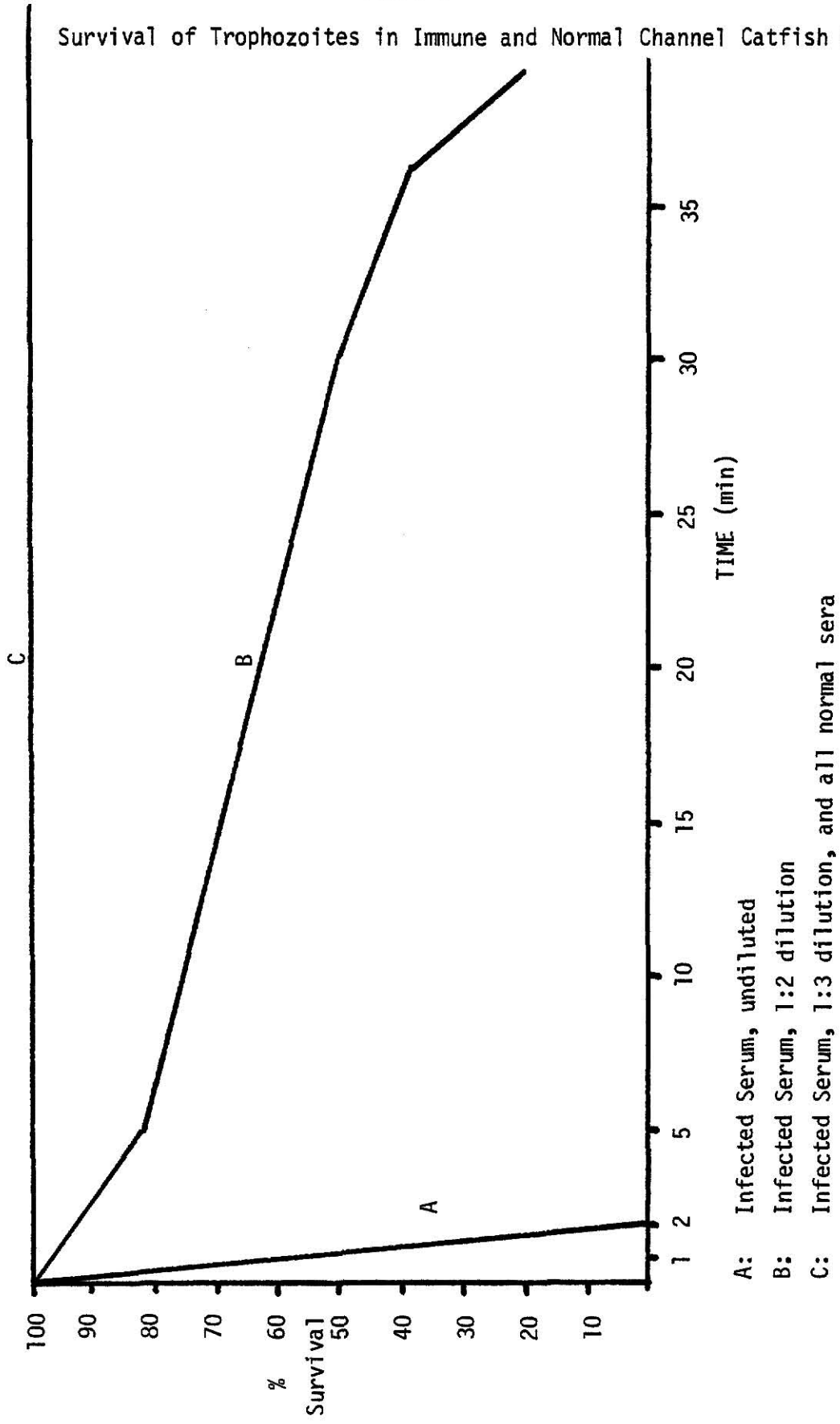
TABLE 15

Bio-Rad Assay Results

Standard		$O.D._{595}$	Protein
1.35 mg/ml		0.015	
1.01		0.014	
0.61		0.010	
0.20		0.006	
Unknowns			
Normal	1	0.013	0.91 mg/ml
	2	0.022	1.88
	3	0.017	0.34
Immune	1	0.011	0.71
	2	0.016	1.24
	3	0.013	0.91

Figure 8

Survival of Trophozoites in Immune and Normal Channel Catfish Sera



protective efficiency of mucus is the greatest and I. multifiliis is most abundant (42, 48). A problem would arise in early spring and late fall when ambient temperatures are below the optimum for fish immune response but the parasite is still able to infect the fish. At these times, an integrated system using natural and chemical controls would be necessary.

