Use of Fish Cell Cultures for the Study and Cultivation of Microsporidia

by

S. Richelle Mader Monaghan

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Biology

Waterloo, Ontario, Canada, 2011

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be electronically available to the public.

Abstract

Microsporidia are a group of obligate intracellular fungal parasites that infect a wide range of vertebrates and invertebrates, and are of economic and academic interest. Some areas of their economic impact are in aquaculture where they can infect salmon and other fish species. In agriculture they have been considered as control agents for insect pests, but more importantly as likely contributing to colony collapse disorder of bees. As an academic topic, microsporidia are fascinating because they are the smallest and simplest eukaryotic cells and require eukaryotic host cells in order to complete their life cycle. Therefore one research avenue that moves forward both economic and academic interests is to use cultures of animal cells to support the growth and development of the microsporidia life cycle, including the production of spores.

Although the use of animal cell cultures for studying the microsporidia of insect and mammals has a fairly large literature, fish cell cultures have been employed less often but have had some successes as reviewed in this thesis. Very short-term primary cultures have been used to show how microsporidia spores can modulate the activities of phagocytes. The most successful microsporidia/fish cell culture system has been relatively long-term primary cultures of salmonid leukocytes for culturing *Nucleospora salmoni*s. Surprisingly, this system can also support the development of *Enterocytozoon bienusi*, which is of mammalian origin. Some modest success has been achieved in growing *Pseudoloma neurophilia* on several different fish cell lines. The eel cell line, EP-1, appears to be the only published example of any fish cell line being permanently infected with microsporidia, in this case *Heterosporis anguillarum*. These cell culture approaches promise to be valuable for describing the growth and development of the microsporidia and for documenting the responses of fish cells to infection.

In this thesis, cell lines from warm water fish, goldfish, fathead minnow and zebrafish, and a coldwater species, rainbow trout, were explored as potential cellular hosts of two microsporidia species that have never been grown or associated with fish before. One is *Anncaliia algerae*, which is an aquatic microsporidium that most commonly infects mosquitoes. This microsporidia is one of the easiest species to grow in mammalian cells, with the rabbit kidney cell line, RK 13, being the most documented culture system. The

other is *Nosema apis*, which is a pathogen of bees and for which few cell culture systems exist.

The ability of warm water fish cell lines to support the life cycle of *A. algerae* was investigated first. Spores were purified from RK-13 cultures and added to cell lines from three warm water species as well as to an insect cell line. The cell lines were GFSK-S1 and GFB3C- W1 from goldfish skin and brain respectively, ZEB2J from zebrafish embryos, FHMT-W1 from fathead minnow testis, and Sf9 from ovaries of a fall armyworm moth. All cultures were maintained at 27 °C. Infection was judged to have taken place by the appearance of sporonts and/or spores in cells and occurred in all cell lines. Spores were also isolated from ZEB2J cultures and used to successfully infect new cultures of ZEB2J, RK-13 and Sf9. These results suggest that cells of a wide range of vertebrates support *A. algerae* growth in vitro and fish cells can produce spores infectious to cells of mammals, fish and insects. As ZEB2J was the most characterized of the fish cell lines and supported good *A. algerae* growth, this cell line was used in further studies described below to compare the efficacy of antimicrosporidial drugs and to test whether fish cells could support *N. apis* growth, but first *A. algerae* growth at lower temperatures was explored with cell lines from a coldwater fish.

Cultures of cell lines from rainbow trout gill, RTgill-W1, and brain, RTbrain-W1, at 9, 18 and 21°C were evaluated for their ability to support the development of *A. algerae*. For up to 8 days after the addition of spores, living and DAPI stained cultures were examined by phase-contrast microscopy, allowing the identification of the meront, sporont, and spore stages in cultures at 18 and 21 °C. Meronts and sporonts were both spindle-shaped, but relative to meronts, sporonts were darker under phase contrast and brighter after DAPI staining. Spores were egg-shaped, phase- bright and intensely DAPI stained. These stages could not be identified conclusively in cultures at 9 °C, but their appearance at 18 °C sets a new low temperature for the growth of this species. The growth of *A. algerae* at room temperature allowed living cultures to be observed conveniently and videoed with a proprietary instrument, the Riveal microscope (www.quorumtechnologies.com). With this microscope, the development of *A. algerae* life cycle stages at room temperature was confirmed plus for the first time meront division and intracellular germination were captured on video. Spore germination in the absence of host cells and in response to 3 percent

hydrogen peroxide was also observed by Riveal microscopy and for first time an abnormal germination phenomenon was clearly documented: polar tubes were extruded but the spore bodies retained the nuclei.

ZEB2J cultures that had been infected with *Anncaliia algerae* spores were used as an in vitro test system to evaluate the curative actions of albendazole, fumagillin, and three fluoroquinolones; ciprofloxacin, norfloxacin, and ofloxacin. For each drug at concentrations above 50 μg/ml, the viability of ZEB2J cell declined sharply so concentrations of 10 and 20 μg/ml were studied. At these concentrations the drugs had little effect on the morphology and germination *A. algerae* spores. Each of the fluoroquinolones failed to prevent *A. algerae* from infecting ZEB2J cells and from growing to the same extent as in untreated ZEB2J cultures. Adding albendazole or fumagillin to cultures did not prevent *A. algerae* from infecting ZEB2J cells but impeded the growth and accumulation of *A. algerae* life-cycle stages. However, albendazole treatments caused a significant fraction of the ZEB2J cells to have nuclear abnormalities. Fumagillin reduced the intensity of infections within a ZEB2J cell, although the number of infected cells in a culture was not reduced. Over 5 days of infection with *A. algerae* the accumulation of ZEB2J cells in cultures was reduced but fumagillin treatment restored the accumulation to control levels. These results suggest that fumagillin has some potential as a treatment for *A. algerae* infections.

ZEB2J was exposed to *Nosema apis* spores from the western honey bee (*Apis mellifera*). Bees were collected from hives that had been naturally infected and confirmed polymerase chain reaction (PCR) to have *N. apis*. Frozen bees were crushed in water to yield a mixture of bee parts, pollen grains, yeast, and microsporidial spores. The mixture was filtered and then centrifuged through Percoll to produce a pellet of spores that was resuspended in L-15 with 10 percent fetal bovine serum (FBS). Aliquots of this were added to ZEB2J cultures. Cultures were observed periodically for up to 24 days with a combination of phase contrast microscopy and of fluorescence microscopy, usually after staining with 4',6-diamidino-2-phenylindole (DAPI). Although earlier life cycle stages were not observed, structures that were concluded to be either sporonts, sporoblasts and/or spores were seen, but these were in less than 5 percent of the fish cells. These *N. apis* life cycle stages had grown in ZEB2J because some appeared to be inside the cells and often they were arranged around the nucleus of the host cell rather than being randomly distributed in cultures. Despite

repeated rinsing over a three week period, all cultures were ultimately lost due to yeast from the original spore preparations over growing the fish cell cultures.

The overarching observation of this thesis is that fish cells in culture have been shown for the first time to support the growth *A. algerae*, and possibly *N. apis*. This suggests that the cells of vertebrates might support the growth of a wide range of microsporidia species that normally are associated with insects. In turn this suggests restriction of a microsporidial species to a particular animal group is unlikely accomplished at the cellular level but through physiological systems expressed at the organismal level and disturbances in these systems might lead to infections in new groups of animal hosts. The overarching observation of this thesis has two general implications for future studies. Firstly, for studying the expression of antimicrosporidia mechanisms in fish cells, the ZEB2J/*A. algerae* co-culture system promises to be useful. Secondly, for microsporidia species that are difficult to grow in culture, cell lines from a wide range of vertebrate and invertebrate species should be explored and one possibility for *N. apis* is fish cells.

Acknowledgements

It is my pleasure to thank the following individuals for providing me the guidance and inspiration to complete my PhD. I owe my deepest gratitude to my co-supervisors, Drs. Lucy Lee and Niels Bols. Specifically, I would like to thank Dr. Lee for granting me the freedom to experiment, and for supporting my setbacks while celebrating my successes. I would like to thank Dr. Bols for his seemingly untiring enthusiasm for research. His excited response to results, and subsequent challenging line of questioning were both equally inspiring and demanding. To both of my supervisors, I thank them for their valued advice and their vital role in the development of this thesis. I am grateful to my committee members Drs. Brian Dixon, Bill Taylor, Simon Jones and Gary Dmitrienko for contributing their time and ideas to this work.

There are many people I would like to extend my appreciation to who have also contributed their expertise, collaboration and/or technical or administrative support along the way including: Mr. Aaron Frenette, Mr. Dale Weber, Mr. Geoffery Williams, Dr. Michael Duffy, Dr. Michael Kent, Ms. Linda Zepf, Ms. Gena Braun and Dr. Peter Takvorian. Additionally, thank you to Quorum Technologies for use of the Riveal microscope, and to NSERC and University of Waterloo for providing funding through scholarships for this work.

I'd like to thank both former and current lab members: Jaclyn MacDonald (for your advice), Bounmy Inthavong (for your genuineness), Colin Way (for your crazy stories), Bryan Sansom (for your brainstorming, coffee and philosophy), Sophia Bloch (for understanding), Nguyen Vo (for being an inspiration, and for your patience and willingness to help), Rebecca Rumney (for our UNB road trip), and Robbie Smith (for your upbeat whistling in the lab, and stellar driving ability through two hurricanes). It was my pleasure sharing the 3rd floor office with some of my labmates as well as Oana, Yvonne, Scott, Matt and Mike, and I thank them for helping me to remember how to laugh – even when things were not working out. Thank you to the so many others who I worked with and met along the way.

To my Mader and Monaghan families, I am grateful for the love and encouragement you have given me. Lastly, I am indebted to my dear friend Jody Dupuis who has always found ways to support me, even when I didn't know I needed it.

Dedication

This thesis is dedicated to my husband, Mike, whose companionship, encouragement and support have been of tremendous importance to all of my successes over the past 17 years. Mike, my gratitude to you is immeasurable.

I also dedicate this thesis in honor of my son, Jonah, and in memory of my daughter, Hope. Jonah, thank you for sharing your brilliant personality and lighting up my life. May you always have dreams and goals you are excited about pursuing. Hope, your memory inspires me to face challenges with utmost grace. I hope I have, and continue to do so. Thank you both for being my greatest teachers.

TABLE OF CONTENTS

List of Figures

List of Tables

List of Abbreviations

A549	pulmonary cell line
AB	alamar blue or resazurin
AB/AM	antibiotic/antimycotic
AIS	aquatic invasive species
ANOVA	one-way analysis of variance
AO	acridine orange
ATCC	American Type Culture Collection
CaCo-2	human intestinal cell line
CCD	Colony Collapse Disorder
CCL-37	RK-13 cell line ordering number at ATCC
CCO	channel catfish ovary
CHSE-214	chinook salmon embryo cell line
CHQ5B	human muscle fibroblast cell line
CF	carboxyfluorescein
CFDA-AM	5-carboxyfluorescein diacetate acetoxymethyl ester
CMT-93	mouse intestinal cell line
CW	calcofluor white
d	day(s)
DAPI	4’,6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
h	hour(s)
HC	host cell
HN	host nucleus
E6	strain of Vero African green monkey kidney cell
EP-1	Japanese eel epithelial cell line
EPC	carp epithelioma cell line

FBS	fetal bovine serum
FHM	fathead minnow
FHMT-W1	fathead minnow testis cell line
FISH	fluorescence in situ hybridization
GB	granular body
GFB3C-W1	goldfish brain cell line
GFSK-S1	goldfish skin cell line
GI	gastrointestinal
IIIV	invertebrate iridescent virus
IPL-LD-657	silkmoth cell line
L-15	Leibovitz-15 media
LPS	lipopolysaccharide
MB III	mouse lymphosarcoma cell line
MDCK	Madin-Darby canine kidney cell line
MRC-5	human lung fibroblast cell line
MetAP-1	methionine aminopepdidase-1
MetAP-2	methionine aminopepdidase-2
MetAPs	methionine aminopepdidases
Mos55	mosquito larva cell line
MSP	mass spectrometry-based proteomics
MTT	3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
N	nucleus
n	sample size
p	p-value
PBLE	American eel peripheral blood leukocyte cell line
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEA	peritoneal exudates adherent cell line
p.i.	post infection
PKD	proliferative kidney disease
PLP	polaroplast primordium

PPG	posterior pre-germination
P/S	penicillin/streptomycin
PTP	polar tube precursors
RFUs	relative fluorescence units
RK-13	rabbit kidney epithelial cell line
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
RTbrain-W1	rainbow trout brain cell line
RTgill-W1	rainbow trout gill cell line
RTS-11	rainbow trout monocyte/macrophage cell line
SD	standard deviation
Sf9	fall armyworm ovarian cell line
SJD.1	zebrafish caudal fin fibroblast
SPC	sporophorocyst
SPV	sporophorous vesicle
Sua.40	Suakoko mosquito strain larva cell line
TEM	transmission electron microscopy
TNP-40	fumagillin analog
Vero	African green monkey kidney cell line
wk	week(s)
ZEB2J	zebrafish embryo cell line

Glossary of Terms

This glossary of terms outlines relevant terminology of *Anncaliia algerae* and *Nosema apis* microsporidian life cycles.

Anchoring Disc: A structure at the anterior pole of a microsporidian spore that physically connects the polar filament to the rest of the spore.

Anterior pole: On the longitudinal axis of a microsporidian spore, the end of the spore that ejects the polar filament. In ovid shaped spores, the apex is the anterior pole.

Diplokaryotic: the possession of two nuclei per parasite; a feature of some microsporidian species including *Anncaliia algerae* and *Nosema apis*.

Extracellular Infective Phase: The phase of a microsporidian life cycle where the parasite has reached maturity as a mature spore, is released from the host (through digestion, elimination, decomposition, etc), and is capable of infecting a new host.

Germination: The process that mature spores can undergo whereby the polar filament is ejected from the spore, and under normal conditions the spore contents, or sporoplasm, travel through the polar tube. This process is a mechanism of transmission if the polar tube injects the sporoplasm into a nearby cell.

Intracellular Proliferative Phase: The growth and development of meronts and sporonts within a host cell and leading to spore maturation, synonymous with proliferative stage.

Mature Spore: The last developmental life cycle stage of microsporidia that is smaller than earlier developmental phases.

Merogony: A life cycle phase of microsporidia in which meronts/schizonts are developing and dividing.

Meront: The earliest stage of microsporidian growth that occurs within a host cell developing from an ejected sporoplasm and developing into a sporont; synonymous with schizont. A meront is a product of merogony in the microsporidian life cycle.

Polar filament: The helically coiled structure within a microsporidian spore, but sometimes used synonymously with polar tube.

Polar tube: The ejected polar filament from a microsporidian spore, but sometimes used synonymously with polar filament.

Posterior pole: On the longitudinal axis of a microsporidian spore, the end of the spore opposite to the end in which the polar filament is ejected during germination. In an ovid spore the posterior pole is opposite the apex. Using Riveal microscopy, a posterior pre-germination (PPG) vacuole is apparent prior to germination as grey area at the posterior pole.

Posterior pre-germination vacuole (PPG): Using Riveal microscopy, a visible organelle or region in the posterior pole of an ungerminated spore that indicates germination is imminent.

Posterior vacuole: A membrane bound organelle situated in the posterior pole of some species of microsporidian spores and thought to play a role in hydraulic pressure promoting spore germination.

Proliferative stage: A generalized term to refer to microsporidia in merogony and sporogony life cycle phases. It is a phase of development in which meronts and/or sporonts are replicating and lead to parasite maturation.

Schizont: The earliest stage of microsporidian growth that occurs within a host cell developing from an ejected sporoplasm and developing into a sporont; synonymous with meront. A schizont is the product of the merogony in the microsporidian life cycle.

Spore coat: A protective structure enveloping a spore and consisting of three layers; the exospore, endospore and spore coat membrane (superficial to deep). Using TEM, the exospore is electron-dense, the endospore is electron lucent, and the spore coat membrane has a typical membrane appearance.

Sporoblast: A developing microsporidian that has already undergone its last division as a sporont, but has yet to become a mature spore. The organization and development of the polar filament begins when a microsporidian becomes a sporoblast.

Sporogony: A life cycle phase of microsporidia in which sporonts are developing and dividing.

Sporoplasm: The contents of a microsporidian spore generally referring to the contents that have been ejected from the spore through the polar tube, but can also refer to ungerminated spore contents.

Sporont: A sporont is a microsporidian growth stage which develops from a meront and becomes a sporoblast after its last division. Using light microscopy sporonts and meronts are morphologically similar but sporonts appear denser/darker than meronts. Using TEM the sporont is distinguished from meronts with a thick, continuous, electron dense material surrounding the parasite that is thinner and discontinuous in a meront. This electron-dense material is the initial formation of outer spore coat. A sporont is a product of sporogony in the microsporidian life cycle.

Chapter 1

General Introduction

Overview

The use of animal cell cultures as tools for studying the microsporidia of insects and mammals is briefly reviewed, along with an in depth review of the literature on using fish cell cultures to study the microsporidia of fish. Fish cell cultures have been used less often but have had some success. Very short-term primary cultures have been used to show how microsporidia spores can modulate the activities of phagocytes. The most successful microsporidia/fish cell culture system has been relatively long-term primary cultures of salmonid leukocytes for culturing *Nucleospora salmonis*. Surprisingly, this system can also support the development of *Enterocytozoon bieneusi*, which is of mammalian origin and has limited success growing in vitro. Some modest success has been achieved in growing *Pseudoloma neurophilia* on several different fish cell lines. The eel cell line, EP-1, appears to be the only published example of any fish cell line being permanently infected with microsporidia, in this case *Heterosporis anguillarum*. These cell culture approaches promise to be valuable in understanding and treating microsporidia infections in fish, which are increasingly of economic importance.

1.1 Introduction

Historically, animal cell cultures have been essential for studying viral and microbial pathogens of humans and animals. Some pathogens require animal cells in order to complete their life cycle, making animal cell cultures, or the in vitro approach, the most convenient avenue for producing and studying the pathogen. This is most famously true of viruses. Cell lines have been crucial for viral detection and for producing viruses, which then can be characterized by a variety of biochemical and morphological features and used in vaccines (Enders et al. 1949, Hsiung 1989). Animal cell cultures have allowed the single-cell reproductive cycle of viruses to be dissected and have contributed to studies of viral pathogenesis and development of therapeutic agents. Less well known is that some single-cell eukaryotic pathogens also require animal cells to complete their life cycle, and their study is aided by the use of animal cell cultures (Wittner 1999). This is the case with microsporidia. Microsporidia survive only by living in other cells and are found outside of host cells only as spores. In this chapter, the past and future value of in vitro approaches to studies of the microsporidia infecting fish is reviewed after a brief overview of the biology of microsporidia and the in vitro success achieved with economically important microsporidia of insects and with clinically important microsporidia of humans.

Microsporidia classification

Microsporidia are currently included in the Fungi (Hibbett et al. 2007). Although organisms now known as microsporidia were originally identified as fungi, they were reclassified as protozoans by the end of the nineteenth century (Nageli 1857, Pasteur 1870), and this designation was accepted for over 100 years until molecular techniques determining phylogeny returned microsporidia to the Fungi (Hirt et al. 1999, Keeling and Fast 2002, Keeling et al. 2000). The phylum Microsporidia encompass over 1,200 species and almost 150 genera (Franzen and Muller 2001, Wittner 1999). They infect every major animal group, from invertebrates to all classes of vertebrates.

The type of host in which they have been found to infect has long been used as an informal categorization of microsporidia. For this classification, the principal groups have been insect

and human microsporidia. These have been identified and studied because of their economic and clinical relevance. Less intensively investigated, but also of economic importance, are fish microsporidia. Amphibians, reptiles, and birds are susceptible to microsporidia, but the research is comparatively much less on these microsporidial infections (Snowden and Shadduck 1999). In the future, these informal categories may be difficult to maintain because growing evidence, including in vitro approaches, demonstrates that several microsporidia have low host-specificity (Coyle et al. 2004, Lores et al. 2003, Rinder et al. 2000, Sutherland and Stelzig 2004).

Cellular life cycle of microsporidia

Diversity is found among the numerous microsporidian genera with respect to their life cycle in animal cells. Despite the variations, a generalized life cycle can be described (Fig. 1). Microsporidia are obligate intracellular pathogens but have an extracellular infective spore phase of development. The spore contains a specialized structure, the polar filament, which under certain conditions, will eject from the spore and inject infective sporoplasm into the host cell. The injection of sporoplasm commences the proliferative, intracellular phase of the parasite's development. The intracellular proliferative phases of the life cycle are characterized by the developmental stages of merogony and sporogony. Merogony, or the stage of meront development, originates with the infective sporoplasm of a germinated spore and typically occurs in direct contact with host cytoplasm. Meronts proliferate, often through binary fission, and differentiate into sporonts during sporogony. Sporogony is characterized by a number of morphological changes. A thickening of the electron dense plasmalemma surrounding the parasites, for example, is one indication of sporont development (Lom et al. 2000). As well, this stage can occur in direct contact with host cytoplasm but may occur within a membranous envelope that develops of host, parasite, or host-parasite origin (Cali and Takvorian 1999, Lom and Dyková 2005). The developing spore is designated as a sporoblast at its last sporont division and, through metamorphosis, condenses to become a mature spore smaller than its developmental predecessors (Cali and Takvorian 1999). Liberation of mature spores into the extracellular environment occurs when the host cell dies.

Economically important microsporidia of insects

Since the mid nineteenth century, microsporidia have been known as pathogens of economic importance. The initial impetus for scientific research of microsporidia was due to an epidemic threatening the silk industry of Europe. Characterized by black speckling of the silk worm, Pasteur identified the causative agent as *Nosema bombycis* and is credited with devising methods to reduce the impact of the disease (Pasteur 1870). Pasteur's work is believed to be the first scientific investigation of the pathogenic nature of microsporidia. The apiculture industry is also negatively affected by microsporidiosis. Until recently, *Nosema apis* was exclusively the cause of nosemosis in the European honeybee (*Apis mellifera*) (Ellis and Munn 2005). Unfortunately, another microsporidian, *Nosema ceranae*, is an emerging pathogen of the European honeybee (Bromenshenk et al. 2010, Chen et al. 2008, Williams et al. 2008). The cost of nosemosis to the apiculture industry is unclear. However, the economics of pollination services have been estimated to provide a 600% to 700% return on investment for farmers, indicating the potential decrease in profitability if pollinations services are not optimal (Olmstead and Woolen 1987, Kevan and Phillips 2001).

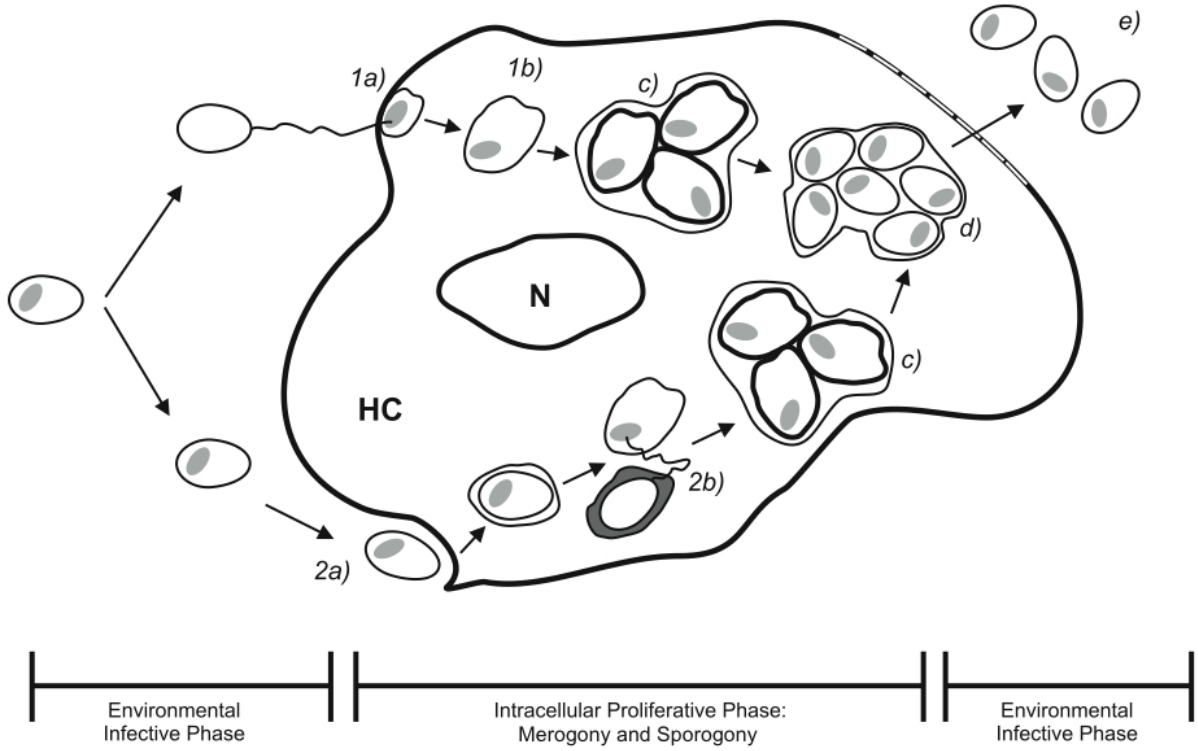

Figure 1.1 Life cycle of generalized microsporidia in culture

Environmental infective phase *(left)*: Spores can germinate and inject infective sporoplasm (*1a*) or undergo phagocytosis (*2a*) to gain entry into the host cell. Intracellular proliferative phase (*middle*): Merogony typically occurs in direct contact with host cytoplasm (*1b*). With phagocytosis, sporoplasm evades lysosomal destruction (*2b*). Sporogony may (*c*) or may not occur with a membrane. Spore maturation occurs through metamorphosis (*d*). Environmental infective phase (*right*): Liberation of infective spores (*e*), accompanied with host cell death. *HC* host cell, *N* nucleus of host cell.