Two possible mass immunization techniques have not been tried. First, irradiation of tomites to permit infection but stop the parasite before it reaches maturity parallels other successful immunization techniques. Gould, et. al. (18), and Levin and Evans (39) used cobalt-60 and X-irradiation, respectively, on Trichinella spiralis. When fed to white rats, an intestinal infection was established, but larval production was reduced. Resistance to subsequent infection was conferred. Jarrett and Sharp (32) irradiated Dictyocaulus viviparus and produced resistance in calves. In preparing irradiated tomites, the number of tomites per dose and the irradiation to which they would be exposed would need to be determined to ensure the fish is sufficiently exposed to the tomite antigen without being killed. In a pond, not all the fish would have to be resistant to protect the population. In making a majority of the fish resistant, mucus secreted would be concentrated enough to protect the whole population.

The second immunization technique was used by Anderson, et. al. (3), to protect rainbow trout (Salmo gairdneri) from Yersinia ruckeri and Aeromonas salmonicida. Simply adding the antigen to ambient water induced antibody-producing cells. Ground trophozoite antigen may be able to induce a similar response.

Serum immunoglobulins have been demonstrated in fish (4, 7, 31, 40, 55, 62). They were specific for the antigen to which the fish had been

exposed and were able to work with complement in order to eliminate the antigen. The ability of the fish to mount an immune response was correlated to ambient temperature, optimum response occurring at optimum temperature for the host species (62). O'Neill (55) demonstrated an upper limit in the extent of the immune response to a specific maximum.

Serum components able to agglutinate I. multifiliis were found in fish exposed to sublethal numbers of trophozoites or injected with antigen prepared from the trophozoites. These fish were all able to resist subsequent exposure to lethal numbers of parasites (4). Beckert (7) tested serum from immunized fish and found it to be lethal to trophozoites and tomites, but not to cysts. Ouchterlony double diffusion plates showed a precipitin reaction between immune sera and the trophozoites or tomites, indicating a serum compoment in immune fish reacts with I. multifiliis surface molecules.

The serum experiment confirmed Beckert's (7) observation that immune sera kill trophozoites and tomites. This experiment also demonstrated that it is a component not present in normal sera, since normal sera did not kill the parasite. If the lethal component is present in mucus, it could be characterized, and ways found to promote its production to protect fish from I. multifiliis infection.

AKNOWLEDGEMENTS

I wish to thank my major advisor, Merle F. Hansen, for his patient support, advice, and encouragement, without which this project could not have been done. I also thank my advisory committee, L. Scott Rodkey, G. William Fortner, and J. Kelley for their invaluable help. Thanks also to other laboratories in the Division of Biology that provided equipment and assistance: T.C. Johnson, R.A. Consigli, P.P. Wong, H.E. Klaassen, and G.W. Conrad. Thanks also to J.L. Copeland, Department of Chemistry, for advice on designing the controlled cooling system. M.D. Wang and M.B.McNerney assisted in laboratory work. S.P. Seymour was very helpful in providing I. multifiliis cultures. Most of all, I thank my parents, Alfred and Margaret Beeler, Ron Taylor, and my roommates Lois, Judy, Helen, and Sherry for patiently and enthusiastically encouraging me in this effort. My puppy, Heidi, kept me sane through the hours of work by keeping me company in the laboratory.

This project was funded by an Agricultural Experiment Station grant (#R028). Personal support was provided by the Division of Biology through a graduate teaching assistant appointment.

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APPENDIX I

Pellet Fish Diet

Ingredient	Amount per ton
Fish meal, menhaden, minimum protein 60%	240 lb.
Blood meal, minimum protein 80%	200 lb.
Soybean meal, solvent, toasted, dehulled, 50% protein	400 lb.
Dried distillers solubles or dried fermentation solubles	160 lb.
Wheat shorts, 12% protein, 12% fat, 12% fiber or better	700 lb.
Wheat mill dust (passing U.S. #80 mesh)	150 lb.
Dehydrated alfalfa, reground 17% protein pellets	70 lb.
Mineralized, iodized salt	20 lb.
Vitamin premix (below)	10 lb.
Pellet binder	50 lb.