Culturing microsporidia of insects

The breakthrough uniting cell culture techniques and microsporidia research came in 1937 with insects (Trager 1937). A silkworm cell culture was successfully infected with *N. bombycis*. The infected cells were reported to have been filled with spores and behaved similarly to uninfected cells. Trager (1937) speculated that the failure of previous attempts to

grow microsporidia in culture were likely a result of the addition of too few spores. His speculation has been supported by subsequent research: The optimal spore-to-cell ratio to infect a culture is 10:1 or greater (Jaronski 1984). Despite the initial success in 1930s, the common use of insect cell cultures in microsporidia research did not begin until the 1960s with the development of insect cell lines. Since then, insect cell lines have been used for two distinct purposes. One is as a basic research tool to study the life cycle of microsporidia. The other purpose is practical and is to develop large-scale cultures that produce spores that can be used as biological control agents for insect pests and for medically important insects (Jaronski 1984). A unique feature of some combinations of insect cell lines and microsporidia species is that the cultures can be subcultivated over 70 times and continue to produce spores (Iwano and Kurtti 1995, Kurtti et al. 1994). *Nosema locustae*, now reassigned to the genus *Paranosema*, is a fatal parasite of over 90 species of grasshoppers and is the only commercially approved microsporidial pesticide in the USA (Canning 1953, Henry and Onsager 1982, Sokolova et al. 2003). Although propagation of *P. locustae* has be achieved in vitro, the spore yield is higher in vivo, which is the commercial source (Becnel 2006, Khurad et al. 1991).

Clinically important microsporidia

Human microsporidiosis was first documented in 1959 (Matsubayashi et al. 1959). Over the next 25 years, incidents of microsporidia infections in humans were rare (Ashton et al. 1973, Marcus et al. 1973, Margileth et al. 1973, Sprague 1974), but in the 1980s, microsporidia were found to be the causative agent of the often fatal diarrheal disorder of AIDS patients (Desportes et al. 1985, Dobbins and Weinstein 1985, Modigliani et al. 1985). Since this discovery, microsporidiosis has been found not only to affect AIDS patients but can also be an opportunistic infection of immunodepressed individuals and potentially individuals with seemingly intact immune responses (Didier and Weiss 2006, Sharma et al. 2011). There are 14 species of microsporidia known to infect humans (Didier 2005). *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are the most commonly identified human microsporidial infections (Didier and Weiss 2006, Kotler and Orenstein 1999). Although intestinal disease is the most prevalent consequence of human microsporidia, they can cause a broad range of conditions including keratoconjunctivitis, myositis, and

encephalitis (Kotler and Orenstein 1999). Once only a concern of economic importance, microsporidia suddenly had significant clinical relevance, and this led to research on these microsporidia in animal cell cultures (Visvesvara 2002).

Culturing microsporidia of human diseases

Although there are some notable exceptions, species of microsporidia in human diseases have been cultured effectively in vitro since 1990. The first success was with *Vittaforma corneae* (Shadduck et al. 1990) and made use of the approaches used years earlier to culture *Encephalitozoon cuniculi*, which infect rabbits and was grown in cultures of a mouse lymphosarcoma cell line (MB III) (Morris et al. 1956). Now, at least a half dozen species infecting humans opportunistically have been cultured, including *Encephalitozoon hellem* and *E. intestinalis* (Didier et al. 1991, Visvesvara 2002, Visvesvara et al. 1995a). This has been done with different mammalian cell lines, and Visvesvara (2002) has compiled a list of microsporidia species, cell lines, and media that have been used together to obtain microsporidia growth in vitro. However, success is not a certainty. For the same species of microsporidia, some isolates can be maintained in culture, whereas other isolates cannot. Most significantly, at least one clinically important species, *E. bieneusi*, has failed to be cultured despite repeated attempts with different isolates. Only short-term cultivation with very low spore production was achieved with *E. bieneusi* (Visvesvara et al. 1995b). For other species, successfully infected cell cultures often can be maintained for several months to over a year. In old cultures, spores were seen by phase contrast microscopy attached to the plastic growth surface among patches of disrupted mammalian cells, and in some cases, spores that had extruded polar tubules were visible as spermatozoan-like structures (Visvesvara 2002). The microsporidia/cell culture systems have been used for a variety of purposes.

The first use is to produce the pathogen in amounts to allow identification and study. Microsporidial organisms are often present in very small numbers in samples such as in urine (Visvesvara 2002). Cell cultures allow their numbers to be increased substantially, which permits their identification by a variety of techniques, such as polymerase chain reactions (PCR) analysis of DNA extracted from the cultures. Thus, cell cultures are an aid to clinical identification. Cell cultures also provide a source of pathogens for infecting animals in vivo

and sufficient microsporidia for biochemical and molecular biology studies (Belkorchia et al. 2008).

A second use of microsporidia/cell culture systems is to study the early events of infection, including initial adherence, kinetics, and route of uptake. This is perhaps the most common use of microsporidia cultures, with them being examined by a variety of techniques and being the subjects for different experimental purposes. They have been used to show that the initial adherence of spores to host cells is mediated by sulfated glycosaminoglycans and is augmented by Mn^{++} and Mg^{++} but not by Ca^{++} (Hayman et al. 2005, Southern et al. 2007). Adherence of *Encephalitozoon* spp. was rapid (3-6 h) (Fischer et al. 2008). Both scanning electron microscopy and transmission electron microscopy (TEM) have been used to visualize the traditional route of infection, polar tube penetration, and deposition of the sporoplasm into the cytoplasm (Schottelius et al. 2000, Takvorian et al. 2005). The advantages for the microscopist are that the events of infection can be closely timed and the presence of infected cells usually can be assured. In vitro studies have shown that besides the traditional route of infection, two additional routes were possible (Couzinet et al. 2000, Takvorian et al. 2005). One was the phagocytosis of spores followed by germination and movement of the sporoplasm from the phagosome to the cytoplasm. The other possibility was that sporoplasm may be released extracellularly into a microenvironment close to the host cell and subsequently internalized into the host cytoplasm by phagocytosis or a type of endocytosis (Takvorian et al. 2005).

Thirdly, microsporidia/cell culture systems can be utilized to study the cell biology and kinetics of the intracellular stages of microsporidia. For this, TEM is perhaps the most successful approach (Hollister et al. 1996, Lowman et al. 2000). The biochemical processes and signaling pathways of the proliferative phase that leads to meronts (merogony) and of meront conversion into spores (sporogony) and sporont differentiation into sporoblasts remain to be studied in detail, likely because of the complexity involved in teasing out these processes within a host cell that is also changing. An example of this would be determining the origin, fate, and fusion of the parasitophorous vacuole (Fasshauer et al. 2005, Ronnebaumer et al. 2008). However, procedures have been developed for isolating sporogonial stages of *E. cuniculi* in cultures with Madin-Darby canine kidney cells (Taupin et al. 2006b). This opens up the possibility of applying many biochemical methods, including

proteome studies. The in vitro systems are very amenable to cytochemical and immunocytochemical investigation. RNA in situ hybridization has been used to follow the level of messenger RNA for endospore-destined protein (Taupin et al. 2006a). Fluorescence microscopy allowed the identification and localization of a microsporidian cytoskeletal component, actin (Bigliardi et al. 1999). The in vitro approach can be used to determine the timing of intracellular events and to study the effect of variables, such as temperature, on proliferation and sporogony. The replication kinetics of *E. intestinalis* in a mouse intestinal cell line (CMT-93) was studied by real-time PCR (Wasson and Barry 2003). Elevated temperature was shown to impede proliferation and the onset of sporogony for *B. algerae* in rabbit kidney cells (Lowman et al. 2000).

A fourth use of microsporidia/cell culture systems is to characterize the responses of the host animal cells to microsporidia, which would include not only innate cellular mechanisms for protecting the host against infection but also modulation of the host cell to protect the pathogen and perhaps disseminate it. These responses are more conveniently studied in vitro than in vivo, but such studies are in their infancy, with only a few cellular processes examined to date. Treatment of mouse macrophages in primary culture but not of a mouse macrophage cell line with interferon gamma inhibited *E. cuniculi* replication (Jelinek et al. 2007). On the other hand, the reorganization of microtubules and induction of multinucleation in green monkey cell line E6 by *Vittaforma corneae* might be a way for the parasite to be protected from the host immune response (Leitch et al. 2005). To date, this has only been documented in vitro. Another focus is the induction of chemokines in macrophages in vitro and the migration of naïve monocytes by *Encephalitozoon* spp. (Fischer et al. 2007). This type of study aims to understand the dissemination of the pathogen within the host. Finally, the modulation of the cell cycle and cell death has been examined (del Aguila et al. 2006). Overall, these types of studies have the potential to provide unique insights into the regulation of cellular processes and to open up new avenues of treatment.

As a final application, microsporidia/cell culture systems provide convenient platforms for discovering and studying treatments that can be used to kill microsporidia. This approach was used to screen a variety of drugs for their therapeutic potential and to identify albendazole and fumagillin as effective at inhibiting the growth of *E. cuniculi* in vitro but at the same time causing little harm to the human cells, which in this case were MRC5 (Beauvis

et al. 1994). Similar systems have been used to evaluate the antimicrosporidial activity of other classes of compounds, including fluoroquinolones (Didier et al. 2005, Didier et al. 2006). Although animals ultimately will have to be used to test the efficacy of therapeutics, the advantage of the in vitro approach is in providing preliminary screens of effectiveness rapidly and inexpensively. Furthermore, animal cell culture systems can be used to study the killing of microsporidia by chlorine and ultraviolet light (John et al. 2003, Wolk et al. 2000).

Microsporidia of fish

Microsporidiosis has been identified in fish for over a century (Moniez 1887). Since that time, there are at least 156 documented microsporidia species in 14 genera recognized in fish (Lom 2002). See Appendix A for a table of microsporidia infecting fish. Microsporidiosis is highly destructive to infected tissue resulting in high mortality rates in fish (Becker and Speare 2007, Shaw and Kent 1999). Some genera of microsporidia in fish are known to cause hypertrophic growth whereby a unique host-parasite complex develops called a xenoma or a xenoparasitic complex (Lom and Dyková 2005). The size of these hypertrophied cells are often 400 to 500 μm in diameter but have been described to be as large as 13 mm (Canning and Lom 1986, Shaw and Kent 1999). Xenoma-inducing microsporidia tend to be more host-specific than non-xenoma-inducing species (Lom and Dyková 2005). This is certainly true of *Heterosporis* sp., which is considered as one of three pathogens on the Great Lakes Commission Priority Invasive Species List (Great Lakes Commission 2005, Sutherland et al. 2004).

Economic importance of microsporidia of fish

Microsporidiosis has substantial consequences to the profitability of aquaculture and commercial fishing. World aquaculture production has experienced an average annual growth of 8.8% since 1970 and continues to outpace all other animal food-producing sector growth (Food and Agriculture Organization of the United Nations (FAO) 2007). With 75% of marine fish stocks at or above sustainable yields, aquaculture can anticipate future pressure to supply demand (UN Atlas of the Oceans 2000). Disease in wild and farmed fish increases these pressures on aquaculture production. For example, Becker and Speare (2007) suggest that mortality of farmed Chinook salmon from microsporidia has, in part, influenced the need to dramatically increase production to compensate for reduced returns. Additionally,

high mortality rates from microsporidia have contributed to the weakening or collapse of several fisheries (Shaw and Kent 1999). Therefore, the economic impact of microsporidia has a compounding effect and has the potential to cause economic hardships in these industries.

Sub-lethal infections can additionally impact commercial fishing and aquaculture. Microsporidia belonging to *Glugea*, *Loma*, *Nucleospora*, and *Heterosporis* genera are responsible for a number of diseases in economically important fish. Pathological expression of microsporidia varies by species of parasite and the tissue infected. Symptoms can include leukemia-like conditions, emaciation, disfigurement from xenoparasitic growths or tissue necrosis, and growth inhibition (Lom and Dyková 2005, Shaw and Kent 1999). For example, farmed salmonids in particular are susceptible to *Loma salmonae* and *Nucleospora salmonis*. *L. salmonae* causes xenoma growths on gill tissue and promotes respiratory failure, whereas *N. salmonis* results in leukemic symptoms. *Heterosporis anguillarum* causes morphological changes in the Japanese eel with necrotic depressions of trunk musculature (T'sui and Wang 1988). The resulting disfigurement is called “Beko Disease” whereby muscle tissue undergoes liquefaction and is replaced with developing spores. *Glugea* spp. cause disfigurement with xenoparasitic growths in other economically important fish, such as in ayu and winter flounder (Cali and Takvorian 1991, Lee et al. 2004). Reductions in the fitness of these fish correspond to a reduction in catch value. Consequently, sub-lethal microsporidial infections also impact the fishing and aquaculture industries.

Microsporidia can have an economic impact in the laboratory as well as in capture fisheries and aquaculture. The use of fish as models in biomedical research has dramatically increased in the last decade, largely lead by the development of the zebrafish (*Danio rerio*) model (Ackermann and Paw 2003). Two important microsporidian diseases afflict laboratory fishes; *Pseudoloma neurophilia* of zebrafish and *Glugea anomala* of stickleback species (Kent and Fournie 2007). Indeed. *P. neurophilia* is the most common pathogen in zebrafish research facilities.

1.2 Fish cell culture

As for cells from mammals, two general types of cultures can be used to study fish cells in vitro: primary cultures and cell lines. The two are interrelated because cell lines are developed from primary cultures. They differ in their life span. Primary cultures are initiated

directly from the cells, tissues or organs of fish and typically last only a few days, but exceptions exist. The extreme is hemopoietic cultures from the rainbow trout spleen, which can be maintained for a year or more (Ganassin and Bols 1996). By convention (Schaeffer 1990), the primary culture ends and the cell line begins upon splitting or subcultivation of the primary culture into new culture vessels. In the case of mammalian cell lines, some can be propagated only a limited number of times, finite cells, whereas others can be grown indefinitely, continuous cell lines. Most fish cell lines appear to be continuous (Bols et al. 2005). Primary cultures or cell lines have been developed from most tissues and organs of fish (Bols and Lee 1991). Many mammalian cell lines express functional properties of mature cells or can be triggered to differentiate into more mature cells, whereas the differentiation status or capacity of piscine cell lines is largely unexplored (Bols et al. 2005), although some B lymphocyte and macrophage cell lines have been developed (Ganassin and Bols 1998, Miller et al. 1994). Relatively recent lists of fish cell lines have been published along with their availability in repositories (Bols et al. 2005, Fryer and Lannan 1994).

Microsporidia of fish in fish cell cultures

Very short-term primary cultures have been used to study the interactions of microsporidia spores with cells of the innate immune system, macrophages and neutrophils. The cultures usually have been used within 48 h and the focus has been on the study of phagocytic and respiratory burst capabilities of the phagocytes. With such cultures, head kidney macrophages from the ayu were shown to phagocytize G*lugea plecoglossi* spores by recognizing concanavalin A-reactive glycoproteins on the spore surface (Kim et al. 1999). The modulation of the macrophages behaviour might aid the establishment of *G. plecoglossi* infection. Another example is a comparison of short-term cultures of peritoneal exudates adherent (PEA) cells from turbot that have been injected intraperitoneally with either sodium thioglycolate or spores of *Tetramicra brevifilum* (Leiro et al. 2001). For peritoneal cells from fish injected with microsporidian spores, more neutrophils were found among the PEA cells and these cells made less ROS in response to *T. brevifilum* spores. Thus again the microsporidian spores seemed capable of impairing the respiratory burst of phagocytes and this could aid infection. Cultures of macrophages from Chinook salmon and Atlantic salmon were compared for their ability to phagocytize *L. salmonae* spores (Shaw et al. 2001).

Phagocytosis was higher in Atlantic salmon macrophages. This suggests a possible cellular basis for the Atlantic salmon being resistant and Chinook salmon being susceptible to the parasite.

Perhaps the most successful microsporidia/fish cell culture system has been obtained not with cell lines but with relatively long-term primary cultures of salmonid mononuclear leukocytes and less frequently head kidney stromal epithelial cells (Table 1.1) (Desportes-Livage et al. 1996, Wongtavatchai et al. 1995, Wongtavatchai et al. 1994). These cultures supported the growth of *Nucleospora salmonis*. The basal medium was Iscove's modified Dulbecco's medium supplemented with fetal bovine serum, concanavalin A, lipopolysaccharide (LPS), and human recombinant interleukin 2. The source of infection was leukocytes from microsporidia-infected fish. Leukocytes from microsporidia-infected Chinook salmon and rainbow trout were co-cultured with leukocytes from non-infected fish to start the cultures. Cells from the initial cultures could be subcultured by adding them to new cultures of leukocytes from healthy fish. This subculturing could be done up to 17 times for almost a year. The cultures retained *N. salmonis* stages from early meronts to mature spores. Two mechanisms were postulated to explain the spread of the parasite among the cultured cells: microsporidia could be transferred from mother cell to two daughter cells upon division of the host cell or could directly penetrate uninfected host cells. As the cultures required the periodic addition of uninfected leukocytes, the latter mechanism appeared to be in operation.

Inocula from these cultures were able to cause in vivo a disease that was identical to the disease observed in naturally infected Chinook salmon (Wongtavatchai et al. 1995). Although cultures stored at 4 ºC in water or at -70 ºC in medium without a cryoprotectant lost infectivity, the cells of *N. salmonis* infected leukocytes cultures could be cryopreserved in liquid nitrogen with a cryoprotectant and still retain their infectivity (Wongtavatchai et al. 1994). Interestingly, cultures with many spores and cultures with few spores were both infective in fish (Wongtavatchai et al. 1995). Possibly, the prespore stages from cultures rapidly sporulated upon injection and these gave rise in vivo to infectious spores, which started the infection in fish. Alternatively, as well as spores, early proliferative forms *of N. salmonis* from cell cultures could be infective to Chinook salmon.

The *N. salmonis*/salmonid leukocyte cultures allowed the developmental stages of the microsporidia to be examined by TEM (Desportes-Livage et al. 1996, Wongtavatchai et al. 1995). The most unique feature was that all developmental stages were in direct contact with the host nucleoplasm. TEM revealed the differentiation of the polar tube precursors (PTP), the polaroplast primordium (PLP), and the granular body (GB) and their assembly into the extrusion apparatus of the spores.

Salmonid leukocyte cultures have been used to show that *N. salmonis* causes the leukocytes to release mitogenic factors that stimulates the proliferation of uninfected mononuclear cells (Wongtavatchai et al. 1995). The significance of this observation is that these factors could explain the principal pathological feature of the disease. Most *N. salmonis* infections have been observed in Chinook salmon, and a characteristic feature of the disease is the excessive proliferation of mononuclear leukocytes. The nature of the factor(s) responsible for this has yet to be determined, but the cell culture system could be a key to unraveling them.

Several fish cell lines have been investigated for their capacity to support the growth of fish microsporidia. Spores of a fish microsporidia of the genus *Glugea* sp. were internalized in 12 h by cells of the Chinook salmon embryo cell line (CHSE-214) and sporoplasms and meronts were seen in parasitophorous vacuoles (Lores et al. 2003). However, development stopped by 48 h. Another fish microsporidia that has been studied is *P. neurophilia*, which, in research facilities, is a common pathogen of zebrafish (*D. rerio*) and is found in the central nervous system (Watral et al. 2006). Spores were collected from brains and anterior spinal cords of infected zebrafish and added to cultures of channel catfish ovary (CCO), zebrafish caudal fin fibroblast (SJD.1) carp epithelioma (EPC) and fathead minnow (FHM), which were maintained at 28 ºC. Aggregates of eight or more spores per cell developed, Fig 1.2. However, further development was limited and requires more investigation.

The only other fish microsporidial pathogen that has been grown in vitro is by far the most unique of the in vitro fish microsporidia systems and perhaps in all of in vitro microsporidia work. This is the eel (*Anguilla japonica*) epithelial-like cell line, EP-1, which seems to be persistently infected with *Pleistophora anguillarum*, now *H. anguillarum* (Kou et al. 1995). EP-1 was developed from infected tissues of eel elvers (young eels) that had 12 d previously been immersed in a suspension of *H. anguillarum* spores. The spores had been obtained from

infected tissue of naturally infected eels. Several cell lines arose from the infected elver tissues, but only EP-1 was characterized. EP-1 seemed immortal as the cells were passaged over 223 times and had a heteroploid karyotype. Merogonial stages of *H. anguillarum* were visualized in EP-1 by TEM, and merozoites were detected by Feulgen staining and indirect immunofluorescent staining with antisera against *H. anguillarum* spores. The appearance of spores in EP-1 cultures was not specifically noted. However, after the 140th passage, EP-1 cells were injected into uninfected elvers, and the young eels developed the muscle pathology of an *H. aguillarum* infection. Therefore, this in vitro system seemed to be producing *H. anguillarum* merozoites that were capable of continuing in vivo sporogonial reproduction and causing disease in the natural host, eels. This unique persistently infected cell line may prove to be an important tool to advance our knowledge of fish microsporidial pathogens and how to study microsporidia in vitro (Monaghan et al. 2008).

To date, the culturing of fish microsporidia in fish cell cultures has not achieved the success that has been accomplished with insect and mammalian microsporidia. This is not likely due to the lack of cell lines from fish because as documented in the first section below, microsporidia from one animal group can infect in vitro the cells from a very different group of animals. A possibility is that specific differentiated cell types from fish are needed to grow fish microsporidia. However, as documented in the second section below, microsporidia often appear less fastidious about the cell type in vitro.

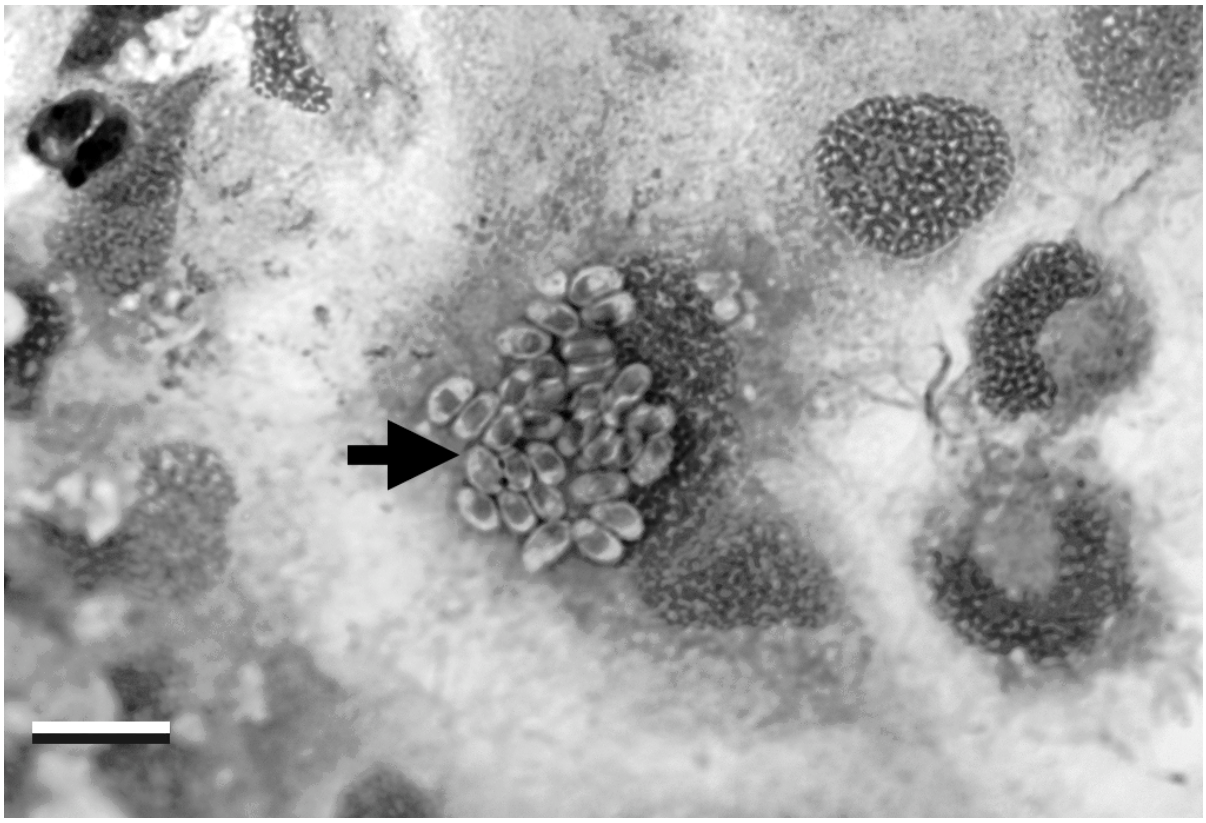

Figure 1.2 *Pseudoloma neurophilia* spores in EPC cells at 5 d post-inoculation
Arrow indicates spores. DifQuick stain. Micrograph from M. Kent. Bar = 10 μm.

Host animal group specificity in vitro

The precise in vivo specificity of a microsporidial species for a host animal group is difficult to state definitively. Microsporidia from diverse animal groups are being found to cause disease in animal species widely different from the hosts in which they were discovered initially. Microsporidia from mammals, insects, and even fish have been implicated in human infections. The occurrence of the four most common human microsporidial pathogens in non-human mammals raises questions regarding possible reservoirs for these parasites and their zoonotic potential (Mathis et al. 2005). A 56-year-old woman died of myositis initiated apparently from a mosquito bite and caused by the "insect" microsporidia *Anncaliia algerae* (Coyle et al. 2004). A fish microsporidia is thought to be the cause of myositis in an immuno-compromised man (Ledford et al. 1985). The in vitro results suggest that microsporidia can initiate at least some infection steps in cells from a wide range of vertebrates and invertebrates.

Table 1.1 Fish cell culture systems used in the cultivation of microsporidia from fish

Characteristics of the Fish Cell Cultures			Characteristics of the Microsporidia		Microsporidia in fish cell culture		Reference
Type of culture	Species	Tissue Origin	Species	Normal Host	Initiating Infection in vitro	Microsporidia Growth	
Primary culture: Leukocytes	Chinook Salmon	Peripheral blood	*Nucleospora salmonis*	Fish (salmonids)	Leukocytes from infected fish (co-cultivation)	Spore development in leukocyte cell nucleus	Wongtavatchai *et al.* 1994; Wongtavatchai *et al.* 1995
Primary culture: Leukocytes	Chinook Salmon	Infected kidney	*Nucleospora salmonis*	Fish (salmonids)	Leukocytes from infected fish (co-cultivation)	Spore development in leukocyte cell nucleus	Desportes-Livage *et al.* 1996
Primary culture: Epithelial-like	Rainbow Trout	Kidney	*Nucleospora salmonis*	Fish (salmonids)	Leukocytes from infected fish (co-cultivation)	Spore development in epithelial cell nucleus	Desportes-Livage *et al.* 1996
Cell line: CHSE-214; Epithelial-like	Chinook Salmon	Salmon embryo	*Glugea* sp.	Fish (many species)	Spores from infected fish (inoculation)	Proliferation of microsporidia stopped after 48 hours in culture	Lores *et al.* 2003
Cell lines: CCO; Fibroblast SJD.1; Fibroblast EPC; Epithelial-like FHM; Epithelial-like	Channel Catfish Zebrafish Carp Fathead Minnow (respectively)	CCO; Ovary SJD.1; Fin EPC; Skin FHM: Connective tissue and muscle	*Pseudoloma neurophilia*	Fish (zebrafish)	Spores from infected fish (inoculation)	Develops aggregates of approximately 8 spores per cell	Watral *et al.* 2006
Cell line: EP-1; Epithelial-like	Japanese eel	Infected tissues of elvers	*Heterosporis anguillarum*	Fish (eels)	Not done; began by exposing elvers to spores	Meront development	Kou *et al.* 1995

Many examples exist of microsporidia from insects being studied in mammalian cell cultures. The first successful cultivation of an insect-derived microsporidian, *A. algerae*, in mammalian cell culture (pig kidney) was subsequently followed by cultivation of microsporidian insect isolates in other mammalian cell cultures including rat, mouse, and rabbit (Ishihara 1968, Smith and Sinden 1980, Undeen 1975). More recently, in vitro approaches using insect-derived microsporidia in mammalian cell culture have furthered our understanding of parasite development and factors that influence it (Franzen et al. 2005a, Lowman et al. 2000, Takvorian et al. 2005, Trammer et al. 1999). For example, temperature-imposed constraints to microsporidia growth from insect isolates have been evaluated in a number of in vitro studies using mammalian cells. *Tubulinosema ratisbonensis*, known only to infect fruit flies in vivo, was isolated and able to infect human lung fibroblasts (MRC-5) at 31 ºC and 37 ºC, yet was unsuccessful in monkey kidney cells (Vero) (Franzen et al. 2005a). *T. ratisbonensis* grew at both temperatures, but when compared, proliferation was reduced at 37 ºC. Likewise, Lowman et al. (2000) determined that the incubation of *A. algerae* spores at temperatures ranging from 29 ºC to 37 ºC did not prevent microsporidia growth but found that higher temperatures inhibited the rate of growth.

Another example of a species of microsporidia from invertebrates interacting in vitro with cells from mammals is *Ameson michaelis*, which infects blue crabs. When placed in culture media with various cell types, *A. michaelis* was able to inject sporoplasm into not only epithelial cells and hemocytes of the blue crab but as well into human erythrocytes and mouse cells of various types (leukemia EL4 cells, macrophages, and neuroblastoma C1300 cells) (Weidner 1972). In these experiments, proliferation of *A. michaelis*, the first infection step, showed no restriction in vitro between cells of different species and types, but subsequent steps must have been limiting.

A few examples exist of microsporidia from mammals being studied in fish cell cultures (Table 1.2). *E. cuniculi*, of mammalian origin, was reported to develop spores in the cytoplasm of a fathead minnow cell line (Bedrnik and Vávra 1972). Also interesting was that this was accomplished at 18 ºC, a temperature far cooler than any mammalian host. *E. bieneusi* was cultivated in a primary culture from rainbow trout kidney cells (Desportes-Livage et al. 1996). This is of particular interest as *E. bieneusi* has been very difficult to culture in mammalian cells (Visvesvara 2002). Desportes-Livage et al. (1996) observed early

E. bieneusi development to be organized with cisternae of the host endoplasmic reticulum. Meronts and sporonts were also found in close association with the host nucleus. In desquamating cells, mature spores were observed, which indicates probable discontinuation of development (Desportes-Livage et al. 1996). The last example relates to *Glugea* sp. collected from the livers of Greater Sand Eels (*Hyperoplus lanceolatus*), which was unproductive in fish culture, but successful at proliferating in a mosquito larvae cell line (ECACC90100401) (Lores et al. 2003). Meront development was observed within 12 h post-infection (pi) and sporogony at 48 pi, and after 7 d, a variety of developmental stages were observed demonstrating the ability of *Glugea* sp. to continuously proliferate in the mosquito larvae cell line (Lores et al. 2003).

Currently, no examples exist of microsporidia from fish being studied in mammalian cell cultures. Temperature would be anticipated to prevent microsporidia from cold-water fish being studied in mammalian cells, but this might not be a barrier for microsporidia from warm-water fish. The advantages of using mammalian cells would be that more cell lines expressing differentiated functions exist, several in vitro differentiation systems are well defined, and more antibodies and molecular probes would be available to study the host cell responses.

Table 1.2 Examples of microsporidia cultivated in animal cell culture systems different from the class or group of the apparent natural host

Characteristics of the Animal Cell Cultures			Characteristics of the Microsporidia		Microsporidia in Animal Cell Culture		Reference
Type of culture	Species	Tissue Origin	Species	Normal Host	Initiating Infection in vitro	Microsporidia Growth	
Cell line: FHM; Epithelial-like	Fathead minnow	Connective tissue and muscle	*Encephalitozoon cuniculi*	Mammals; including humans	Spores from unspecified mammalian origin (inoculation)	Spore development	Bedrnik and Vavra, 1972
Primary culture: Epithelial-like	Rainbow Trout	Kidney	*Enterocytozoon bieneusi*	Mammals; including humans	Spores from human duodenal biopsies (inoculation)	Spore development in host cell cytoplasm	Desportes-Livage et al., 1996
Cell line: ECACC 90100401; Epithelial-like	Mosquito	Larvae	*Glugea sp.*	Fish; various species	Spores from infected fish (inoculation)	Mature spores within 72 h post-infection	Lores et al., 2003
Cell line: RK13; Epithelial-like	Rabbit	Kidney	*Brachiola algerae*	Insect; but can infect humans	Spores isolated from mosquito (inoculation)	Mature spores within 72 h post-infection	Lowman et al., 2000
Cell line: MRC-5; Fibroblasts	Human	Lung	*Tubulinosema ratisbonesis*	Fruit flies	Spores isolated from fruit flies (inoculation)	Mature spores within 72 h post-infection	Franzen et al., 2005a

Host tissue or cell specificity in vitro

The precise in vivo specificity of a microsporidial species for a host tissue or cell type is difficult to state definitely. While *Encephalitozoon* spp. are the primary cause of disseminated microsporidiosis in humans, other species of microsporidia appear to infect only specific tissue in their hosts (Mertens et al. 1997, Tosoni et al. 2002). For example, of the 14 species of microsporidia that infect humans, three are exclusively known to infect the eye, while three other species infect the eye and one other site (Didier and Weiss 2006). As well, *Pleistophora ronneafiei* and *Brachiola vesicularum* are two species known to infect only muscle tissue (Cali and Takvorian 2003, Didier and Weiss 2006). Interestingly, even among the same genus of microsporidial pathogens, such as *Brachiola*, there can be a broad range of clinical presentations. *B. connori* demonstrates low-tissue specificity as the causative agent of system infections, while *B. vesicularum* demonstrates high tissue specificity. On the other hand, *E. bieneusi* is rarely found infecting tissue other than the epithelium of the small intestine (Didier and Weiss 2006). Nonetheless, mechanisms providing the ability for some species to invade a variety of tissue types while others are found in limited tissue types remain unclear.

Despite hints of apparent tissue tropism in vivo, the same microsporidia in vitro appear to be able to infect cells of different tissue origin and of stage of differentiation. For example, Franzen et al. (2005b) studied the internalization of four microsporidian species in seven different cell lines in vitro and observed that phagocytic cells were more proficient at internalizing spores than non-phagocytic cells. Comparing the non-phagocytic cells, the only clear result was that a pulmonary cell line, A549, was ten times less efficient than its counterparts at internalizing the spores. Furthermore, the intestinal cell lines were not only effective at internalizing spores that commonly affect the gastrointestinal tract but were also effective at internalizing spores of microsporidian species that typically do not cause gastrointestinal microsporidiosis. Because internalization patterns in the cell lines could not be correlated to clinical presentations, it was suggested that access to tissue types through various routes of infection are likely responsible for apparent tissue specificity rather than tissue tropisms (Franzen et al. 2005b).

Another example of an apparent loss of tissue tropism in vitro is with *Nucleospora salmonis* from rainbow trout (Desportes-Livage et al. 1996). In vivo *N. salmonis* was found only in leukocytes from blood and from hematopoietic tissues (kidney and spleen). By contrast, in vitro *N. salmonis* developed in epithelial cells of long-term head kidney cultures, and the yield of spores per cell was higher for the epithelial cells than for leukocytes.

1.3 Discussion

Insect and mammalian cell culture systems pioneered the in vitro study of microsporidia leading to important observations on their infectivity and development and an evaluation of possible treatments and disinfection procedures. Fish microsporidiology in fish cell culture also could make advancements in these areas and these in turn could help in understanding how to diagnose and control microsporidia infections in aquaculture. For example, cell cultures could be a source of material for the development of vaccines. However, besides their practical value, microsporidia/fish cell culture systems might uncover some unique cell processes. One is the possibility that for some fish microsporidia, fluctuating temperatures rather than constant temperatures are needed for all stages to be expressed in vitro. Another is the process behind the induction of xenomas, which are exceptional animal cells because of their enormous size and are most commonly found with fish microsporidia. A final example is the possible ability of fish microsporidia to immortalize animal cells, which are hinted at in the work on *H. anguillarum*.

In the following chapters, the use of fish cell cultures to study microsporidia is demonstrated by growing *A. algerae* in several fish cell lines, and by using microscopy methods to evaluate the life cycle and possible control agents of the parasite (Chapters 2 to 4). Additionally, in Chapter 5, fish cells are used to demonstrate the first evidence that *Nosema apis* can grow in a vertebrate cell line. Prior to this work, fish cells had been used with various success to grow fish derived microsporidia for four microsporidian species: *N. salmonis* (Desportes-Livage et al. 1996, Wongtavatchai et al. 1995, Wongtavatchai et al. 1994), *P. neurophilia* (Watral et al. 2006) *H. anguillarum* (Kou et al. 1995), and *Glugea* sp. (Lores et al. 2003) (Table 1.1). Further, fish cell cultures were used in two other investigations to grow to mammalian derived microsporidia, *E. cuniculi* and *E. bieneusi* (Bedrnik and Vávra 1972, Desportes-Livage et al. 1996) (Table 1.2). With the over 1200

species of microsporidia (Wittner 1999), and 156 species of microsporidia known to infect fish (Lom 2002), there are clearly extensive areas of research to explore in this field. The goal of this work is to demonstrate the value fish cell cultures have in the study of microsporidia, and ultimately to use this experience to grow microsporidia that are known to infect economically important fish, in species/tissue specific fish cell cultures.

Chapter 2

In vitro Growth of Microsporidia *Anncaliia algerae* in Cell Lines From Warm Water Fish

Overview

Anncallia algerae is an aquatic microsporidium that most commonly infects mosquitoes but can be grown on the rabbit kidney cell line, RK-13. Spores were purified from RK-13 cultures and added to cell lines from warm water fish and from an insect. The cell lines were GFSK-S1 and GFB3C-W1 from goldfish skin and brain respectively, ZEB2J from zebrafish embryos, FHMT-W1 from fathead minnow testis, and Sf9 from ovaries of a fall armyworm moth. All cultures were maintained at 27ºC. Infection was judged to have taken place by the appearance of sporonts and/or spores in cells and occurred in all cell lines. Spores were also isolated from ZEB2J cultures and used to successfully infect new cultures of ZEB2J, RK-13 and Sf9. These results suggest that cells of a wide range of vertebrates support *A. algerae* growth in vitro and fish cells can produce spores infectious to cells of mammals, fish and insects.

2.1 Introduction

Microsporidia are a diverse group of fungi that are obligate intracellular parasites of other eukaryotes, including invertebrates and vertebrates (Keeling and Fast 2002). Informally, they can be categorized in several ways. One is to describe them as being insect, mammalian, or fish microsporidia based on the host from which they were originally derived. Another is to classify them as being terrestrial or aquatic based on whether spores survive and transmit from host to host in an aquatic or terrestrial environment and have different underlying germination mechanisms (Undeen and Vander Meer 1999). In humans, microsporidia rarely cause diseases, and when they do, the patients are usually immunocompromised (Didier 2005). By contrast, several animal diseases of considerable economic importance have been attributed to microsporidia, including diseases that impede aquaculture of fish and have been a factor in the weakening or collapse of several fisheries (Becker and Speare 2007, Shaw and Kent 1999).

Anncaliia algerae, which was first known as *Nosema algerae* and then as *Brachiola algerae* before being moved to the genus *Anncaliia* (Franzen et al. 2006), is an aquatic and insect microsporidium with an exceptional host range. Originally the species was discovered as a parasite of *Anopheles* mosquitoes (Vávra and Undeen 1970) and subsequently was shown to infect a variety of other insect species (Undeen and Maddox 1973). For mammals, the potential for an association was demonstrated first experimentally with mice, and then surprisingly, clinical cases emerged with humans. When spores were injected into the feet and tails of mice, limited infections were observed (Trammer et al. 1997, Undeen and Maddox 1973), but after the ocular administration of spores to immunodeficient mice, severe liver infections arose (Koudela et al. 2001). In humans, *A. algerae* was identified initially in eye scrapings of an immuno-competent person (Visvesvara et al. 1999) and then in skeletal muscle of a woman undergoing treatment for arthritis with immunosuppressive drugs and who ultimately succumbed to the infection (Coyle et al. 2004).

Whether *A. algerae* infects aquatic vertebrates such as fish is unknown, but one approach for assessing this prospect is to determine whether they can infect fish cells in vitro. *A. algerae* has been successfully grown in insect and mammalian cell cultures, with the rabbit kidney epithelial cell line RK-13 being particularly useful for this purpose (Lowman et al.

2000), but fish cells have not been examined. Generally, animal cell cultures have been valuable tools for studying the interactions between microsporidia and mammalian cells (Visvesvara 2002), but for fish microsporidia, the in vitro approach has been used less frequently and with less success (Monaghan et al. 2009). Therefore, in order to evaluate the prospects of *A. algerae* infecting fish and of using *A. algerae* as a general model for studying the cellular pathology of fish microsporidial infections, we infected cell lines from zebrafish, goldfish, and fathead minnow with *A. algerae* spores. Cells of warm water fish and an incubation temperature of 27ºC were chosen because *A. algerae* has been shown to develop in vitro at temperatures as low as 25-26ºC (Belkorchia et al. 2008, Undeen 1975) and because cells of warm water fish grow well between 25ºC and 27ºC (Xing et al. 2008). Overall the results suggest that cells from warm water fish support the production of *A. algerae* spores and these spores can infect mammalian, lepidopteran, and other piscine cells in culture.

2.2 Materials and Methods

Cell Lines and Their Growth

The American Type Culture Collection (ATCC, Manassas, VA) supplied the rabbit kidney epithelial cell line RK-13 (CCL-37), and InVitrogen (Carlsbad, CA) was the source of Sf9 from fall armyworm ovary. Although initially grown in vented flasks at 37ºC in an atmosphere of 5% CO2 and 95% air in Modified Eagle's Minimum Essential Medium (ATCC Cat#30-2003) with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St. Louis, MO), RK-13 were switched to Leibovitz-15 (L-15; HyClone) with 2mM L-glutamine and 10% FBS. With this medium, the RK-13 could be grown in an atmosphere of air in non-vented flasks just as was done for the other cell lines. RK-13 was routinely grown at 37ºC, but confluent cultures could be maintained without any obvious change in cell number for at least 10 d at 27ºC. The Sf9 cells were grown at 27ºC in Grace's Insect Culture Medium (HyClone) with 2mM L-glutamine, 500 mg/L calcium chloride, 2,800 mg/L potassium chloride, 3,330 mg/L lactalbumin hydrolysate, 3,330 mg/L yeastolate, and 10% FBS. Penicillin/streptomycin was used in all animal cell culture media at 100 IU/m

The fish cell lines were developed in the laboratories Dr. Niels Bols (University of Waterloo) and Dr. Lucy Lee (Wilfrid Laurier University). ZEB2J is a zebrafish blastula cell line (Xing et al. 2008), and GFSK-S1 is from goldfish skin (Lee et al. 1997). The two other

fish cell lines, GFB3C-W1 from goldfish brain and FHMT-W1 from fathead minnow testis, have yet to be formally described in the literature. All fish cell lines are predominantly epithelial-like and were grown at 27ºC in L-15 supplemented as described above for RK-13.

Production and Purification of A. algerae spores from RK-13 Cultures

Initially, *A. algerae* spores were obtained from the ATCC as *B. algerae* (PRA-168). These were used to begin the routine production of spores in RK-13 cultures. RK-13 was used because of the success other have had with this cell line and *A. algerae* (Lowman et al. 2000, Takvorian et al. 2005). The initial ATCC sample was thawed, centrifuged at ~200*g,* and resuspended in L-15 with 2 mM L-glutamine and 10% FBS. This spore suspension was placed in a confluent culture of RK-13 cells in a 25-cm^2 flask, which was incubated at 27ºC. After 10 d, spores were seen over the majority of the monolayer, and the monolayer was starting to deteriorate. The remaining attached cells were disassociated from the flask using a cell scraper. All contents were removed, placed in a 15-ml test tube, and centrifuged at ~200 x g for 5 min. The supernatant was carefully removed using a Pasteur pipette, and the pellet was resuspended in L-15 with 2 mM L-glutamine and 10% FBS. The suspension was then used to infect other RK-13 flasks at 27ºC. This was repeated until a sufficient supply of spores was acquired to store some and use others to prepare more spores for experimental infection of other cells lines.

For experimental infection of tested cell lines, spores were purified from RK-13 cultures by a method modified from Hester et al. (2002). Purification was done in order to make subsequent microscopic examination of cultures easier: purification eliminated the possibility of RK-13, whether intact or as debris, from being transferred. The RK-13 monolayer was scraped and the contents of a flask placed in the 15-ml test tube, centrifuged at ~1,100 xg for 10 min, and the supernatant removed. The cell/spore pellet was then resuspended in 5 ml sterile water at room temperature for more that 24 h. The water lysed the cells but not the spores. To further liberate spores from any host cells membranes, the spore/cell lysate mixture was pushed through a 25-G needle twice, mixed with 5 ml Percoll (Sigma P1644), vortexed for approximately 5 s, and centrifuged at ~1,800 x *g* for 30 min. The spores pelleted to the bottom of the test tube, while cellular debris was in the Percoll/water supernatant, and removed by aspiration. The spore pellet was resuspended in sterile water. Some aliquots of

the suspension were autoclaved for 75 min at 121ºC. All aliquots were stored at room temperature. Spores were used within 2 wk of purification to infect animal cell cultures.

Infecting Animal Cell Cultures with A. algerae Spores from RK-13 Cultures

Infections were done in 12.5-cm^2 non-vented flasks (Falcon). Each cell line was grown at the temperature appropriate for the cell line to approximately 70-90% confluency for the vertebrate cell lines and to about 30% confluency for the insect cell line, Sf9. As Sf9 cells grew loosely attached to the flask surface and in suspension, the flasks were used at a point when adherent cells were still visible and not blocked from view by cells floating in the medium. Spore suspensions in water were centrifuged at ~200xg, resuspended in L-15 with 10% FBS for the vertebrate cells and in Grace's Insect Medium for Sf9, and counted with a hemocytometer. For spore inoculation, each flask had 3.2 ml of culture medium and received either untreated or autoclaved spores to give 2 x 10^6 spores/ml. Additional flasks received no spores (no spore control). All co-culture combinations and their controls were maintained at 27ºC. After 1-2 d post-infection (p.i.), the medium was removed from each flask and rinsed three times with fresh medium.

Isolating and using A. algerae spores from other animal cell cultures

Spores were also prepared from successfully infected cultures of the fish cell lines and of Sf9 and used to infect new cultures of each cell line from which they were produced. Additionally, spores from ZEB2J were used to infect RK-13 and Sf9 cultures and spores from Sf9 used to infect RK-13 and ZEB2J cultures. Spores were isolated and used as described above for RK-13. Again, 1 to 2 d p.i., cultures were rinsed and fresh medium added. Cultures were viewed at various times afterwards as described below.

Monitoring animal cell cultures infected with A. algerae spores

A Nikon TE300 inverted microscope with a TE-FM Epi-Fluorescence attachment and Nikon Cool Pix 5400 camera was used to monitor and photograph living and fixed cultures by phase and fluorescence microscopy, respectively. Phase contrast microscopy was used to view living cultures daily. Cell cultures were fixed at various times p.i. in Carnoy's fixative (absolute methanol/glacial acetic acid, 3:1) and stained for DNA with 4',6-diamidino-2-phenylindole (DAPI). For fixation, 1 ml of Carnoy's was added to the medium of a flask

culture and left for 2 min. A bulb pipette was used to remove the fixative and another 1.5 ml of Carnoy's was added for a further 5 min before removal. Afterwards the culture flasks were rinsed in phosphate-buffered saline (PBS) and stained for at least 1 h in 10 µg/ml of DAPI in PBS. Cultures were scored for sporonts and spores by the criteria described in the first section of the "Results."

Comparing fish cell lines for their ability to support A. algerae spore production

Counts were made of *A. algerae* spores in cultures of the four fish cells lines, 2 and 6 d after they had been infected as described above. Counts were done from micrographs of five random fields of DAPI stained cultures from 5 different flasks. The spores were the intensely stained ovoid structures, although sporonts were possibly included in the counts at day 6. The numbers were analyzed using GraphPad InStat (version 3.00 for Windows 95, GraphPad Software, San Diego, CA, www.graphpad.com). A one-way analysis of variances (ANOVA) was used to compare whether the number of spores in different cultures were statistically different ($p<0.05$). For each cell line, an unpaired t-test was used to compare the number of spores at day 6 with day 2 ($p<0.05$).

Day 2 values were the reference points from which to determine whether cultures supported *A. algerae* development over the next 4 d. After spore additions, 2 d were allowed for the spores to distribute and settle over the culture before removal of the medium and rinsing of cultures. Rinsing immediately before the day 2 count meant that the spores being counted were those that had adhered to cells or plastic and potentially could infect cells. However, some spores would have infected cells during the first 2 d and would not likely be counted because of the weak DAPI staining of meronts (see "Results"). To determine the extent of this problem, parallel cultures received autoclaved spores before being rinsed. At day 2, the counts from these cultures were compared to the counts from cultures receiving untreated spores and were found not to be significantly different (unpaired t test, $p>0.05$). this suggests that the day 2 counts are an appropriate reference point from which to judge whether cultures supported spore production over the next 4 d.

Viability of fish and mammalian cells in cultures infected with A. algerae

The cells in control and infected cultures were compared in several ways. For the adherent cell lines, cells were removed from the growth surface with TryplE (InVitrogen) as described by Servili et al. (2009) or trypsin as described previously (Bols and Lee 1994). Sf9 cells grew loosely over the growth surface and could be removed by scraping. Trypan blue was used to determine viability in cell suspensions. The proportion of viable cells and their size distribution were determined with an automated cell counter, The Countess (InVitrogen), according to the manufacturers instructions. Statistical analysis was done with GraphPad as described earlier.

2.3 Results

RK-13 cultures: the spore factory

Signs of infection with *A. algerae* were seen clearly by phase contrast microscopy in RK-13 cultures at between 24 to 48 h p.i. Prior to being added to cultures, spores were ovoid, phase-bright structures, and RK-13 cells had the conventional phase-contrast appearance of epithelial cells in culture (Fig 2.1A). However, between 24 to 48 h p.i., spindle-shaped structures appeared in the cytoplasms of many RK-13 cells. These structures were similar to those described by others in *A. algerae* infected animal cell cultures. They resemble the "developing stages" visualized by phase contrast microscopy in human foreskin fibroblast cultures (Belkorchia et al. 2008) and Giemsa-stained structures that were termed "meronts" and "sporonts" in pig kidney cell cultures (Undeen 1975) and "proliferative stages" in RK-13 cells (Lowman et al. 2000). With Giemsa staining, multiple nuclei were seen (Lowman et al. 2000, Undeen 1975). However, in our study, multiple nuclei were only occasionally visible by phase contrast microscopy and rarely by fluorescence microscopy after DAPI staining for DNA. DAPI stained spores strongly and the spindle-shaped structures weakly. Yet, DAPI staining and the ability to view the same microscope field by fluorescence and phase contrast helped localize spindle-shaped structures that were barely visible and hard to find by phase contrast microscopy alone. These are interpreted to be meronts (Fig 2.3 "M"). The spindle-shaped structures that were much darker under the phase contrast microscope are interpreted to be sporonts (Fig 2.3 "S"). As these can be scored unequivocally, the appearance of phase-dark, spindle-shaped structures was a clear indication that a culture had been successfully

infected. After 6 to 12 d p.i., the cytoplasms but not the nuclei of RK-13 cells became full with egg-shaped, phase-bright spores (Fig 2.1B). Cultures with this appearance were used as the source from which to isolate and purify spores, which were suspended in H_2O and used in the following experiments.