Vitamin Premix (on finely ground soybean meal carrier)

	guaranteed potency per ton of feed
Vitamin A activity (from palmitate in gelatin beadlets)	5,000,000 IU
Vitamin D_3 activity	1,000,000 IU
Alpha tocopherol acetate	20 g
Menadione sodium bisulfite	20 g
Choline chloride	1000 g
Niacin	50 g
Riboflavin	10 g
Pyridoxine	5 g
Thiamine	5 g
D-calcium pantothenate	20 g
Biotin	200 mg
Folic acid	1000 mg
Vitamin b-12	20 mg
BHT antioxidant	10 mg

STUDIES ON *ICHTHYOPHTHIRIUS MULTIFILIIS* FREEZING APPARATUS, CRYOPRESERVATION LABORATORY CULTURE, AND INTERSPECIFIC TRANSFER

by

CATHERINE REVELL BEELER

B.S., University of California, Davis, 1978

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

PARASITOLOGY

Interdepartmental Program

Division of Biology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1981

Ichthyophthirius multifiliis, a lethal fresh water fish ectoparasite, is a major cause of mortality in fish hatcheries, farm ponds, and tropical fish. It is endemic in tropical and temperate regions worldwide. The life cycle is completed in 3 to 4 days at optimal temperatures of 21 to 24°C, and can take as long as a month at 4°C. The pathology of infection is similar to other ectoparasitic diseases. The fish gradually become weak, produce massive quantities of epithelial mucus and leukocytes, and die of toxic nitrogenous waste accumulation, osmoregulatory failure, and disruption of gas exchange through the gills. The parasite is cultured by in vivo serial passaging in previously unexposed (naive) fish. The fish must be kept in flow-through tanks to prevent accumulation of immune mucus secretions and be large enough to carry an infection to term while small enough to succumb to infection.

To control freezing, an apparatus was constructed to control heat flux between the liquid nitrogen and the ethanol cooling medium in which the cell tubes were suspended. A Dewar flask was modified so a vacuum pump could be attached and the relationship between cooling rate and the vacuum drawn from the flask was calculated. To cool at -1°C/min, 23.75"Hg vacuum was drawn for 5 tubes or less, or 23"Hg for 10 tubes or more.

I. multifiliis cells were harvested 4 to 6 hours after the host was pithed. They were concentrated by centrifugation, cooled on ice, and 1-ml aliquots placed in glass screw-cap tubes. 0.75 ml fetal bovine serum (FBS) and 0.25 ml of a 40% DMSO-20% glucose solution in biologically conditioned water (BCW) were cooled on ice and added to each tube. The tubes were incubated at -5°C, seeded to initiate extracellular freezing, and incubated 5 to 30 min before placing them in the controlled cooling unit. They were cooled at -1°C/min from -5 to -45°C, then plunged in liquid

nitrogen and stored. Cells in 5% DMSO were thawed slowly in a 2°C ice slush and incubated 1 hour before washing 2 or 3 times with BCW. Cells in 6% DMSO required methyl cellulose (2.5%) in the freezing solution and could be thawed rapidly with 1 hour incubation or thawed slowly and incubated for 2 hours. Cells in 7.5% DMSO did not seem to require methyl cellulose and could also be thawed rapidly or slowly. Survival ranged from 11 to 75%, averaging 37%, 5% DMSO survival was 37%, while 6% DMSO had 26% survival. 7.5% DMSO cell survival ranged from 17 to 75%.

I. multifiliis was transferred from tropical fish species to channel catfish in three parasite generations. Mollys, guppies, brown discus, and Plecostomus all carried the parasite which was able to infect the channel catfish immediately, but took three generations to kill the host. This supports the hypothesis that in temperate regions, the parasite has a wide host range and cannot be divided into subspecies based on host specificity.

Some catfish became immune to I. multifiliis. They resisted attempts to reinfect them up to 6 months after immunity was detected. They also protected naive fish from infection when placed in the same aquarium. Sera from immune fish killed trophozoites within 2 min while sera from normal fish had no effect on the parasite. A specific substance, possibly IgM, is induced in fish exposed to I. multifiliis.

The cryopreservation of I. multifiliis will allow more complete studies into the antigenic structure, in vitro culture, and treatments for ichthyophthiriasis.