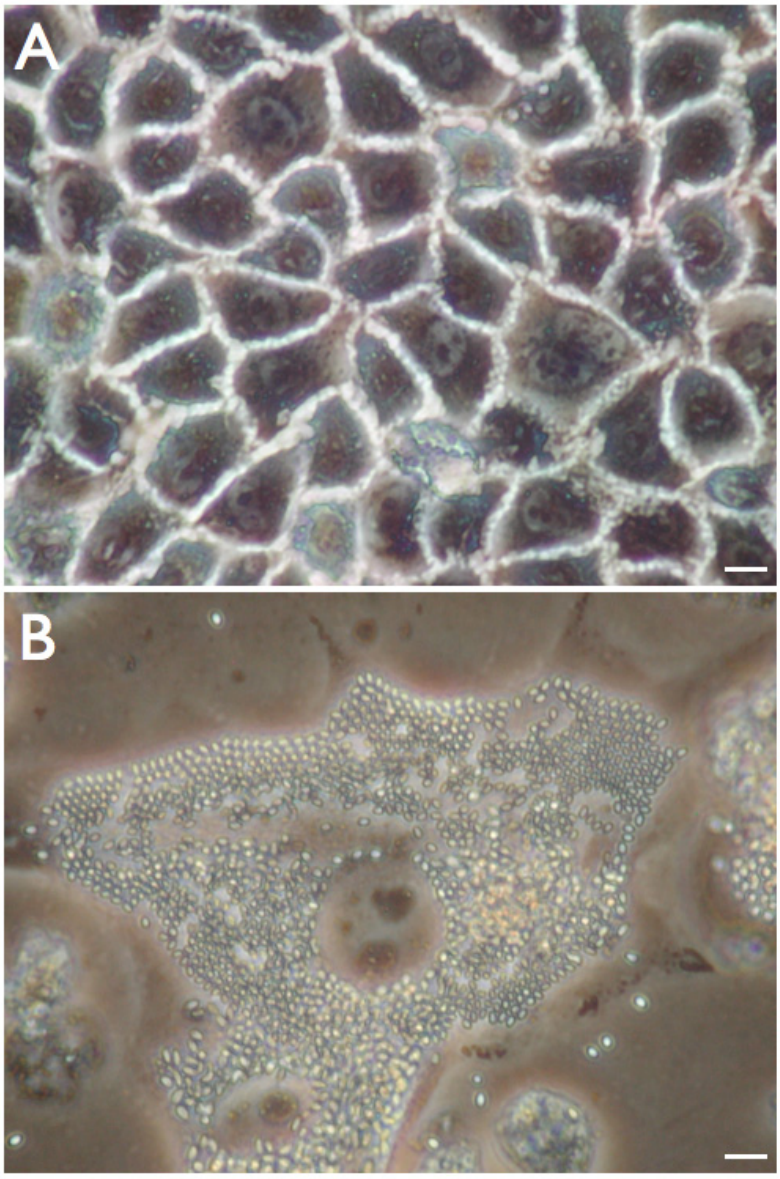

Figure 2.1 The *A. algerae* spore factory in RK-13 cultures

Phase contrast appearance of RK-13 cultures without infection (A) and 11 d after infection with *A. algerae* spores (B). Cultures were maintained in L-15 with 10% FBS at 27°C. Scale bar = 10 µm.

Spores

Immediately upon preparation, all the spores appeared phase bright but this changed as early as 5 min after the addition of purified spores to culture vessels with medium (Grace's or L-15 with FBS) but no animal cells. The phase bright spore coat became phase dark and a polar filament became visible (Fig 2.2). In some cases, ejection of the polar filament was witnessed and seen to completely eject to the point of resistance, coil around the terminal sporoplasm, and forcefully pull the empty spore coat in its direction. These events occurred in seconds and are interpreted to be germination. However, spore germination was not synchronous and took place erratically over several hours. After approximately 5 h, no further germination was observed, even though many un-germinated spores were still present. When such a culture was fixed and incubated with DAPI, the un-germinated spores fluoresced much more strongly than the germinated spores. Some spore preparations were immediately autoclaved to create dead spores as controls in selected experiments. The autoclaved spores all appeared phase bright and stained strongly with DAPI. The autoclaved spores could be stored for days at 4ºC in water or at 27°C in culture medium without any noticeable change in their phase contrast appearance and DAPI staining.

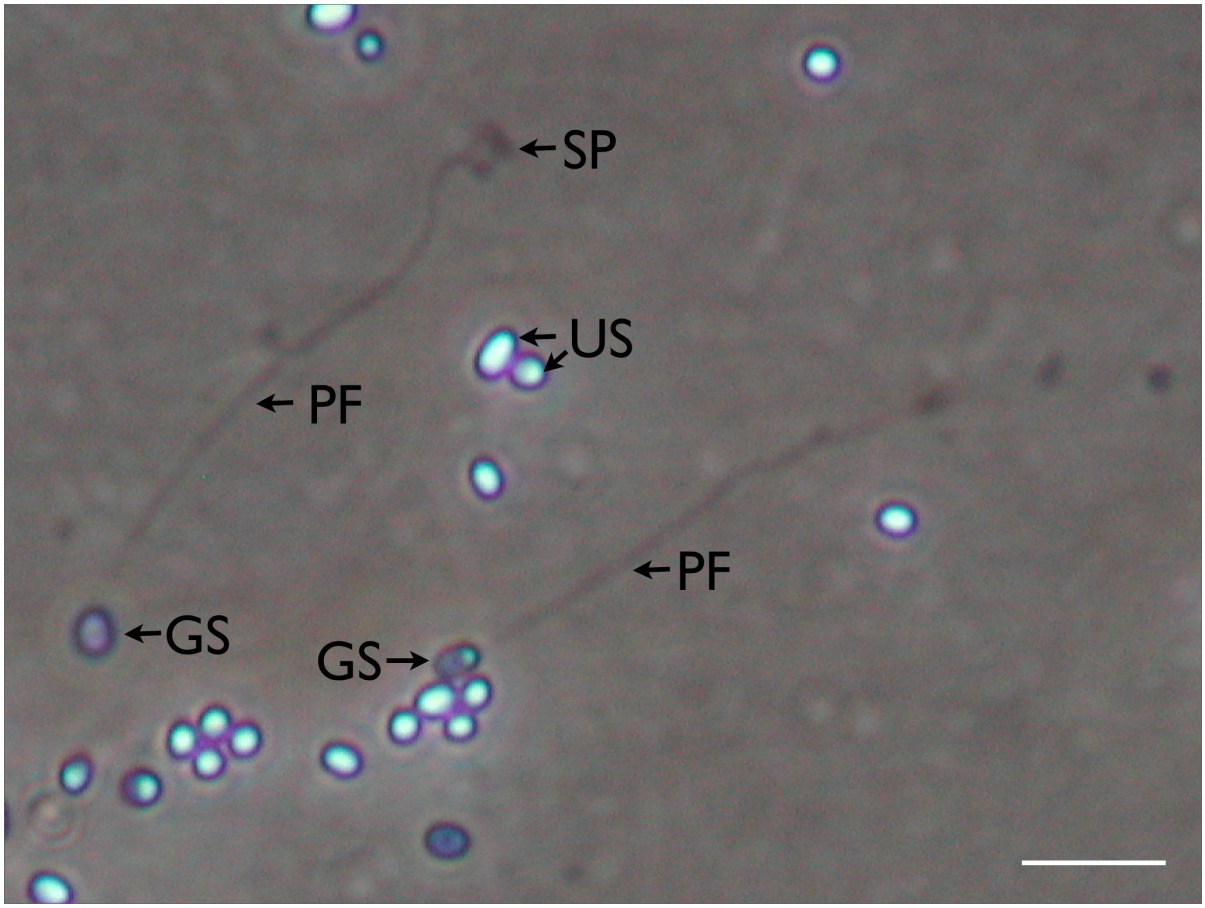

Figure 2.2 Phase contrast appearance of germinated and ungerminated *A. algerae* spores in L-15 with 10% FBS

Ungerminated spores (US) are generally phase bright using phase contrast microscopy. Polar filaments (PF) and sporoplasms (SP) can be observed from germinating spores (GS), and are generally darker in appearance using phase contrast microscopy. Spores are uniform but may appear more circular when observing them superiorly or inferiorly, in contrast to longitudinally. Scale bar = 10 μm.

Infecting fish cell lines

Spores from RK-13 cultures were added to cultures of several fish cell lines: GFSK-S1 and GFB3C-W1 from goldfish skin and brain, respectively, ZEB2J from zebrafish blastula and FHMT-W1 from fathead minnow testis. Between 12 and 48 h after the addition of spores, sporonts clearly were visible in the cytoplasm of some cells in all cultures. As in RK-13, these were spindle-shaped phase-dark structures that stained diffusely with DAPI (Fig 2.3). Several of these structures often lay roughly parallel to one another within the cytoplasm. Such structures failed to appear in cultures that had been killed with Carnoy's fixative prior to the addition of spore or in living cultures that received autoclaved spores. After 3 d p.i., ovoid, phase-bright spores were seen in the cytoplasm of many cells in cultures of these fish cell lines (Figs 2.4 and 2.5). These cultures were processed as was done with RK-13, including a water lysis step, to isolate spores. The spores were added to new cell cultures of the same cell lines. Additionally, spores from ZEB2J cultures were inoculated into cultures of RK-13 and of the insect cell line, Sf9. Again, sporonts and spores became clearly visible in cells of the fish and rabbit cell lines. As discussed below, only spores were seen in Sf9 cultures. Overall, the results suggest that *A. algerae* spores can be produced in fish cells in culture, and these spores are infectious to piscine, mammalian, and insect cells.

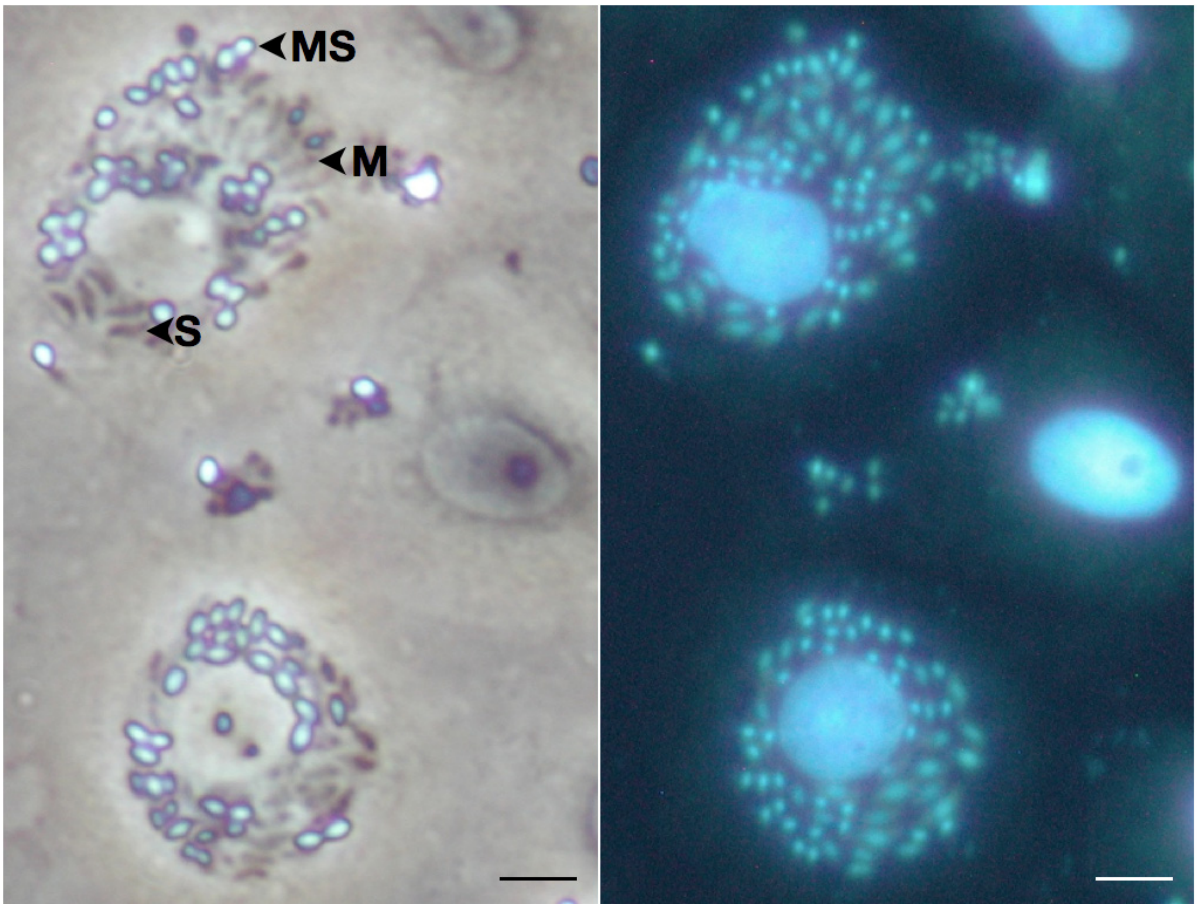

Figure 2.3 DAPI staining of spindle-shaped early developmental stages and ovid later developmental stages in ZEB2J 4d p.i.

Meronts (M) and sporonts (S) can be distinguished from mature spores (MS) (left) using 10 µg/ml DAPI (right). Scale bar = 10 µm.

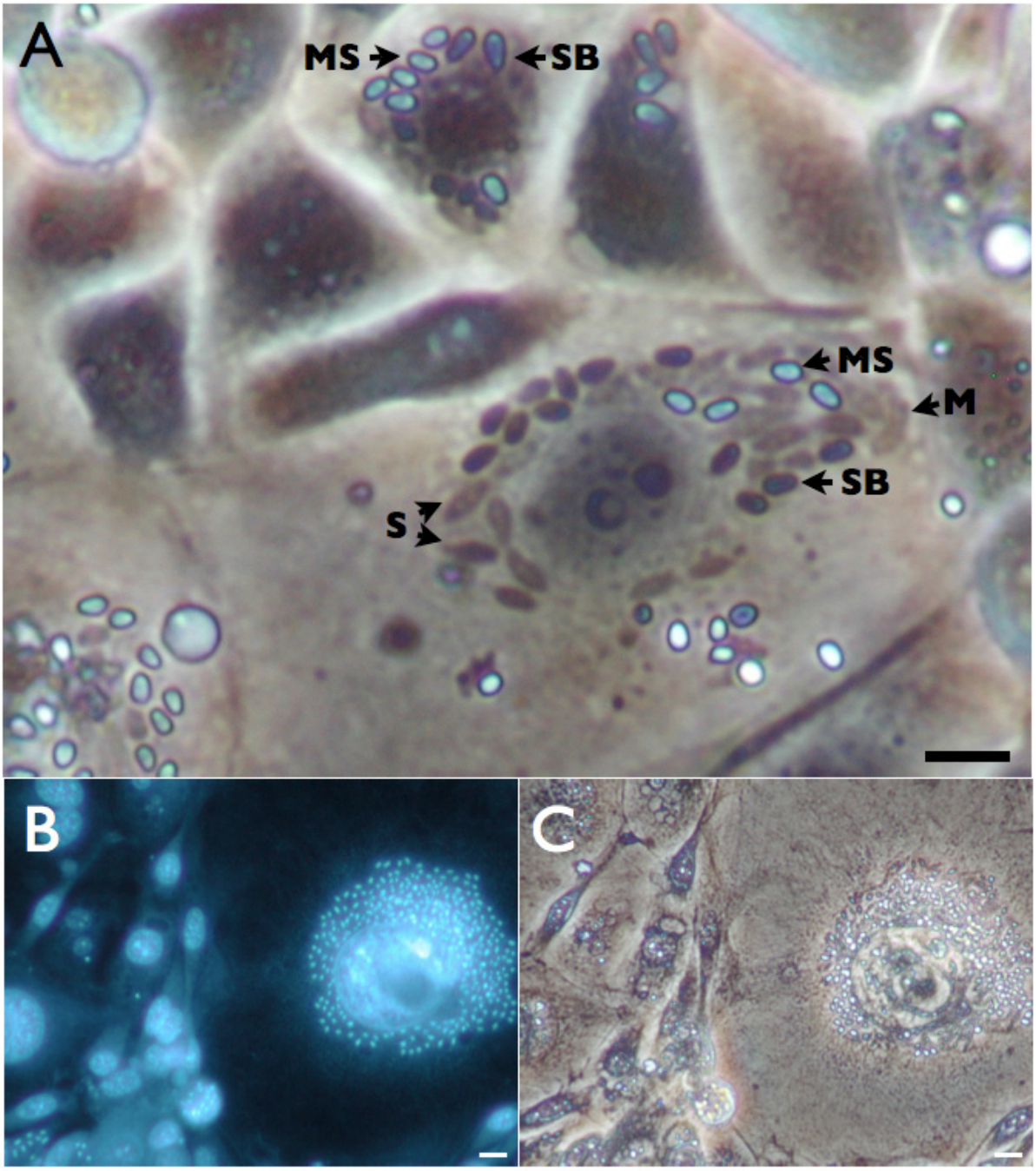

Figure 2.4 Phase contrast and fluorescence microscopy views of zebrafish cell culture 3 to 4 d after infection with *A. algerae* spores

Phase contrast micrograph of ZEB2J at 3 d p.i. (A). *M* meronts, *S* sporonts, *SB* sporoblasts, and *MS* mature intracellular spores. Fluorescent (B), and phase contrast microscopy (C) ZEB2J day 4 p.i. with 10 µg/ml DAPI. A giant host cell nucleus surrounded by over 280 spores developing with the host cell cytoplasm. Scale bar = 10 µm.

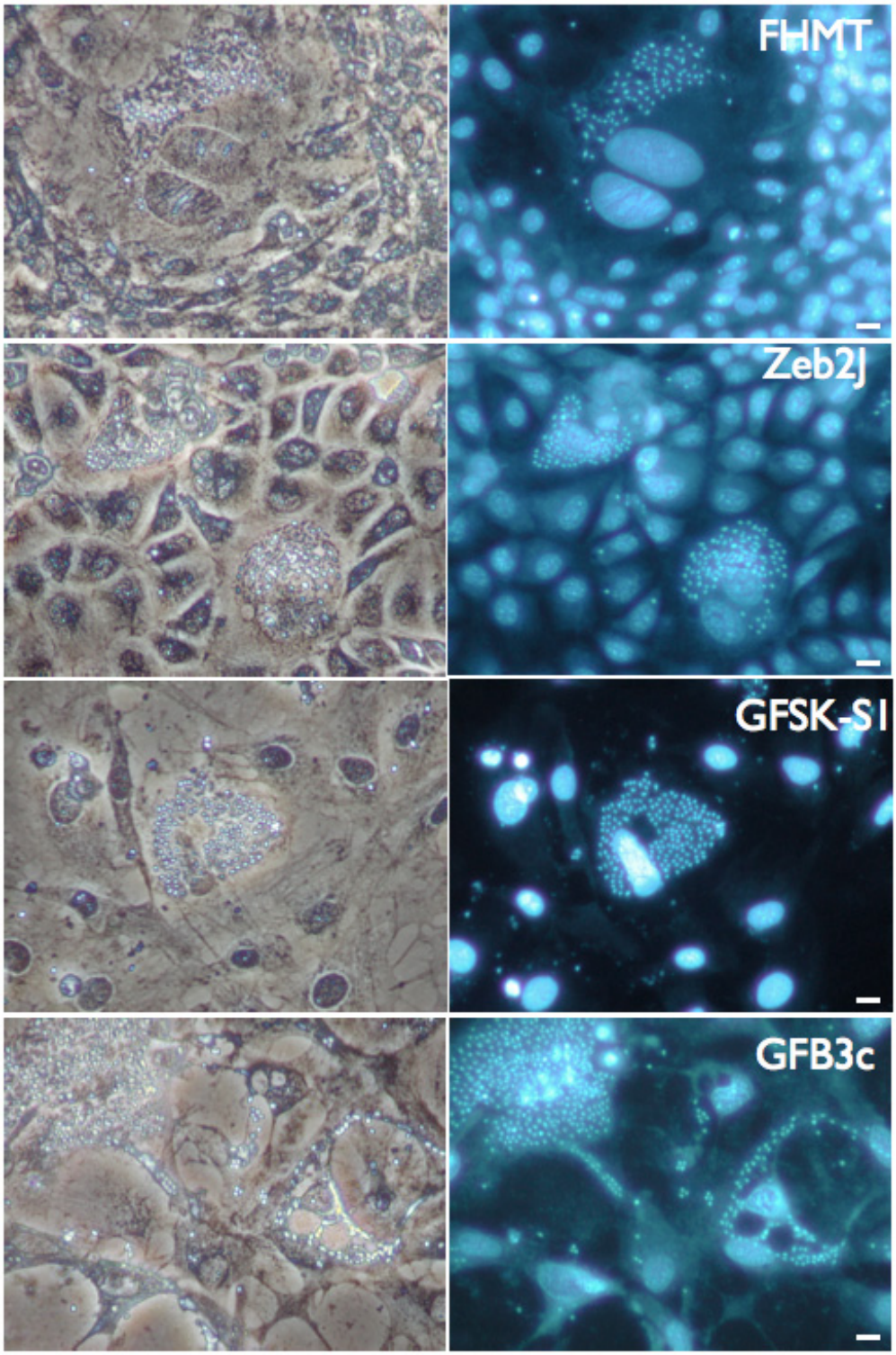

Figure 2.5 Phase contrast and fluorescence microscopy views of several warm water fish cell cultures 4 d after infection with *A. algerae* spores

Growth occurred in all inoculated fish cell lines and could be clearly observed using 10 µg/ml DAPI. Scale bar = 10 µm.

Infecting the lepidopteran cell line, Sf9

Spores from RK-13 cultures were also introduced to cultures of the insect cell line Sf9. However, approximately 1 d p.i., the insect cells started floating compared to the uninfected control culture. This prevented the easy visualization of microsporidial developmental stages, such as sporonts, inside the Sf9 cells. After 6 d p.i., DAPI staining and phase contrast microscopy revealed structures that are interpreted to be spores associated with Sf9 cells (Fig 2.6). Spores were isolated from these cultures by the same method as was done with RK-13 and used to infect ZEB2J and RK-13 cultures as well as new Sf9 cultures. Over approximately a week, cells showed the development of sporonts and spores in the ZEB2J and RK-13 cultures and spores in Sf9 cultures. These results suggest that cell cultures of the armyworm support the production of *A. algerae* spores that are infectious in vitro for insect, fish and mammalian cells.

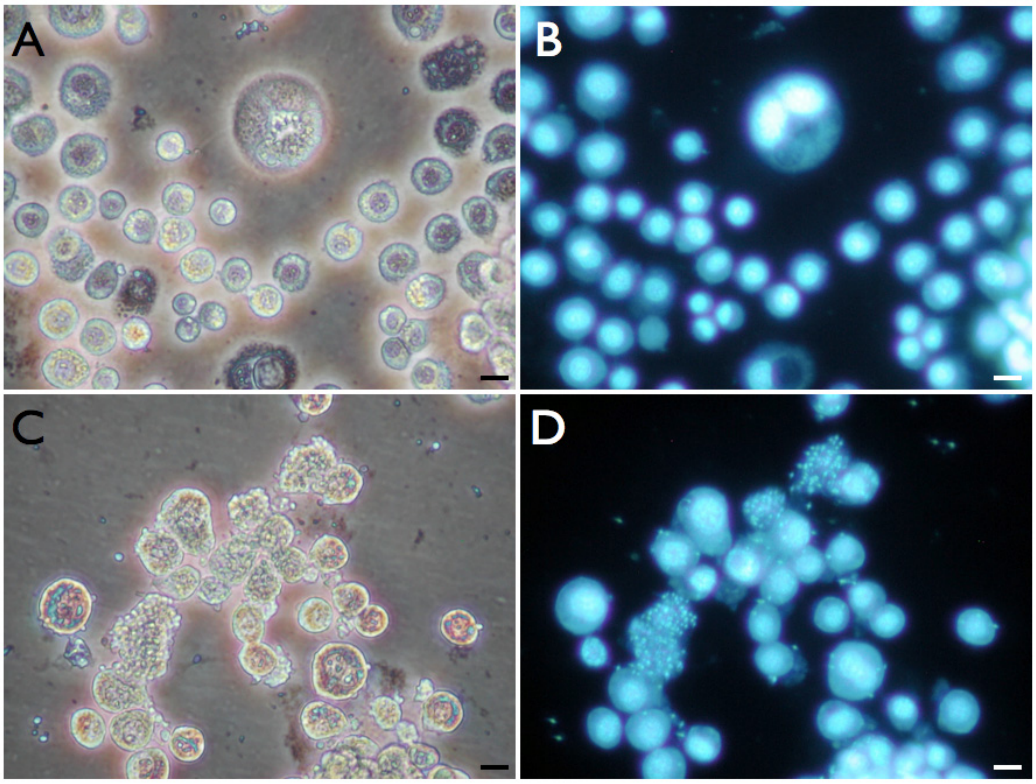

Figure 2.6 Phase contrast and fluorescence microscopy views of Sf9 cell cultures 4 d after infection with *A. algerae* spores

Uninfected Sf9 cells phase and corresponding fluorescent (*A* and *B*), and infected Sf9 cells (C and D) 4 d p.i. with 10 µg/ml DAPI. Scale bar = 10 µm.

Comparing fish cell lines for their ability to support A. algerae spore production

For each fish cell line, more spores were present in cultures 6 d after infection with *A. algerae* spores than after 2 d (Fig 2.7). Spores were ovoid structures that stained intensely with DAPI. The percent increase was 164.6 for GFB3C-W1, 204.3 for FHMT-W1, 397.8 for ZEB2J and 795.0 for GFSK-S1. Between the four fish cell cultures, spore numbers were not significantly different at day 2 (ANOVA, $p>0.05$) but were at day 6 ($p<0.05$). At day 6, spore numbers in GFSK-S1 were higher than in GFB3C-W1 and FHMT-W1 cultures (Tukey, $p<0.05$). These results suggest that each cell line supported *A. algerae* development, but one cell line GFSK-S1 supported spore production better than at least two other cell lines.

When autoclaved spores were added to cultures, the number of spores at day 6 was significantly less than the number at day 2 for each cell line (unpaired *t* test, $p<0.05$). The percent decrease in spore counts was 84.3 for FHMT, 86.7 for ZEB2J, 84.0 for GFSK-S1, and 64.6 for GFB3C. These results suggest that in vitro fish cells degraded *A. algerae* spores.

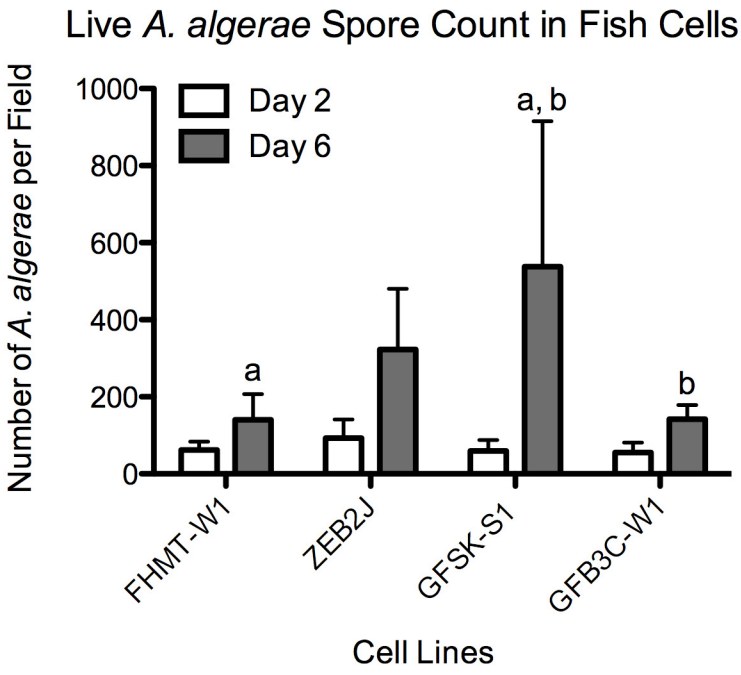

Figure 2.7 Comparing fish cell lines for their ability to support *A. algerae* spore production

Two days after the inoculation of cultures of four fish cell lines (FHMT, ZEB2J, GFSK-S1, GFB3C) with spores, the cultures were rinsed thoroughly. One set of cultures was fixed immediately (open bars); the other set of cultures were fixed 4 d later (solid bars). The cultures were stained with DAPI and scored for sporonts/spores. The means with SD (n=5) are plotted. For each cell line, the values for 2 and 6 d p.i. were compared by an unpaired t test and were significantly different ($p<0.05$). An ANOVA was used to compare values at day 6 between cell lines and was significant ($p<0.05$). Cell lines that have the same symbol are significantly different from one another (Tukey-Kramer Multiple Comparisons Test, $p<0.005$).

Viability of fish and mammalian cells in cultures infected with A. algerae

Cells in cultures of fish and rabbit cell lines showed little or no change in viability during the first 6 d p.i. with *A. algerae* spores and production of new spores. Over this time frame, the epithelial-like- and fibroblast-like-infected cells remained adherent to the culture surface even when full of spores. All infected fish cell cultures developed the appearance of some

giant cells with mature and developing spores within, but of the fish cell cultures, this seems most apparent in ZEB2J. When trypsin or TryplE was added to control and infected cultures, cells rounded and detached from the surface of both culture types over a similar time frame, and in infected cultures, cells with and without spores rounded and detached similarly. Cell suspensions prepared by trypsin or TryplE treatments of control and infected cultures had a similar number of viable cells as judged by Trypan Blue exclusion and size distributions. In control and *A. algerae*-infected cultures of ZEB2J, the percentages of viable cells were respectively 97.5±2.12% (n=2) and 98.5±0.707% (n=2; t test $p>0.05$) and the mean diameters of the cells were 22.8±0.778 µm (n=2) and 21.0±2.19 µm (n=2; t test $p>0.005$). In control and *A. algerae*-infected cultures of RK-13, the percentages of viable cells were, respectively, 99.5±0.707% (n=2) and 97.5±0.707% (n=2); t test $p>0.05$) and the mean diameters were 21.3±1.98 µm (n=2) and 21.0±2.83 µm (n=2; t test $p>0.05$). When cell suspensions were added to new culture surfaces, cells from control and infected cultures attached and spread similarly over the time frame of 24 h as did cells from the infected cultures with or without spores. Therefore, *A. algerae* caused no gross changes in fish and rabbit cell cultures over the first 6 d p.i. of spore production.

Viability of armyworm cells in cultures infected with A. algerae

In cultures infected with *A. algerae* for 7 d, Sf9 cells showed a change in behaviour but not in viability or in size. Normally, Sf9 cells grew loosely attached to the culture surface, but more cells were detached and floating in cultures several days after the introduction of *A. algerae* spores. However, at 6 d p.i., the number of viable cells as judged by Trypan blue exclusion was unchanged from control cultures, and the size distribution of Sf9 cells was similar in control and infected cultures. In control cultures and *A. algerae*-infected cultures, respectively, the proportions of viable cells were 86.0±4.24% (n=2) and 87.5±4.95% (n=2; t test $p>0.05$) and the mean diameters were 17.4±0.071 µm (n=2) and 17.0±1.06 µm (n=3; t test $p>0.05$).

2.4 Discussion

The current study extends the range of animal cells that are known to support *A. algerae* growth to the cells of warm water fish. Growth was supported in cell cultures from goldfish,

zebrafish, and fathead minnow at 27ºC. The growth of cells from coldwater fish, such as rainbow trout, is restricted to temperatures below 24ºC (Bols et al. 1992) so whether cells of coldwater fish support *A. algerae* spore development requires further research. However, the warm water fish cells join a variety of insect and mammalian cells in culture that at between 25ºC and 37ºC become infected with *A. algerae*. These include cells from Lepidoptera and mosquitoes (Belkorchia et al. 2008, Streett et al. 1980), frogs (Smith et al. 1982), and mammals, including humans, rabbits, monkeys, pigs, and rats (Belkorchia et al. 2008, Cali et al. 2004, Lowman et al. 2000, Moura et al. 1999, Smith et al. 1982, Takvorian et al. 2005, Trammer et al. 1999). Thus, at the cellular level *A. algerae* appears to be promiscuous in its choice of hosts.

The tissue of origin for the cells supporting *A. algerae* growth in vitro is wide but hints of possible tissue or cell preferences in vertebrates exists. Common cell types supporting infection have been kidney epithelial cells (Lowman et al. 2000, Takvorian et al. 2005, Undeen 1975); African Green monkey kidney (Kucerova et al. 2004) and fibroblasts from human lung, muscle, and foreskin (Belkorchia et al. 2008, Scanlon et al. 1999, Trammer et al. 1999) and from *Xenopus* (Smith et al. 1982). Also used successfully to grow *A. algerae* have been cells or cell lines from rat brain and skeletal muscle (Cali et al. 2004, Smith et al. 1982, Smith and Sinden 1980), human intestine (Leitch and Ceballos 2008), and liver (Smith et al. 1982). For the warm water fish, cell lines from embryo, brain, testis, and skin became infected with *A. algerae*, but the skin cell line, GFSK-S1 appeared to be better at supporting the production of *A. algerae* life cycle stages at 6 d post-infection. Other hints of cell type preference were seen in the poor infection of differentiated human intestinal epithelial cells (Leitch and Ceballos 2008) and in the severe infection of the liver but not in the other tissues of immunodeficient mice (Koudela et al. 2001). In the future, the in vitro approach might allow the delineation of possible tissue or cell selectivity.

The ability of cells lines from warm water fish to support the production of infectious *A. algerae* spores raised the possibility that this microsporidia species might be transmitted between an aquatic insect and fish and subsequently between fish species as a rare event. As in the case of humans (Visvesvara et al. 2005), the immune system of the fish would likely have to be impaired to allow an initial infection to be established. Establishment might occur in skin or in the gastrointestinal (GI) tract. Microscopic wounds have been demonstrated in

skin and fins of fish (Kiryu and Wakabayashi 1999) and could be a route for microsporidia spores to enter fish from water. In at least one study, ditch water was found to contain spores of the genus *Nosema* (Avery and Undeen 1987), which is the genus to which *A. algerae* belonged formerly, and the spores might have arisen from aquatic insects, like mosquitoes. Interestingly, *A. algerae* appeared to grow best on the goldfish skin cell line. Another route might be through fish feeding on infected insects or fish and transmission occurring in the GI tract. For this route, the fish GI tract would have to allow spore germination and sporoplasm penetration into the epithelial cells. Although no clinical cases have been seen to involve the GI tract, *A. algerae* has been shown to infect the human intestinal cell line, CaCo-2 (Leitch and Ceballos 2008).

The cell culture observations for approximately a week suggest that an *A. algerae* infection might cause chronic rather than acute effects in many hosts, although this would likely depend on the species. In Sf9 cultures, most cells remained viable, whereas Streett et al. (1980) observed cell lysis in other lepidopteran cell cultures infected with *A. algerae*. For fish cells, despite supporting spore production, the cultures showed little change, with the cells staying adherent and viable. One possible change was an increase in the number of giant cells in infected cultures, but when the size distribution was compared for cells detached from control and infected cultures, no difference was found. This requires further investigation. Despite supporting spore production, the cells showed little change, staying adherent and viable. The ease with which these cultures of *A. algerae* and fish cell lines can be established, maintained, and monitored should allow future studies on the general interactions between microsporidia and fish cells. These would include describing chronic changes in the molecular and cellular activities of fish cells and the response of cultures to potential therapeutic agents.

Chapter 3

Cell Lines from Cold Water Fish Support Growth of *Anncaliia algerae*

Overview

Cultures of rainbow trout gill and brain, RTgill-W1 and RTbrain-W1, were used to evaluate their ability to host the growth of *Anncaliia algerae* at 9, 18 and 21 °C. There was no conclusive evidence of parasite growth at 9 °C, and in both cell lines 21 °C allowed for more *A. algerae* growth than 18 °C. In both cell lines maintained at 21 °C , sporonts were visible in the cytoplasm 4 days p.i., followed by the mature spores 6 days p.i., while with cultures maintained at 18 °C these developmental stages were observed 6 and 8 days p.i, respectively. The appearance of these developmental stages was the same regardless of cell type. However, proliferation after 21 days was significantly greater in RTbrain-W1 than RTgill-W1. Using a novel real-time live cell (Riveal) microscopy (www.quorumtechnologies.com), proliferation and spontaneous germination of *A. algerae* grown at 18 ºC in RTgill-W1 for 10 days were observed over a 2.5 h period. Percoll gradient purified *A. algerae* spores grown at 18ºC were live stained with the fluorescent stain DAPI and stimulated to germinate using 3% hydrogen peroxide. Using Riveal microscopy, the ejection of sporoplasm was regularly observed, but some spores were visualized to germinate without ejecting the sporoplasm.

3.1 Introduction

Microsporidia comprise a varied group of obligate intracellular fungal parasites with an exceptional host range infecting most animal phyla. Hosts include important marine and freshwater food fish in the gadid and salmonid families. Rainbow trout (*Oncorhynchus mykiss*) are susceptible to microsporidial infection by *Loma salmonae* (Bader et al. 1998, Beaman et al. 1999, Becker and Speare 2004). Disease from this parasite is often reported on the gills, but can affect a variety of tissues resulting in increased mortality and heavy economic losses for aquaculture (Lovy et al. 2008, Morrison and Sprague 1983, Ramsay et al. 2003). *Anncaliia algerae*, previously in the genera *Nosema* and *Brachiola*, is a non-xenoma forming microsporidium typically known to infect mosquitoes and mosquito larvae. Rainbow trout could be exposed to this aquatic microsporidian through environmental spores, and by consuming infected mosquitoes and mosquito larvae, though no infection of any fish species with *A. algerae* has been described. Previous reports suggest that *A. algerae* proliferates at temperatures between 25 and 38°C (Belkorchia et al. 2008, Lowman et al. 2000, Trammer et al. 1999), well above the environmental temperatures in which rainbow trout thrive.

Studies on the effects of temperature on *A. algerae* growth have primarily focused on its upper proliferative limits. Although initially thought to be a microsporidian parasite of mosquitoes exclusively, culture of *A. algerae* in mammalian cells at physiologically relevant temperatures accurately predicted the possibility that mammalian body temperatures were within the proliferative temperature range of the parasite. The growth of *A. algerae* in pig kidney cultures at 26 and 35ºC was the earliest demonstration that development of the parasite occurs in mammalian cells (Undeen 1975). *A. algerae* was also shown to develop in the extremities of experimentally infected athymic mice (Trammer et al. 1997), and grow in human muscle fibroblasts (CHQ5B) at temperatures up to 38 ºC (Trammer et al. 1999). Subsequently, several mammalian cell lines have supported the growth of this parasite (Belkorchia et al. 2008, Kucerova et al. 2004, Lowman et al. 2000, Moura et al. 1999, Scanlon et al. 1999), and disease in the deep tissue, vocal cords, and cornea of humans has been attributed to *A. algerae* (Cali et al. 2010, Coyle et al. 2004, Visvesvara et al. 1999).

There has been less priority given to determining the lowest temperature in which *A. algerae* will grow. Undeen's initial work demonstrated that *A. algerae* grew at 26 ºC in pig

kidney cells, but the percent of germinated spores from this culture was reduced compared to spores grown at 35 ºC (Undeen 1975). Development of *A. algerae* in two mosquito cell lines (Mos55 and Sua4.0) at 25 ºC is also described, but infection took 10 to 15 days to detect and few spores were produced (Belkorchia et al. 2008). *A. algerae* has recently been determined to be able to grow at 27 ºC in cells derived from warm-water fish (Monaghan et al. 2011). Demonstrating the parasite's ability to also grow in cells from cold-water fish provides an ideal and important in vitro system in which to study the effects of lower temperatures on *A. algerae* growth.

In fish, water temperature is an important factor in the prevalence of microsporidial infections (Becker and Speare 2004, Becker et al. 2006, Takvorian and Cali 1984). For example, *Loma salmonae* was found to infect rainbow trout at temperatures between 9 and 20ºC, but xenoma formation was not observed at temperatures above and below this range (Beaman et al. 1999). Seasonal increases in water temperature have also been found to increase *Glugea stephani* infection in winter flounder (*Pseudopleuronectes americanus*) (Takvorian and Cali 1984). At lower temperatures (10 ºC) growth of *G. stephani* in English sole (*Parophrys vetulus*) was arrested, but infection continued to develop once temperatures were increased to 19-20 ºC (Olson 1981).

Microsporidia interactions with host cells are often evaluated using transmission electron microscopy (TEM). Although useful for microsporidial research, a principal disadvantage of TEM is the need to extrapolate host-parasite interactions from fixed preparations. In this study we utilize Riveal Contrast microscopy by Quorum Technologies (www.quorumtechnologies.com) in order to document the developmental stages, growth and germination of *A. algerae* in living RTgill-W1, a rainbow trout gill cell line (Bols et al. 1994). Riveal Contrast is a brightfield imaging technique that provides increased resolution and contrast of live specimens while providing colour information from a sample without having to add foreign contrasting agents. Using this technique, high-resolution of features within nanoparticle range (100 nm) can be achieved. DAPI stained cultures were also used to document differences in the number of spores at infection sites within cultures at the various temperatures.

3.2 Materials and Methods

Fish Cell Lines and their growth

The rainbow trout cell lines were developed in the laboratories of the authors. RTgill-W1 and RTbrain-W1 are derived from rainbow trout gills and brain, respectively (Bols et al. 1994). These cell cultures, similar to the species from which they were derived, can be maintained at relatively low temperatures (9 to 21 ºC). Lowman et al. (2000) previously determined that the optimal growth of *A. algerae* is 29 ºC, but rainbow trout cells do not survive at these temperatures, and cells from even warm water fish generally do not succeed if maintained at 29 ºC. The zebra fish embryo cell line, ZEB2J, (Xing et al. 2008) that can tolerate a temperature of 27 ºC, has already demonstrated that it can support *A. algerae* growth at that temperature (Chapter 2) (Monaghan 2011), and was used as a positive control by demonstrating that the spore inoculate used contained viable spores and were capable of growing in an already established culture system. All fish cell lines are predominantly epithelial-like and were grown in 25 cm^2 flasks (Falcon) to approximately 80-90% confluency at 21 ºC in Leibovitz-15 (L-15; HyClone) with 2 mM L-glutamine and 10% FBS.

Infecting fish cell lines with A. algerae

A. algerae spores were originally obtained from the ATCC as *B. algerae* (PRA-168), grown in the rabbit kidney epithelial cell line RK-13, and purified suspensions were achieved using the spore growth and purification methods described in Chapter 2. Falcon 12.5 cm^2 flasks of confluent RTgill-W1 and RTbrain-W1, and ZEB2J were inoculated with either viable or autoclaved spores at approximately 3 x 10^3 spores, a relatively low number. There were 15 flasks of RTgill-W1 and RTbrain-W1 with 5 flasks maintained at each temperature. Two additional flasks of each cell line and for each temperature received no spores as an uninoculated control. Two days after inoculation, all media was removed, cell cultures were rinsed twice and replaced with spore free L-15 media as described. Rainbow trout co-culture combinations and their controls were maintained at 9, 18 and 21ºC, while ZEB2J co-cultures and controls were kept at 27 ºC.

Comparing A. algerae growth in fish cells

A Nikon TE300 inverted microscope was used to monitor and photograph living and fixed cultures by phase and fluorescence microscopy, respectively. Phase contrast microscopy was used to view living cultures daily. The presence of sporont developmental stages was evidence that the spore inoculate infected the cells. Cultures were examined for the presence of this definitive stage, which appear as spindle shaped, phase dark structures in the cytoplasm (Chapter 2) (Monaghan et al 2011). Cells were also monitored for the first appearance of mature spores, which appear ovid and phase bright. Cell cultures were fixed at 21 days p.i. (3:1 absolute methanol/glacial acetic acid fixative), and DNA stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma). For fixation, 1 ml of fixative was added to the medium of a flask culture and left for 2 min. A bulb pipette was used to remove the fixative and another 1.5 ml of fixative was added for a further 5 min before removal. Afterwards, the culture flasks were rinsed in phosphate-buffered saline (PBS) and stained for at least 1 h in 10 µg/ml of DAPI in PBS. Cultures were examined for loci of infection. Fluorescent photographs were taken, and quantification of *A. algerae* (spores and sporonts) in the foci was conducted using the images and Cell Counter mode in ImageJ 1.42q software (National Institutes of Health, NIH; ImageJ www.imagej.com). Fluorescent and phase micrographs of the same field were compared to verify spore or sporont morphology.

Growth and preparation of A. algerae in RTgill-W1 for Riveal contast microscopy

RTgill-W1 was grown to near confluency on single well slide flasks (Falcon) at 21ºC in L-15 media supplemented as described above. Slide flasks were inoculated with 5 x 10^6 spores of *A. algerae* in media, and maintained at 18ºC. Two days after inoculation all medium was removed and replaced with spore free medium. After 10 days, the flask chamber was removed from the slide, and a coverslip placed over the slide and sealed using a fine line of petroleum jelly applied with a 18G blunt syringe. A Leica DM2500 upright microscope was used fitted with 100x oil immersion objective and condenser lens with a 1.2 numerical aperture. A Hamamatsu C7780 3 chip colour camera was used, and proprietary software algorithms from Quorum Technologies (www.quorumtechnologies.com) provided a means to

extract native colour information from the sample. The algorithms help to present the data in a way that is more easily interpreted than the actual image created in the microscope ocular. Further information on this technique can be found in the United States Patent document # 6,704,140.

Germination of purified spores grown in RTgill-W1

RTgill-W1 infected with 5 x 10^6 *A. algerae* spores were grown in a 25 cm^2 flask (Falcon) at 18°C for 14 days. Cells were lysed with water, and lysate was purified using a Percoll gradient as described in Chapter 2 (Monaghan et al. 2011). Purified spores were kept in a 1.5 ml bullet tube and vital stained with 10 µg/ml DAPI in PBS for more than 12 h. Spores were centrifuged, resuspended in 3% hydrogen peroxide, and placed on a glass slide. A coverslip was sealed using a fine line of petroleum jelly applied with a blunt syringe. Riveal microscopy images were taken using the camera, microscope and software described above.

Statistical analysis

The spore numbers were analyzed using GraphPad Prism (version 5 for Mac OS X, GraphPad Software, San Diego, CA, www.graphpad.com). One-way analyses of variance (ANOVA) were used to compare whether the number of spores in different cultures and at different temperatures were statistically different ($p<0.05$). Tukey's Multiple Comparisions test, an ANOVA post-test, was used to determine significance ($p<0.05$) between means at each temperature. For rainbow trout cell lines maintained at the same temperature, an unpaired t-test was used to determine significant differences ($p<0.05$) between cell lines regarding the number of spores per foci of infection.

3.3 Results

A. algerae growth in cultures of rainbow trout cell lines

Spores from RK-13 cultures were added to cultures of rainbow trout cell lines, RTgill-W1 and RTbrain-W1 from gill and brain respectively, and incubated at 21 °C, 18, and 9 °C and examined over time for sporonts and spores. Sporonts and spores were spindle-shaped phase-dark structures that stained diffusely with DAPI (Fig 3.1). At 4 days after the addition of spores, sporonts clearly were visible in the cytoplasms of some cells in cultures at 21 °C with mature spores observed after 6 days. The appearance of sporonts and mature spores in cultures of both cell lines at 18 ºC occurred on days 6 and 8 p.i., respectively. However, proliferation after 21 days was significantly greater in RTbrain-W1 than RTgill-W1 (Fig 3.2). At 18ºC, there was 60% ±30.3 less in the parasite numbers per infection site in the rainbow trout gill cells compared to the brain cells, and at 21ºC this decrease was 47% ±44.2. The number of *A. algerae* per loci were counted to represent the number of parasites that grew per infective transmission into a cell. However, it was observed that there were more than twice the number of loci at 21 °C as were found in flasks at 18 °C regardless of cell line, and no loci were observed at 9 °C. Cultures maintained at 9ºC did not demonstrate any conclusive parasite development, such as meronts or sporonts, over the 3-week period.

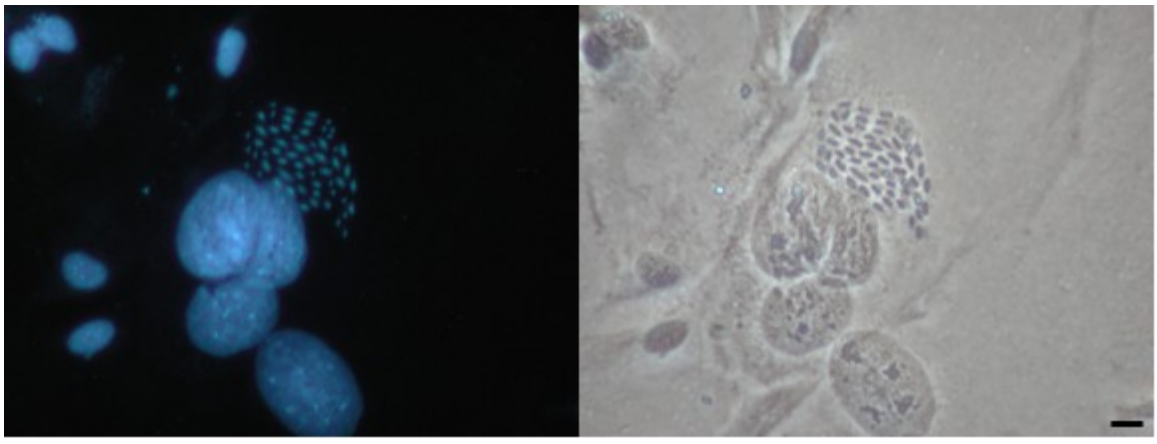

Figure 3.1 Foci of *Anncaliia algerae* growth in RTgill-W1 maintained at 18°C
Left: DNA was stained in fixed rainbow trout cell cultures in order to evaluate the level of infection 21 d p.i. Nuclei of sporonts and spores were visible in the cytoplasm of the RTgill-W1 cells using DAPI. Right: corresponding phase contrast image. Scale bar = 10 μm.

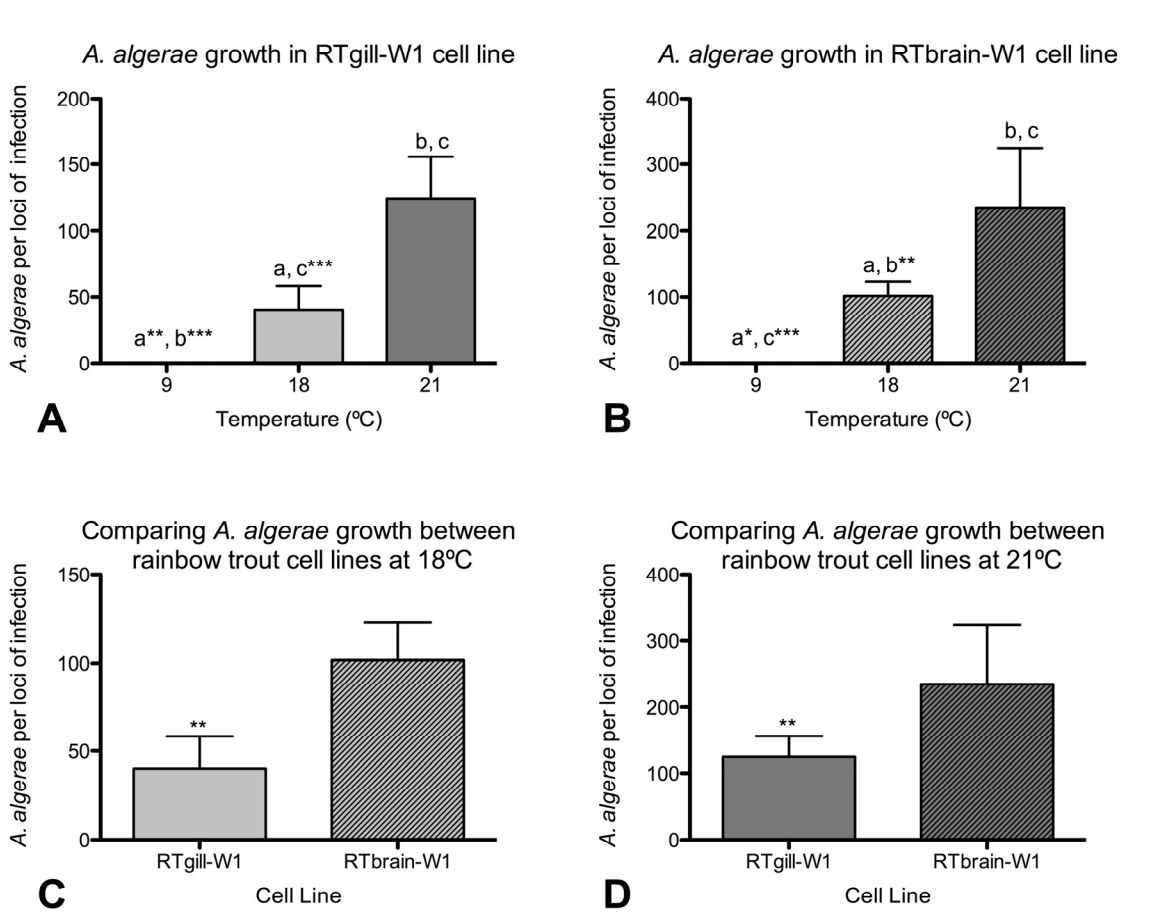

Figure 3.2 Comparing rainbow trout gill and brain cell lines for their ability to support *A. algerae* at 9, 18 and 21°C after 3 weeks

A and B: The mean number of *A. algerae* per infection site increased in both RTgill-W1 and RTbrain-W1 with increases in temperature. The means with SD are plotted (18 ºC n=5, 21 ºC n=5). ANOVA for both A and B indicates that temperature effects the number of *A. algerae* per loci of infection ($p<0.0001$). Significant differences between temperatures were evaluated using Tukey-Kramer's Multiple Comparisons test and indicated with the same lower case letter/symbol **C and D:** RTbrain-W1 supported more *A. algerae* growth than RTgill-W1 at both 18 and 21 ºC. The means with standard deviations are plotted. The differences between RTgill-W1 and RTbrain-W1 at both temperatures were significant with $p=0.0012$ at 18 °C and $p=0.0030$ at 21 °C using Tukey-Kramer's Multiple Comparisons test

Visualization of A. algerae *life cycle stages in RTgill-W1 by Riveal microscopy*

Most stages of the *A. algerae* life cycle that have been visualized by others through bright field light microscopy and transmission electron microscopy of fixed cultures have been identified tentatively for some stages and definitively for others through Riveal microscopy of living cultures (Fig 3.3). From the literature, the order of the life cycle stages can be succinctly summarized as sporoplasm, meront, sporont and sporoblast, followed in vitro by early spore and germinated early spore (Becnel et al. 2005). Although cultures were not examined immediately after infection when the chances of detecting the earliest stage, sporoplasms, would have been highest, the later stages, meronts, sporonts and sporoblasts, were seen as oblong or oval structures with at least one pair of nuclei. A nuclear pair (diplokaryons) was seen as a dark line, usually running perpendicular to the long axis of the oblong, with two very light grey circular structures on each side (Fig 3.3). Meronts, sporonts, and sporoblasts were distinguished by their size and by the darkness of their cytoplasms. Meronts were the largest and lightest of the three stages. Sporonts were slightly smaller and darker than meronts; sporoblasts slightly smaller and darker than sporonts (Fig 3.3 and 3.4). Meronts and sporonts are sometimes referred to collectively as the proliferative phase and some appeared to be dividing. Spores were dark. Nearby sporoblasts and spores in the host cytoplasm were small dark vesicles (Fig 3.4). In some spores internal features could be seen. The most prominent of these was a small, oval grayish white region at one end (Fig 3.4). Spores with these structures were identified as mature spores because they were subsequently seen to germinate. Germinated spores had an obvious polar tube (Fig 3.4). As the small, oval, grayish white regions anticipated germination and appeared at the ends opposite from which the polar tubes were eventually observed, they have been termed posterior pre-germination (PPG) vacuoles.

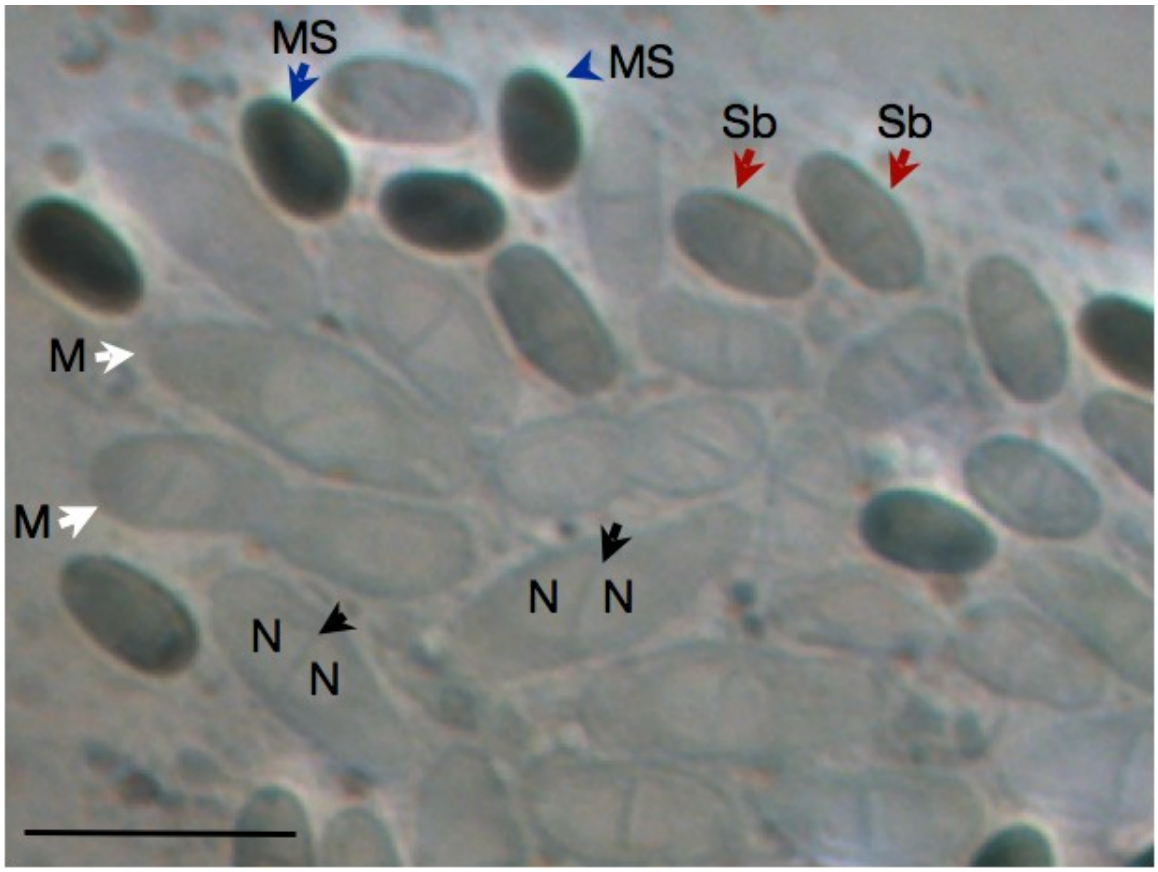

Figure 3.3 Diplokaryia of *A. algerae* and parasite developmental stages

Using Riveal Contrast microscopy, *A. algerae* proliferation is observed in RTgill-W1 cells maintained at 18°C, 10 d p.i. Two nuclei are observed in most developmental phases except in dividing meronts where 4 nuclei are present. Black arrows indicate where nuclear membranes meet, N indicates *A. algerae* nuclei. Meronts (M) are indicated with a white arrow, Sporoblasts (Sb) are indicated with a red arrow, and Mature spores (MS) are indicated with a blue arrow. Scale bar = 7 µm.

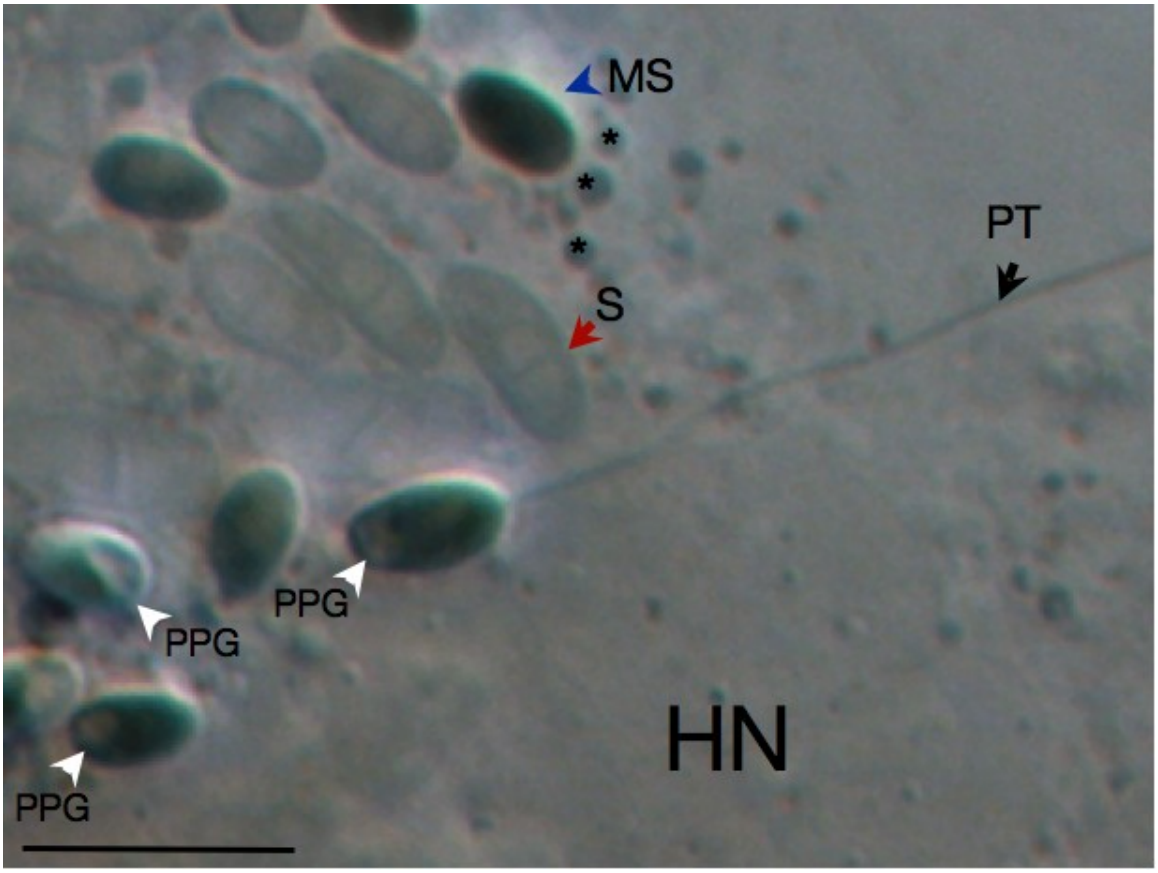

Figure 3.4 Germination of *A. algerae* and developmental phases

The posterior pre-germination vacuole (PPG), can be observed in the posterior pole (white arrowheads) of spores that are about to germinate or are germinating. The black arrow indicates a polar tube (PT) as it is being ejected from a spore situated adjacent to the nuclear membrane of the RTgill-W1 host cell nucleus (HN). A sporont (S) is indicated with a red arrow and mature spore (MS) with a blue arrow. * Indicates granular structures associated with spore development. Scale bar = 7 μm.

Video microscopy of A. algerae *proliferation*

Through video and Riveal microscopy the proliferative phase of the *A. algerae* life cycle could be observed. The best example of this was the cytoplasmic division of a meront. After 10 d at 18 °C flasks were placed on the Riveal microscope stage at room temperature over the period of 2 h and 25 minutes. The meront was initially seen as a sausage-shaped structure with a pair of nuclei at each end (Fig 3.5A). A cytoplasmic furrow developed and the meront cleaved to yield two cells each with a diplokaryon (Fig 3.5D). A video of this can be viewed using the supplementary material in the back pocket of bound copies of this thesis or by searching this thesis at http://uwspace.uwaterloo.ca (see Appendix B).

Video microscopy of A. algerae *spore development and germination*

Video and Riveal microscopy also allowed observations on the intracellular development and germination of *A. algerae* spores and on their extracellular germination in response to hydrogen peroxide. In the fish cells the oval spores at first appeared as uniformly dark but subsequently in some, over the period of 2 h and 25 min the PPG vacuole became visible. The development of a clearly visible posterior vacuole and a slight swelling of the spore anticipated the spontaneous intracellular germination of the spore (Fig 3.5A and 3.5C). At the moment of polar filament expulsion, which took place in less than a second, the PPG vacuoles expanded to fill most of the spore, appearing to push out the polar tube and sporoplasm and leaving behind the dark spore wall surrounding a predominantly grayish white region (Fig 3.5C and 3.5D). This region is referred to as a germination (G) vacuole. A video of this can be viewed using the supplementary material in the back pocket of bound copies of this thesis or by searching this thesis at http://uwspace.uwaterloo.ca (see Appendix B).

A similar sequence of events took place for spores that had been purified from the host cell cultures and triggered to germinate extracellularly with hydrogen peroxide (Fig 3.6 and 3.7). Some of these preparations were also stained with DAPI, permitting transmitted light and fluorescent views of the same field. This allowed visual confirmation that nuclei were expelled from spores and were in sporoplasms (Fig 3.6). Surprisingly, exceptions were found where the polar tube had been expelled but the DAPI still stained the spore for DNA

(Fig 3.7), suggesting a possible 'misfire'. Further investigation using Riveal microscopy can be used to examine various aspects of germination and incomplete germination.

Life cycle of A. algerae in fish cells derived from phase, fluorescent and Riveal microscopy

Using phase, fluorescent and Riveal microscopy, a schematic of the *A. algerae* life cycle as observed in fish cells was developed (Fig 3.8). The diagram depicts the parasite's stages in sequential order of development, as well as characterizes the appearance of growth in the cytoplasm as it was observed. Fluorescent microscopy with DAPI staining and Riveal microscopy allowed for early developmental stages, such as meronts, to be clearly seen. Meronts are spindle or sausage shaped structures in the cytoplasm of host cells (Fig 3.3) that are subtly defined. Sporonts are similar in shape to meronts, but are easily distinguishable using phase contract, fluorescent and Riveal microscopy since the plasmalemma is more well defined and the parasites at this stages are darker using phase contrast and Riveal microscopy. All three microscopy methods could be used to observe later developmental stages, such as sporoblasts and mature spores. However, the superior resolution provided by Riveal microscopy allowed for various details, such as the appearance of *A. algerae* nuclei at various stages and intracellular germination of spores, to be clearly illustrated.

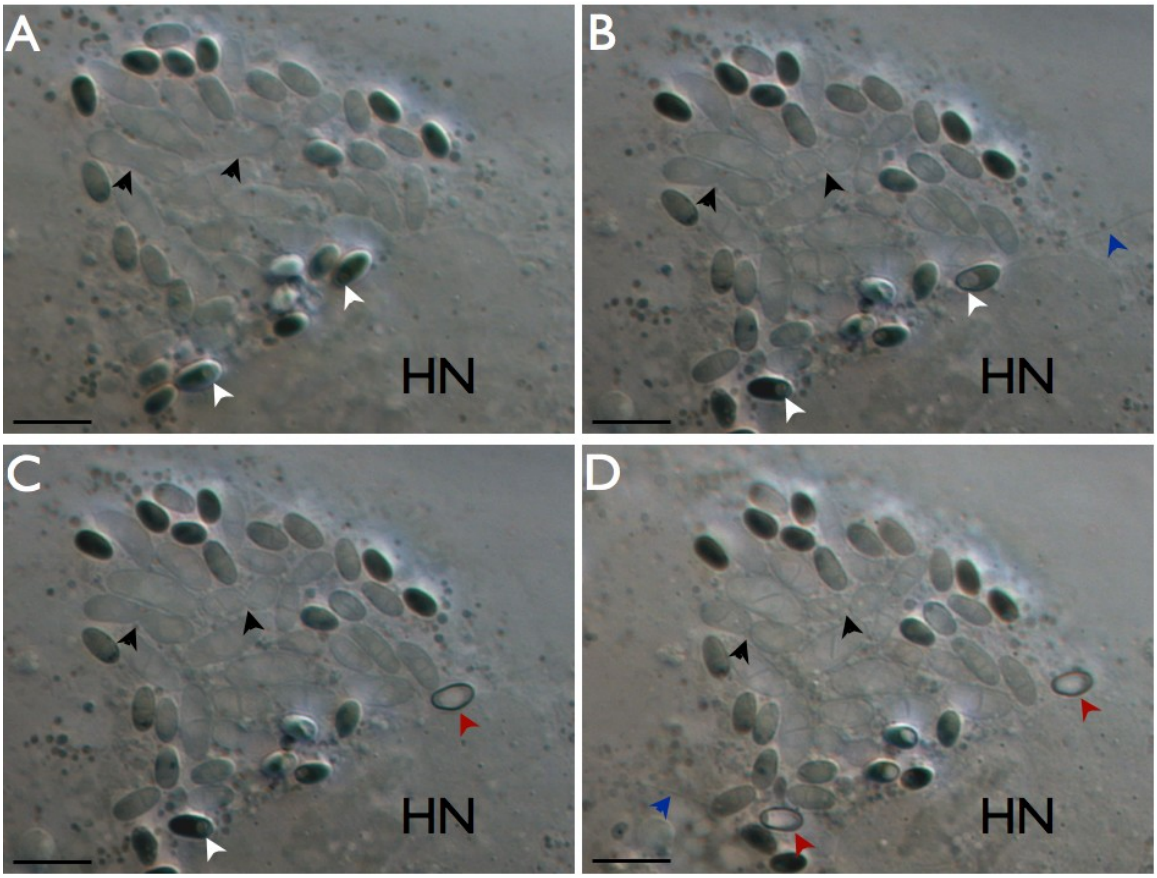

Figure 3.5 Time-lapse *A. algerae* proliferation and germination in RTgill-W1

Black arrows indicate dividing meronts observed over 2 h 25 min; White arrows indicate germination vacuole (G) which are visible in at least two other mature spores in D – indicating imminent germination; Blue arrows indicate extruded polar tubes (B and D), Red arrows denote empty spore coats surrounding germination vacuoles that have expelled all sporoplasm after germination. Scale bar = 7 µm. Riveal microscopy; Quorum Technologies Inc.. A, B, C and D represent images from video at time points 1, 85, 85 (seconds later) and 140 min. To watch video in full (30 times real-time), or a section of video highlighting germination, see Appendix B.

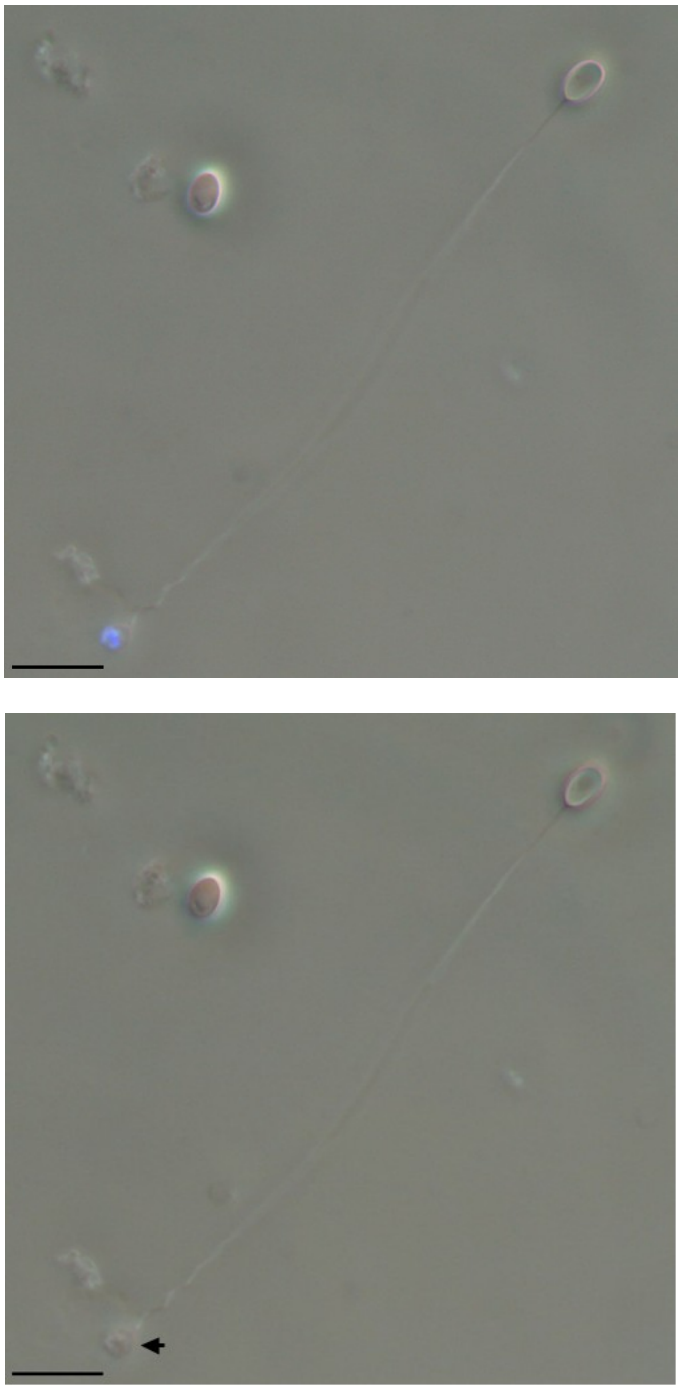

Figure 3.6 Extracellularly germinated *A. algerae* spore grown in RTgill-W1 at 18°C with DAPI stained nuclei in ejected sporoplasm

Riveal microscopy fluorescent (top) and corresponding non-fluorescent Riveal (bottom) micrograph. *A. algerae* spores were live stained with DAPI and germinated extracellularly using 3% hydrogen peroxide. Ejected sporoplasm (arrow) contains parasite nuclei. Scale bar = 7 µm.

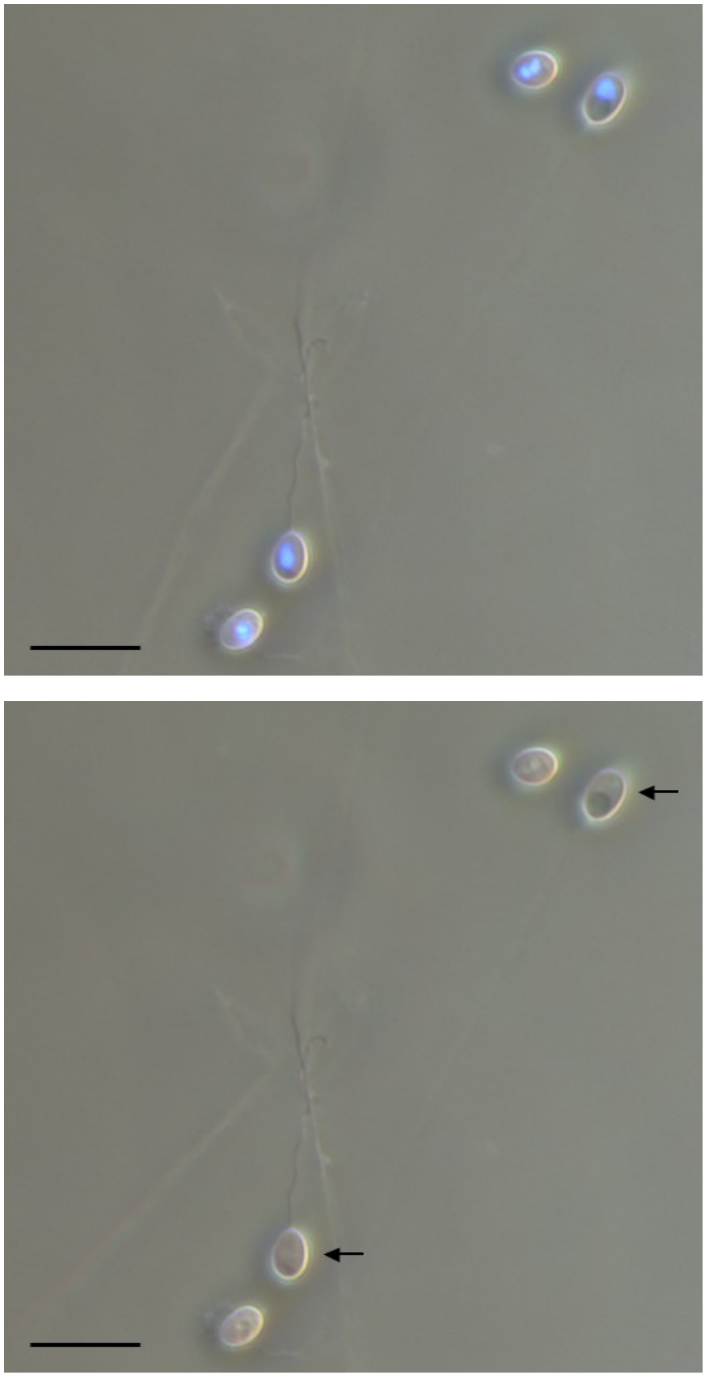

Figure 3.7 Incomplete germination, or misfire, of *A. algerae* spores grown at 18°C

A. algerae spores were live stained with DAPI and germinated with 3% hydrogen peroxide. Occasionally, spores "misfired" and the spore nucleus was not ejected with the polar filament (black arrows). The top Riveal fluorescent micrograph allows for DAPI stained nuclei to be observed, while the bottom panel is the corresponding non-fluorescent Riveal image. Refer to Appendix C for video from which these images were taken. Scale bar = 7 μm.

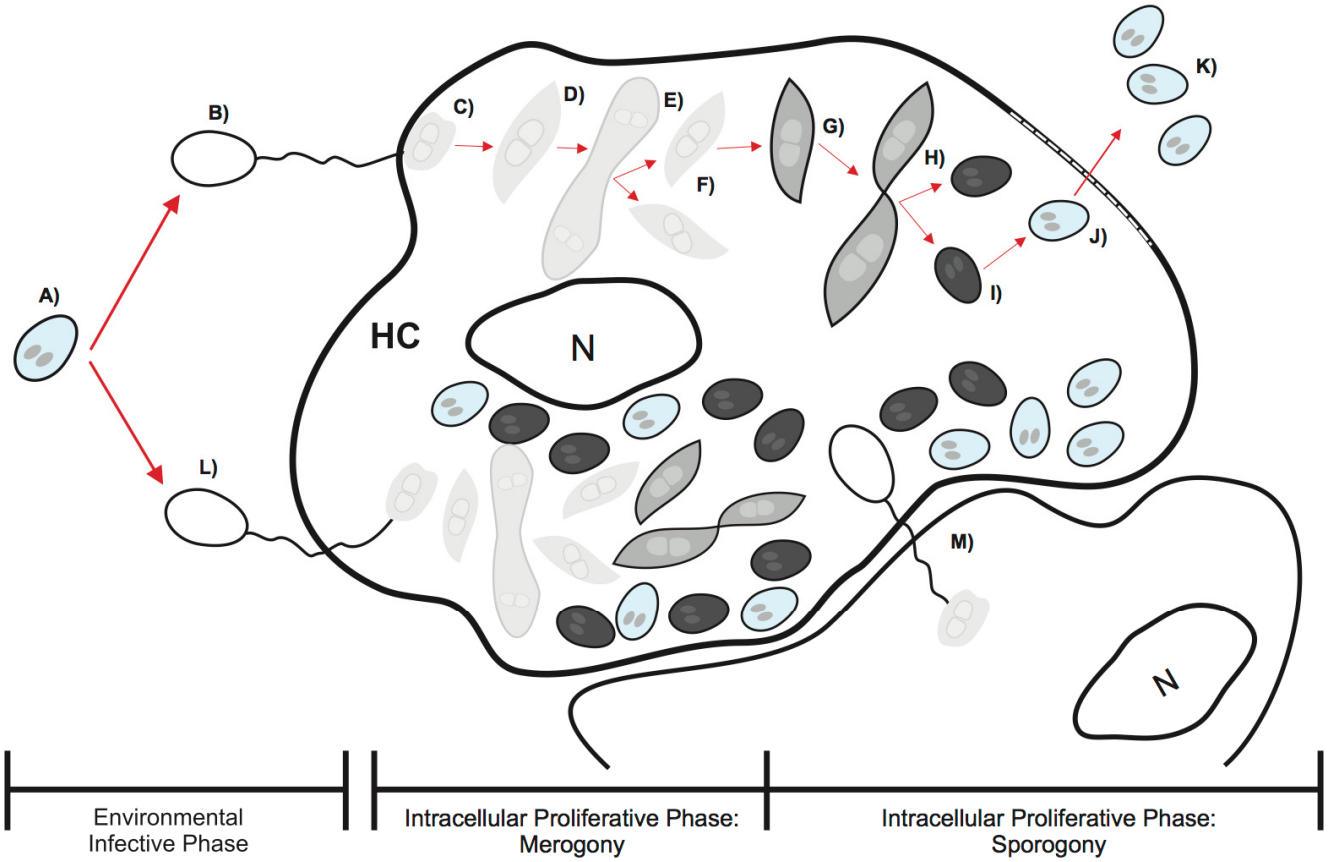

Figure 3.8 A schematic representation of the *A. algerae* life cycle in fish cell cultures as derived from phase contrast, fluorescent and Riveal microscopy observations

Fig 3.8 A schematic representation of the *A. algerae* life cycle in fish cell cultures as derived from phase contrast, fluorescent and Riveal microscopy observations

An *A. algerae* spore that starts the life cycle by germinating and injecting its contents or sporoplasm into the fish cell or host cell (HC) is shown in A. In the series B to K (connected with red arrows) in the top of the HC, the life cycle stages are arranged in the order in which they are thought to occur. In the bottom of the HC that begins with L is approximately the arrangement of the stages in a cell at any particular time. The sporoplasm (C) and meronts (D to F) are only occasionally visible using phase contrast microscopy, but can be observed using fluorescent microscopy in DAPI stained cultures, or Riveal microscopy. Spindle shaped meronts divide by becoming sausage shaped, and the midline narrows to the point that cytokinesis is complete and two new meronts result (F). Changes occur within the meront to become sporonts (G) and are more visible using light microscopy because of their phase dark appearance and more defined plasmalemma. Sporonts undergo division similarly to meronts (H), and their last division results in sporoblasts (I), which appear as phase dark ovids using light and Riveal microscopy. Sporoblasts undergo a metamorphosis to become spores (J) and appear as phase bright ovids using phase contrast microscopy. They are released into the environment by an unknown mechanism. The arrangement of the life cycle stages at the bottom of the diagram of the HC is shown as it is commonly observed within the cytoplasm of a fish cell. Spores are often in association with the nuclear membrane (nucleus = N), and plasma membrane, while earlier developmental stages are more centrally located in the cytoplasm. Intracellular spores can germinate in a living cell (M), and inject sporoplasm into a neighbouring cell to transmit infection in the culture.

3.4 Discussion

Two rainbow trout cell lines, RTgill-W1 and RTbrain-W1, have been shown to support the growth of the *A. algerae* at room temperature, which has allowed living cultures of *A. algerae* and rainbow trout cells to be visualized by a new method, Riveal microscopy. The implications of *A. algerae* growth in rainbow trout cells and the new features of its life cycle revealed by Riveal microscopy are discussed below.

Growth of A. algerae in rainbow trout cell lines

As observed in chapter 2 with cell lines from warm water fish, some cell lines in cold water fish appeared to support the growth of *A. algerae* better than other cell lines, but a pattern to the selectivity has yet to emerge. For rainbow trout cell lines, *A. algerae* grew better in RTbrain-W1 than in RTgill-W1. For warm water fish, a goldfish skin cell line, GFSK-1, supported the best growth. Explanations for the preferential growth in some cell lines over others might lie in differences in either the extracellular and/or intracellular environment. Extracellular matrix proteins and surface receptors might influence the adherence and entry of sporoplasms into host cells and thus the start of an infection. For example, sulfated glycans influenced the adherence of *Encephalitozoon intestinalis* to host cells (Hayman et al. 2005). Intracellular differences that could preferentially impede *A. algerae* development in one cell line over another cell line would include differential expression of antimicrobial defense mechanisms. In some insects, some tissues became infected more frequently by *A. algerae* and produced more spores than in other tissues, but only in some insect species (Staiber 1994, Undeen and Maddox 1973, Vávra and Undeen 1970). For infections in humans, *A. algerae* was found to have grown in epithelial, muscle, and connective tissues (Cali et al. 2010, Coyle et al. 2004, Visvesvara et al. 2005). Therefore, although there might be some selectivity in *A. algerae* infections, this microsporidial species appears capable of infecting most animal tissues.

This work demonstrates that the infective and proliferative temperature range of *A. algerae* is broader than previously reported. Over several years, efforts to elucidate the upper limit of this range have been the primary focus because of its clinical relevance. To investigate the lower limit of this range, rainbow trout cell cultures proved to be a valuable model. Rainbow trout cell lines kept at relevant environmental temperatures were capable of hosting *A. algerae* growth at 18ºC, though the number of spores per infection site was less than in cells maintained at 21ºC. There were no identifiable intracellular sporonts or spores present at 9 ºC. This could indicate that at this temperature *A. algerae* had limited capacity for germination and/or for progression through its life cycle. Interestingly, Undeen (1975) reported that temperatures of 26ºC reduced *A. algerae* spore germination compared to spores at 35ºC. This suggests that temperature may hamper spore infectivity at the germination stage

with inhibition or alteration of germination mechanisms, as well at the intracellular proliferative stage.

The observations here show that rainbow trout cells can support *A. algerae* growth but whether rainbow trout can be infected is unknown as no natural or experimental infections have been reported. *A. algerae* spores have been found in surface water (Avery and Undeen 1987), and *A. algerae* infect mosquitoes (Andreadis 2007, Becnel et al. 2005) which are eaten by rainbow trout. Thus rainbow trout and *A. algerae* could meet in the wild. However, several barriers can be considered as possibly preventing infection. For exposure through the digestive tract, *A. algerae* spores would have to germinate in the gut and infect epithelial cells of the GI tract. For exposure through water, *A. algerae* spores would have to germinate in mucus and infect skin or gill epithelial cells. If epithelial cell infections were established, the next barrier would be the innate and adaptive immune systems of fish. To date little is known about the immune response of fish to microsporidia, but adaptive immunity appears to play an essential role in eliminating infections (Rodriguez-Tovar et al. 2011). A final barrier might be temperature. The temperature preference of rainbow trout varies regionally but growth conditions of the fish are reported to be optimal at mean water temperatures at or below 20ºC, with preferred water temperatures ranging from 10 to 15.6ºC (FERC 2007). If *A. algerae* infection of rainbow trout is possible, lower temperature preferences of the fish may limit the parasite to a degree that allows for immune responses to clear infection. Even so, this research demonstrates that upper preferred temperature ranges of rainbow trout are within the lower temperature ranges of *A. algerae* proliferation, making infection in vivo something to look for in the future.

Riveal microscopy and A. algerae life cycle

For the first time, microsporidia have been examined by Riveal microscopy, a new bright field imaging technique that provides increased resolution and contrast without the use of stains. Usually the intracellular life cycle stages of *A. algerae* have been visualized in the past through Giemsa staining and bright field light microscopy (Undeen 1975, Vávra and Undeen 1970) or through heavy metal staining and transmission electron microscopy (TEM) (Avery and Anthony 1983, Cali et al. 2004, Lowman et al. 2000, Takvorian et al. 2005,

Trammer et al. 1999). Besides these microscopy methods, pores and spore germination have been examined by many additional techniques. These include video-enhanced contrast microscopy (Frixione et al. 1992), freeze-fracture (Cali et al. 2002), phase contrast microscopy and scanning electron microscopy (SEM) (Visvesvara et al. 2005). Riveal, fluorescent and phase contrast microscopy have provided unique insights into several aspects of the *A. algerae* life cycle, and were used to develop a schematic of the development that has been observed in fish cells with this work (Fig 3.8).

Riveal and video microscopy has provided both new and confirmatory information on the proliferative stage of *A. algerae*. For the first time, an event in the proliferative stage has been videoed and timed. This is the cytoplasmic division of a meront, which was found to take approximately 2 h at room temperature in rainbow trout cells. The timing might differ at other temperatures and in other cells. Also, the act of observing the cells might have influenced the timing by raising the temperature. Additionally the meront examined had two diplokaryons and the timing might change in meronts with one diplokaryon. Therefore many examples will have to be recorded in the future to build up an overview of meront division, but this study is a start. Riveal microscopy detected dark, round granules at sites in the host cytoplasm where sporogny was occurring. These resemble the dense spherical material that was seen through TEM by Takvorian et al (2005) in the vicinity of sporognic stages. The authors speculated that these might be remnants of vesiculotubular appendages that were present on earlier developmental stages. Unfortunately vesicuolotubular appendages were not obvious with Riveal microscopy so the origin of these dark, round granules is unknown at this time.

Intracellular germination of *A. algerae* was clearly documented by video and Riveal microscopy. The germination of spores within cells in culture has been observed for several *Nosema* species (Fries et al. 1992, Ishihara 1969, Iwano and Ishihara 1989, Kawarabata and Ishihara 1984, Kurtti et al. 1983) but this is the first observation of the phenomenon with *A. algerae*. However, because of the rapid progression of in vivo infections within individual mosquito larvae, *A. algerae* spore germination has been thought to take place intracellularly (Avery and Anthony 1983). The first spores produced, early spores, might be subtly different from spores coming afterwards and have a different role in disease transmission. Early spores could be acting to quickly spread the infection to other cells and tissues within a

host, whereas the late or environmental spores would transmit the infection between individuals (Avery and Anthony 1983).

What triggers intracellular germination in the current study remains to be determined and could include physiological or artificial stimuli. Physiological variables that have been found to trigger microsporidia spore germination include changes in pH, cations, anions, and osmolarity (Xu and Weiss 2005). Stimuli could be created artificially as the culture is being observed with the microscope. These could include an increase in temperature and osmolarity as the slide slowly dries. In the future, attempts can be made to control this by trying different viewing chambers, but clearly rainbow trout cells are advantageous in that observations can be done at room temperature, which is appropriate for the health of these cells.

The video and Riveal microscopy has provided for the first time visual evidence for a posterior vacuole-like structure in *A. algerae* spores. In the spores of many microsporidia, a posterior vacuole has been identified (Williams 2009). By TEM this often appears as an electron-lucent region. However no such structure has been found in *A. algerae* spores (Cali et al. 2002, Chioralia et al. 1998, Sinden and Canning 1974), although an electron dense amorphous zone posterior to the nucleus has been distinguished (Chioralia et al. 1998). With Riveal microscopy, the posterior region of some spores clearly contained a small, oval, grayish region. This structure slowly became apparent over approximately 2 h at the posterior and then expanded rapidly to almost completely fill the spore, with the concurrent explosive emergence of the polar tube. As this posterior structure became visible just before germination and to distinguish it from the posterior vacuole common in other microsporidia, the name posterior pre-germination (PPG) vacuole is used to describe this before polar tube extrusion and germination (G) vacuole afterwards. The PPG vacuole was not seen for spores that had been purified from cells and stimulated to germinate with hydrogen peroxide. In this case the PPG might have developed too quickly to be detected. Alternatively extracellular germination might be different, either because of differences in the spores or in the environment in which germination takes place, and not involve a PPG vacuole. This might also be why a posterior vacuole-like structure has not been seen in *A. algerae* spores by TEM. However, after germination, TEM did reveal a large vacuole that filled most of the spore (Vávra and Undeen 1970). This might be the G vacuole.

For microsporidia in general, the sudden swelling of the posterior vacuole has been thought to play a pivotal role in generating the force necessary for spore germination (Xu and Weiss 2005), and the PPG of *A. algerae* appears to perform a similar function. The mechanisms by which a posterior vacuole swells and generates expulsive force are still unclear but several ideas have been proposed, usually invoking changes in intrasporal osmotic pressure (Lom and Vávra 1963, Undeen and Frixione 1990, Xu and Weiss 2005) (Findley et al. 2005). Osmotic pressure could increase due to changes in the permeability of the spore coat to water (Lom and Vavara, 1963), in a proton gradient in the posterior vacuole (Dall 1983), and in the degradation of trehalose into a large number of small molecules (Undeen and Frixione 1990). The involvement of the PPG in these processes awaits further research.

Riveal microscopy of DAPI stained spores after germination had been stimulated with hydrogen peroxide clearly illustrated examples of polar tube discharge without nuclear expulsion. Occasional incomplete discharges have been observed previously for *A. algerae* spores (Frixione et al. 1992) but the retention of the spore nuclear DNA is more clearly illustrated in the current study. Spore discharge can be divided into several phases (Frixione et al. 1992, Xu and Weiss 2005). These can be reduced most simply to just two: polar filament eversion and passage of the main sporoplasm mass through the filament. For unknown reasons, these normally coordinated processes become disconnected occasionally in *A. algerae*, possibly because of the complexity of the last step.

Chapter 4

Evaluating three classes of antimicrobial drugs on a unique in vitro combination of microsporidia and host cells: *Anncaliia algerae* and the zebrafish cell line, ZEB2J

Overview

Cultures of the zebra fish embryo cell line, ZEB2J, that had been infected with *Anncaliia algerae* spores were used as an in vitro test system to evaluate the curative actions of albendazole, fumagillin, and three fluoroquinolones; ciprofloxacin, norfloxacin, and ofloxacin. For each drug at concentrations above 50 µg/ml, the viability of ZEB2J cell declined sharply so concentrations of 10 and 20 µg/ml were studied. At these concentrations the drugs had little effect on the morphology and germination *A. algerae* spores. Each of the fluoroquinolones failed to prevent *A. algerae* from infecting ZEB2J cells and from growing to the same extent as in untreated ZEB2J cultures. Adding albendazole or fumagillin to cultures did not prevent *A. algerae* from infecting ZEB2J cells but impeded the growth and accumulation of *A. algerae* life-cycle stages. However, albendazole treatments caused a significant fraction of the ZEB2J cells to have nuclear abnormalities. Fumagillin reduced the intensity of infections within a ZEB2J cell, although the number of infected cells in a culture was not reduced. Over 5 days of infection with *A. algerae* the accumulation of ZEB2J cells in cultures was reduced but fumagillin treatment restored the accumulation to control levels. These results suggest that fumagillin has some potential as a treatment for *A. algerae* infections.

4.1 Introduction

Microsporidia are a group of intracellular parasites capable of infecting a broad range of hosts including mammals, fish and insects and of causing diseases in humans and significant economic losses in agriculture and aquaculture. Therapeutic options to treat microsporidiosis have been elusive (Costa and Weiss 2000, Gross 2003). One method for studying potential antimicrosporidial agents to treat mammals has been to determine the effectiveness of drugs in vitro (Beauvais et al. 1994, Didier 1997, Franssen et al. 1995). This is a quick and inexpensive approach, but this has yet to be used for fish. The demonstration that fish cell lines support the growth of the microsporidia, *Anncaliia algerae,* (Chapter 2) opens up the possibility of testing the efficacy of potential chemotherapeutic drugs on experimentally infected fish cells.

To date the in vitro approach for testing potential cures has been restricted to relatively few combinations of animal cells and microsporidia (Table 4.1). The animal cells have been either insect cell lines or primary cell cultures and cell lines from mammals. The first insect cell line was IPLB from the moth *Heliothis zea* and the infectant was either *Glugea disstriae or Nosema* sp (Kurtti and Brooks 1977). Subsequently, the S9 cell line from the fall armyworm, *Spodoptera frugiperda*, was used with the microsporidia, *Nosema bombycis* (Haque et al. 1993, Sichtova et al. 1993). For mammalian cells, treatments for eliminating *Encephalitozoon cuniculi* have been studied in primary cultures from rabbit choroid plexus and kidney and from canine embryos (Shadduck 1980) and in cultures of three cell lines, Madin-Darby kidney (MDCK) (Beauvais et al. 1994), rabbit kidney RK13 (Franssen et al. 1995), and monkey kidney Vero (E6) (Ditrich et al. 1994, Sobottka et al. 2002). The inhibition of microsporidial growth also has been examined in cultures of MDCK with *Vittaforma corneae* (Silveira and Canning 1995), of RK13 with *V. corneae* and *E. intestinalis* (Didier 1997, Didier et al. 2005), and of Vero with *E. hellem* (Ditrich et al. 1994). The mouse myoblast cell line C2, C12 has been used to study the treatment of *Trachipleistophora hominis* (Lafranchi-Tristem et al. 2001). Neither fish cells nor *A. algerae* have been subjects for in vitro studies of antimicrosporidial agents.

The main drugs studied in vitro for the ability to inhibit microsporidia growth have been fumagillin and albendazole, although recently several others have been explored to a limited degree, such as the fluoroquinolones. Fumagillin is a natural product of the fungus

Aspergillus fumigatus with a rare sesquiterpene carbon skeleton and a range of biological actions and uses for which the first prominent one was the treatment of honeybees for *Nosema apis* (Katznelson and Jamieson 1952). The antimicrosporidial activity of fumagillin has been demonstrated in vitro against *E. cuniculi* in primary mammalian cell cultures (Shadduck 1980) and in cultures of cell lines from dog (MDCK), rabbit (RK13) and monkey (E6) (Beauvais et al. 1994, Franssen et al. 1995, Sobottka et al. 2002), and against *E. intestinalis* and *Vittaforma corneae* in RK13 cultures (Didier 1997, Didier et al. 2006). Albendazole belongs to the benzimidazole compounds, which are dicyclic with benzene and imidazole rings. Antimicrosporidial activity by albendazole has been demonstrated in vitro against *N. bombycis* in S9 cells (Haque et al. 1993), *E. cuniculi* in cultures of MDCK, RK13 and E6 (Beauvais et al. 1994, Franssen et al. 1995), *E.intestinalis* in RK13 (Didier 1997), *E. hellem* in E6 (Ditrich et al. 1994), V. *corneae* in RK13 (Didier 1997, Silveira and Canning 1995), and *T. hominis* in C2,C12 cultures (Lafranchi-Tristem et al. 2001). The fluoroquinolones are fluorinated derivatives of quinolone compounds and have been synthesized and marketed for their antibacterial actions. Fifteen fluoroquinones have been assayed for their ability to inhibit the growth of *E. intestinalis* and *V. corneae* in RK13 cultures and several were very effective, others had little effect, and some were more effective on one microsporidial species than on the other (Didier et al. 2005).

Differences between the host animal cells and the infectant microsporidia appear to account for the antimicrosporidial actions of fumagillin, albendazole and the fluoroquinones. For fumagillin the mechanism of action likely revolves around methionine aminopeptidases (MetAPs), which come in two forms, MetAP-1 and MetAP-2. MetAPs remove methionine from the N-terminus of growing polypeptides, which is a necessary step for other post translation modifications to proceed at the N-terminus and ultimately for the protein to function properly. Fumagillin inhibits MetAP-2 but not MetAP-1 (Sin et al. 1997). Microsporidia, at least as represented by *E. cuniculi*, has only MetAP2, whereas mammalian cells have both MetAP-1 and MetAP-2 and presumably survive in the presence of fumagillin because MetAP-1 still functions (Weiss et al. 2003, Zhang et al. 2005). Albendazole likely is effective because the drug inhibits the polymerization of tubulin from parasites at much lower concentrations than tubulin from mammals (Chatterji et al. 2011). In some microsporidia albendazole blocks the development of intranuclear spindles (Colbourn et al.

1994). These structures are thought to be the only site in microsporidia where microtubules assemble and interfering with their assembly would interfere with spindle formation, which would prevent chromosome segregation and microsporidia growth (Colbourn et al. 1994). For the fluoroquinolones, the inhibition of enzymes necessary for DNA replication in microsporidia but not in mammalian cells likely accounts for their effectiveness. In bacteria some fluoroquinones inhibit two enzymes essential for DNA replication, DNA gyrase and topoisomerase IV. The gene for topoisomerase IV has been identified in the genome of one microsporidia, *Vittaforma corneae* (Didier et al. 2005, Mittleider et al. 2002).

The goal of this chapter was to evaluate different classes of antimicrobial drugs on a new in vitro combination of microsporidia and host cells: *Anncaliia algerae* and the zebrafish cell line, ZEB2J. Neither *A. algerae* nor fish cells have previously been subjects for in vitro studies of antimicrosporidial agents. The agents tested were three fluoroquinoles (ciprofloxacin, norfloxacin, and ofloxacin), albendazole and fumagillin. Evaluating cultures for the efficacy of drug treatments to eliminate microsporidia is difficult, with no standard method yet to emerge. Here two new steps were used. Cultures were stained with the fluorescent DNA dye, 4',6-diamidino-2-phenylindole (DAPI), and ImageJ 1.42q software (National Institutes of Health, NIH) was used as an aid to quantify microsporidia. Fumagillin was found to hold the most promise as a drug to treat *A. algerae* infections.

Table 4.1 Animal and insect primary and cell cultures used to test potential antimicrobial agents against microsporidia

Cell Line or Primary Culture	Origin of Cell Line or Primary Culture	Microsporidia	Drug	Reference
IPLB	*Heliothis zea* (moth)	*Glugea disstriae* or *Nosema* spp.	Fumagillin	Kurtti and Brooks 1977
Primary cultures	Rabbit choroid plexus and kidney, and canine embryos	*Encephalitozoon cuniculi*	Fumagillin	Shadduck 1980
S9	*Spodoptera frugiperda* (fall armyworm)	*Nosema bombycis*	Albendazole	Haque et al 1993
S9	*Spodoptera frugiperda* (fall armyworm)	*Nosema bombycis*	Albendazole	Sichtova et al 1993
MDCK and MRC5	Canine kidney and Human embryonic lung, respectively	*Encephalitozoon cuniculi*	Fumagillin Albendazole and 20 other drugs	Beauvais et al 1994
E6 (Vero)	Money Kidney	*Encephalitozoon cuniculi*	Albendazole	Ditrich et al 1994
RK-13	Rabbit kidney	*Encephalitozoon cuniculi*	Fumagillin	Franssen et al. 1995
MDCK	Canine kidney	*Vittaforma corneae*	Albendazole	Silveira and Canning 1995
RK-13	Rabbit kidney	*Vittaforma corneae* *Encephalitozoon intestinalis*	Albendazole, Fumagillin, and TNP-470	Didier 1997
C2, C12	Mouse myoblast	*Trachipleistophora hominis*	Albendazole	Lafranchi-Tristem et al. 2001
E6 (Vero)	Money Kidney	*Encephalitozoon cuniculi*	Fumagillin	Sobottka et al. 2002
RK-13	Rabbit kidney	*Vittaforma corneae* *Encehpalitozoon intestinalis*	15 Fluoroquinolones	Didier et al 2005
RK-13	Rabbit kidney	*Vittaforma corneae* *Encehpalitozoon intestinalis*	Fumagillin, TNP-470, Ovalicin and derivatives	Didier et al 2006

4.2 Materials and Methods

Chemotherapeutic Drugs

Albendazole, fumagillin, and three fluoroquinolones were tested. Sigma-Aldrich (St Louis, MO) was the source of four chemotherapeutics: albendazole ≥ 98% (Titration by $HClO_4$) (A4673), norfloxacin ≥ 98% (TLC)(N9890), ciprofloxacin >98.0% (HPLC) (17850), and ofloxacin ≥ 99% (HPLC) (O8757). Fumagillin-B dicyclohexylammonium was purchased from Medivet Pharmaceuticals Ltd., Red River, AB. The fumagillin-B was a proprietary formulation of 2.1 mg fumagillin-B per 100 mg dicyclohexylammonium salts from Medivet Pharmaceuticals for use in apiculture in the control of *Nosema apis*. All drugs were in powder form and weighed on an analytic balance. Stock solutions of albendazole and the fluoroquinolones were prepared at 200 times concentration in dimethyl sulfoxide (DMSO) to give concentrations of 2 and 4 mg of each chemotherapeutic per ml. Fumagillin-B was prepared at 20 times concentration in 10% DMSO and 90% L-15 to given concentrations of 200 and 400 μg of fumagillin-B per ml. Stock solutions were added to give final concentrations of 10 and 20 μg/ml in cultures of the fish cell line, ZEB2J.

ZEB2J cultures

ZEB2J was developed from zebrafish blastula (Xing et al. 2008) and was grown routinely at 21 °C in Leibovitz's medium (L-15) (HyClone) with 10 % fetal bovine serum (FBS) and 100 IU/ml of penicillin/streptomycin (P/S). This medium is referred to as growth medium or L-15/FBS. ZEB2J is predominantly epithelial-like. Chemotherapeutic drugs were tested on cultures of ZEB2J to which *A. algerae* spores had been added but first their effect on ZEB2J viability was determined.

Effects of chemotherapeutics on ZEB2J

Two fluorescent indicator dyes, alamar blue (AB or resazurin) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), were used to examine the viability of ZEB2J cultures after exposure to the chemotherapeutics. ZEB2J cells were plated in 96-well plates at approximately 1.5×10^4 cells per well. After 24 h, the growth medium was removed and replaced with fresh growth medium for control wells and with different concentrations of the

chemotherapeutic in growth medium for treatment wells. Plates were placed at 27°C for 5 d. At this point the media were removed and the viability of cultures was evaluated by adding AB and CFDA-AM for 1 h as described previously (Dayeh et al. 2005). AB becomes fluorescent upon reduction by living cells and provides a relative measure of energy metabolism. CFDA-AM is converted to carboxyfluorescein (CF) by cellular esterases and retained by cells with intact plasma membranes. The reduction of AB and retention of CFDA-AM by each well were measured as relative fluorescence units (RFUs) with a Spectra-Max microplate reader. The RFUs in treatment wells were expressed as a percentage of the RFUs in control wells.

Anncaliia algerae spores

The microsporidia, *A. algerae*, was obtained from the American Type Culture Collection (ATCC, Manassas, VA) as spores (ATCC number PRA-168). Also purchased from ATCC was the rabbit kidney epithelial cell line RK-13 (CCL-37). RK-13 was grown routinely at 37 °C in L-15 with 10 % FBS and 100 IU/ml P/S. *A. algerae* spores were grown in and purified from RK-13 cultures at 27 °C as described previously (Chapter 2). The spores were used to infect ZEB2J cultures but first the effect of chemotherapeutics on spore morphology and germination was determined.

Effect of chemotherapeutics on A. algerae spores

Spores were incubated with each drug for 24 h prior to being exposed to 3% hydrogen peroxide to trigger germination. Exposures began by dividing preparations of purified *A. algerae* spores into six equal aliquots and centrifuging them at approx 450 x g for 5 min to get six pellets of spores. These were resuspended in L-15/FBS that had either one of the five chemotherapeutics at 20 µg/ml or no addition (control) and incubated at 27°C for 24 h. At this time a small sample of each spore suspension was taken for observation under a phase-contrast microscope and exposures were terminated. For terminating exposures, the suspensions were centrifuged at 450 x g for 5 min and the supernatants removed. The spores were resuspended in 3% hydrogen peroxide (Life Brand) and incubated at 27°C. After 20 hours, 100 µl of each suspension was placed on a slide and observed by phase contrast

microscopy. Spores that had become phase dark, rather than phase bright, and/or had ejected polar filaments were scored as germinated.

Effect of Chemotherapeutics on ZEB2J cultures with A. algerae spores

Cultures of ZEB2J that had been grown to confluency at 21 °C in 12 well tissue culture plates (Falcon) were infected with *A. algerae* spores and treated for 5 days with chemotherapeutics. For cultures to be subject to chemotherapy, the spores were suspended in L-15/FBS with either 10 or 20 µg/ml of albendazole, fumagillin, ciprofloxacin, norfloxacin or ofloxacin. For cultures without chemotherapy, the spores were suspended in L-15/FBS with 0.5 % DMSO. In all cases these media contained approximately 3.4 x 10^3 spores/ml and 3 ml were applied to each well. Some additional control wells received 3 ml of only L-15/FBS. Plates were incubated at 27°C for 5 days. Each 12 well plate had 3 wells designated for control, and three wells designated for each concentration of drug. The experiments were repeated 3 times. At this point cultures were fixed in Carnoy's and stained with 10 µg/ml of DAPI as described in Chapter 2. Cultures were examined with a Nikon TE300 inverted phase constrast microscope with a TE-FM Epi Fluorescence attachment. The appearance in the cytoplasm of ZEB2J cells of phase- dark, spindle-shaped structures that stained weakly with DAPI were interpreted as sporonts and as a sign that infection had taken place. For the growth of *A. algerae* in cultures with and without treatments all life cycle stages were counted as outlined below.

Quantifying A. algerae infections in ZEB2J cultures with and without chemotherapy

A Nikon Cool Pix 5400 camera was used to photograph DAPI-stained cultures and from the fluorescent micrographs the number of *A. algerae* was counted with the aid of ImageJ 1.42q. A grid was established for the surface of each well so that a total of 6 fluorescent images and corresponding phase contrast micrographs were taken each at a grid intersection. An example of a fluorescent image without and with counting markers is illustrated in Fig 4.1. Dragging and dropping the JPG micrographs on the bottom of the ImageJ tool bar opened images in the software. "Particle Analysis" was selected under "Plugins" at the top of the screen, and "Cell Counter" was then selected from the drop down menu. This action re-opened the image in Cell Counter mode. To begin counting, a marker colour must be selected

from the clearly labeled options. “Infected Cells” were designated a red marker, “Uninfected Cells” a yellow marker, and “*A. algerae*” at any phase of development was designated a green marker (Fig 4.1). The values obtained from the counts of 6 images were averaged to establish a count per field for each well.

Statistical analysis

The numbers were analyzed using GraphPad Prism (version 5 for Mac OS X, GraphPad Software, San Diego, CA, www.graphpad.com). A one-way analysis of variances (ANOVA) was used to compare differences among drugs or among concentrations, and determine statistical significance ($p<0.05$). Tukey-Kramer’s Multiple Comparisions test is an ANOVA post-test used to determine significance ($p<0.05$) between means of every treatment, and every other treatment. An unpaired *t* test was used to determine significance ($p<0.05$) between total cells in uninfected ZEB2J cultures and *A. algerae* infected ZEB2J cultures.

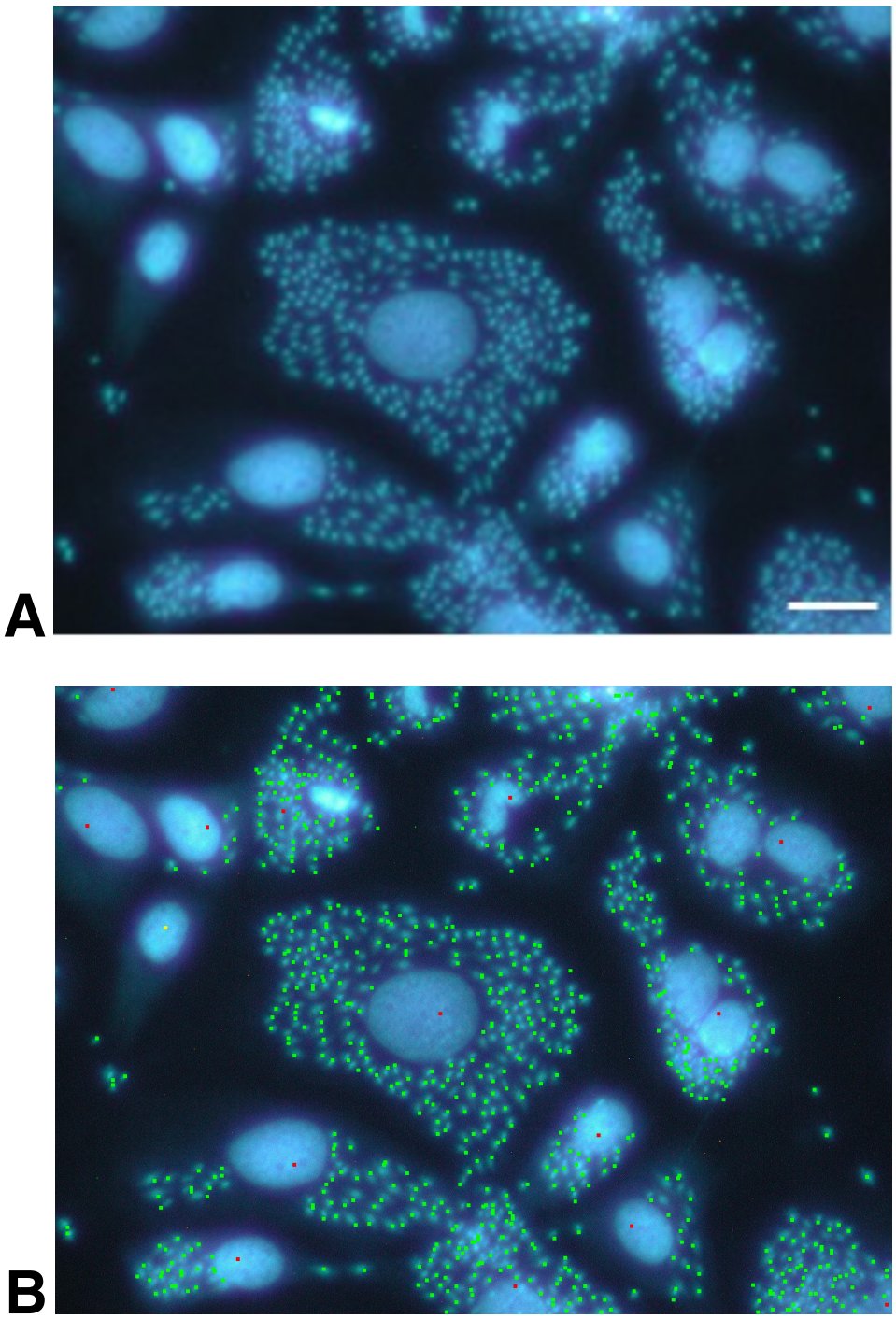

Figure 4.1 Example of how one of six images of a DAPI stained culture was scored

A) Fluorescent DAPI stained nuclei of ZEB2J and *A. algerae* without software enhancement. The host and microsporidia nuclei fluoresce blue unadorned with counter markers. B) Software enhanced image of above micrograph. Red and yellow markers indicate nuclei of infected cells and uninfected cells, respectively. Multinucleated cells were counted as a single cell. Green markers indicated intracellular or extracellular *A. algerae* at any stage of development. Counts for this image: Red= 15, Yellow = 1, Green = 825. Scale bar = 20 µm.

4.3 Results

Effects of chemotherapeutics on ZEB2J

Exposure for 5 days to increasing concentrations of each of the 5 chemotherapeutic agents caused a dose dependent decline in cell viability as measured with either Alamar Blue for energy metabolism or CFDA AM for cell membrane integrity (Fig 4.2). At 1,000 µg/ml cell viability was reduced to near zero as evaluated with either endpoint, but at 20 µg/ml little or no change was observed. With each of the chemotherapeutics at 20 µg/ml, cultures were examined for changes in appearance. Cellular and nuclear shapes were unchanged in fumagillin, ciprofloxacin, norfloxacin and ofloxacin, but cultures with 20 µg/ml of albendazole had a small percentage of cells with irregular nuclear outlines, multiple nuclei and enlarged nuclei (Fig 4.3 and 4.7). Despite this sublethal action of albendazole, the highest concentrations of drugs that had little or no effect on cell viability in ZEB2J cultures, 10 and 20 µg/ml, were studied further.

Effect of chemotherapeutics on A. algerae spores

Exposure to the chemotherapeutics appeared to have little effect on the morphology and germination *A. algerae* spores. After exposure to each of the five drugs at 20 µg/ml for 24 h, little or no change was seen in the overall spore morphology as judge by phase contrast microscopy and in spore nuclear shape as judged by fluorescence microscopy after staining with DAPI. The spores remained as ungerminated spores in these solutions. However, when drugs were removed and the spores exposed to 3% hydrogen peroxide, spore germination was seen (Fig 4.4). Whether the spores had had a prior 24 h drug treatment or not, the germination percentage was near 100%. These results indicated that exposure to albendazole, fumagillin, ciprofloxacin, norfloxacin, or ofloxacin for 24 h did not inhibit the ability of the spores to subsequently germinate.

Effect of fluoroquinolones on ZEB2J cultures with A. algerae spores

Adding fluoroquinolones to cultures did not prevent *A. algerae* from infecting ZEB2J cells and from growing to the same extent as in untreated ZEB2J cultures. Five days after

concurrent infection with *A. algerae* spores and treatment with fluoroquinolones ZEB2J cultures had cells with sporonts (Fig 4.5). The appearance of sporonts provided unequivocal evidence that *A. algerae* infection took place in the presence of ciprofloxacin, nofloxacin and ofloxain at 10 and 20 µg/ml. When the total of all *A. algerae* life cycle stages were enumerated for cultures, little difference was found between control and treated cultures, indicating that *A. algerae* continued to develop and grow over 5 days in the presence ciprofloxacin, nofloxacin and ofloxacin (Fig. 4.6).

Effect of albendazole on ZEB2J cultures with A. algerae spores

Adding albendazole to cultures did not prevent *A. algerae* from infecting ZEB2J cells but impeded the growth and accumulation of *A. algerae* life-cycle stages. Five days after concurrent infection with *A. algerae* spores and treatment with albendazole ZEB2J cultures had cells with sporonts and these sporonts often appeared larger than in untreated ZEB2J cells (Fig 4.7). This suggests that in cultures with albendazole *A. algerae* spores infected ZEB2J cells but subsequent development was impeded. This was seen clearly when the total of all *A. algerae* life cycle stages were enumerated. Cultures with 10 and 20 µg/ml of albendazole had significantly fewer *A. algerae* life-cycle stages than control cultures (Fig 4.8A). Albendazole and control cultures were further compared by counting the number of *A. algerae* life-cycle stages per infected cell and the number of infected cells per field (Fig 4.8 B and C). Albendazole at 10 and 20 µg/ml reduced the number of *A. algerae* life-cycle stages per infected cell but not the number of infected cells per field (Fig 4.8B and C). As noted earlier for ZEB2J cultures without spores but with albendazole (Fig 4.3), cells with irregular nuclear outlines, multiple nuclei and enlarged nuclei were observed in ZEB2J cultures infected with spores and treated with 10 and 20 µg/ml albendazole (Fig 4.7B).

Effect of fumagillin on ZEB2J cultures with A. algerae spores

Adding fumagillin to cultures did not prevent *A. algerae* from infecting ZEB2J cells but impeded the growth and accumulation of *A. algerae* life-cycle stages. Five days after concurrent infection with *A. algerae* spores and treatment with fumagillin ZEB2J cultures had cells with sporonts and these sporonts sometimes appeared thinner than in untreated ZEB2J cells (Fig 4.9). This suggests that *A. algerae* spores infected ZEB2J cells in the presence of fumagillin but fumagillin interfered with subsequent development. When the

total of all *A. algerae* life cycle stages were enumerated, cultures with 10 and 20 µg/ml of fumagillin had significantly fewer *A. algerae* life-cycle stages than control cultures (Fig 4.10 A). Fumagillin and control cultures were further compared by counting the number of *A. algerae* life-cycle stages per infected cell and the number of infected cells per field. Fumagillin at 10 and 20 µg/ml reduced the number of *A. algerae* life-cycle stages per infected cell but not the number of infected cells per field (Fig 4.10 B and C). A study was also done to compare the number of ZEB2J cells in control cultures with number in cultures that had received either spores or spores and fumagillin. Over 5 days of infection with *A. algerae* the accumulation of cells in ZEB2J cultures was inhibited but, fumagillin restored the accumulation to control levels (Fig 4.11).

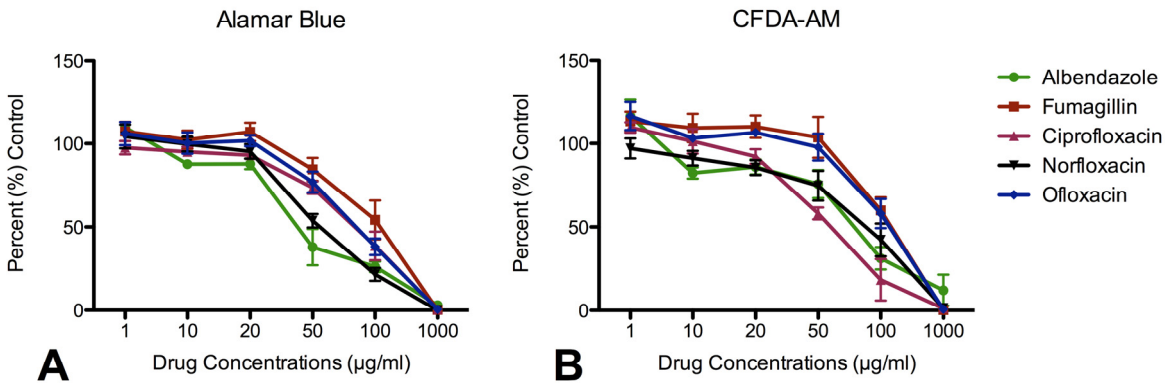

Figure 4.2 Effect of chemotherapeutics on the viability of uninfected ZEB2J cultures
Metabolism of cells was measured using alamar blue fluorescent dye and demonstrated fumagillin is least toxic and albendazole and norfloxacin are most toxic among chemotherapeutics tested (A) at 27°C over 5 days. Cell membrane permeability was measured using fluorescent dye CFDA-AM. Fumagillin was found to least disrupt membrane permeability, while ciprofloxacin, albendazole, and norfloxacin disrupted membrane permeability the most among the chemotherapeutics tested (B).

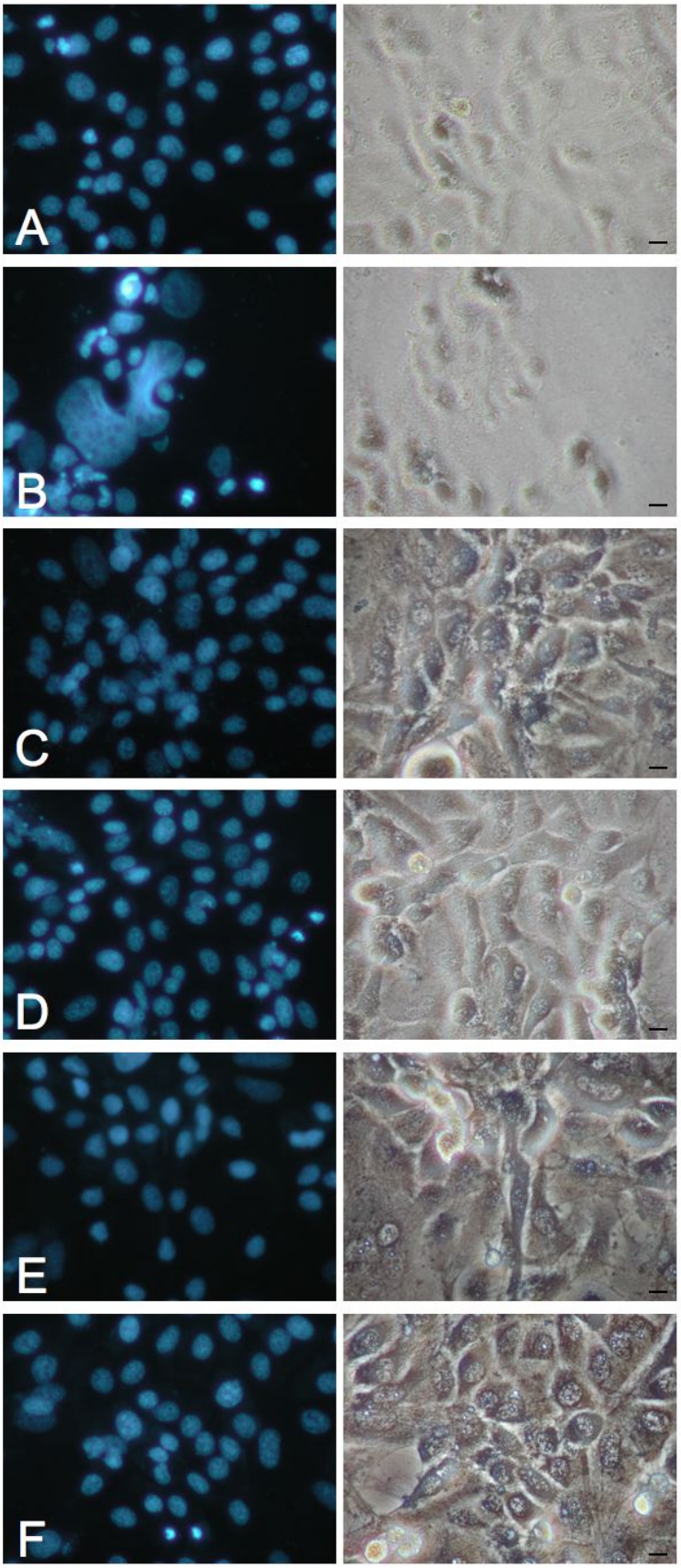
A
B
C
D
E
F

Figure 4.3 Effects of five chemotherapeutic agents on nuclear and cellular morphology of uninfected ZEB2J

On previous page: Media containing 20 µg/ml of drugs tested was placed on uninfected cells for 5 d at 27ºC. After 5 d cultures were fixed in 3:1 methanol to glacial acetic acid and stained with DAPI at 10 µg/ml. From A to F are DAPI images and corresponding phase contrast micrographs for: control, albendazole, fumagillin, ciprofloxacin, norfloxacin, respectively. Scale bar = 10 µm.

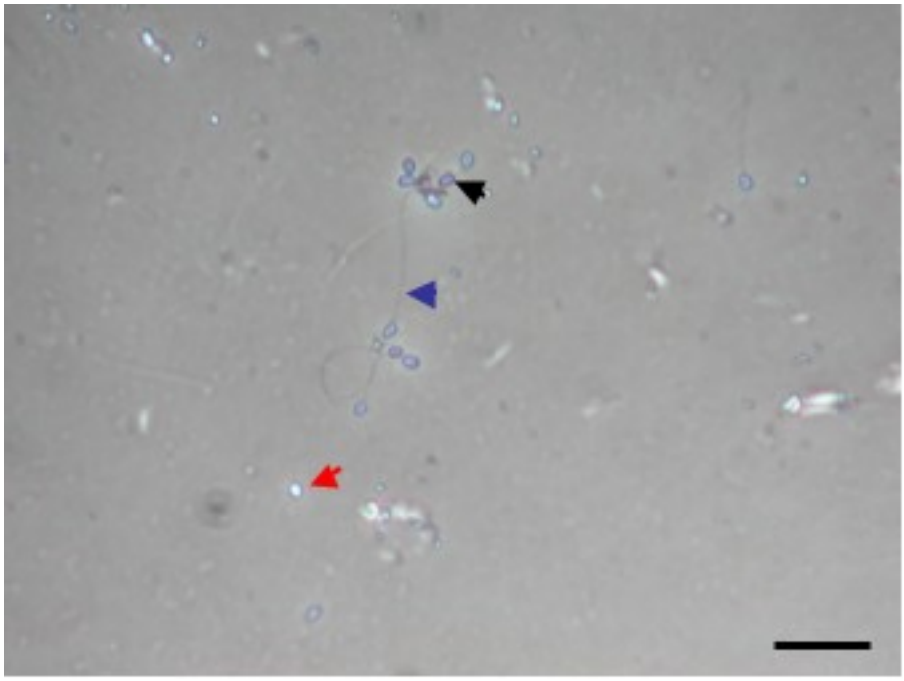

Figure 4.4 *A. algerae* spore suspension after ciprofloxacin and hydrogen peroxide treatments

Most spores germinated after suspension in 20 µg/ml of any drug and then exposed to 3% hydrogen peroxide. Empty spore coats from germinated spores are indicated with a black arrow. Polar filaments often tangle around each other as indicated with a blue arrow, and appear to be a "thick polar filament." Only a few spores did not germinate as indicated with the red arrow. Scale bar = 20 µm.

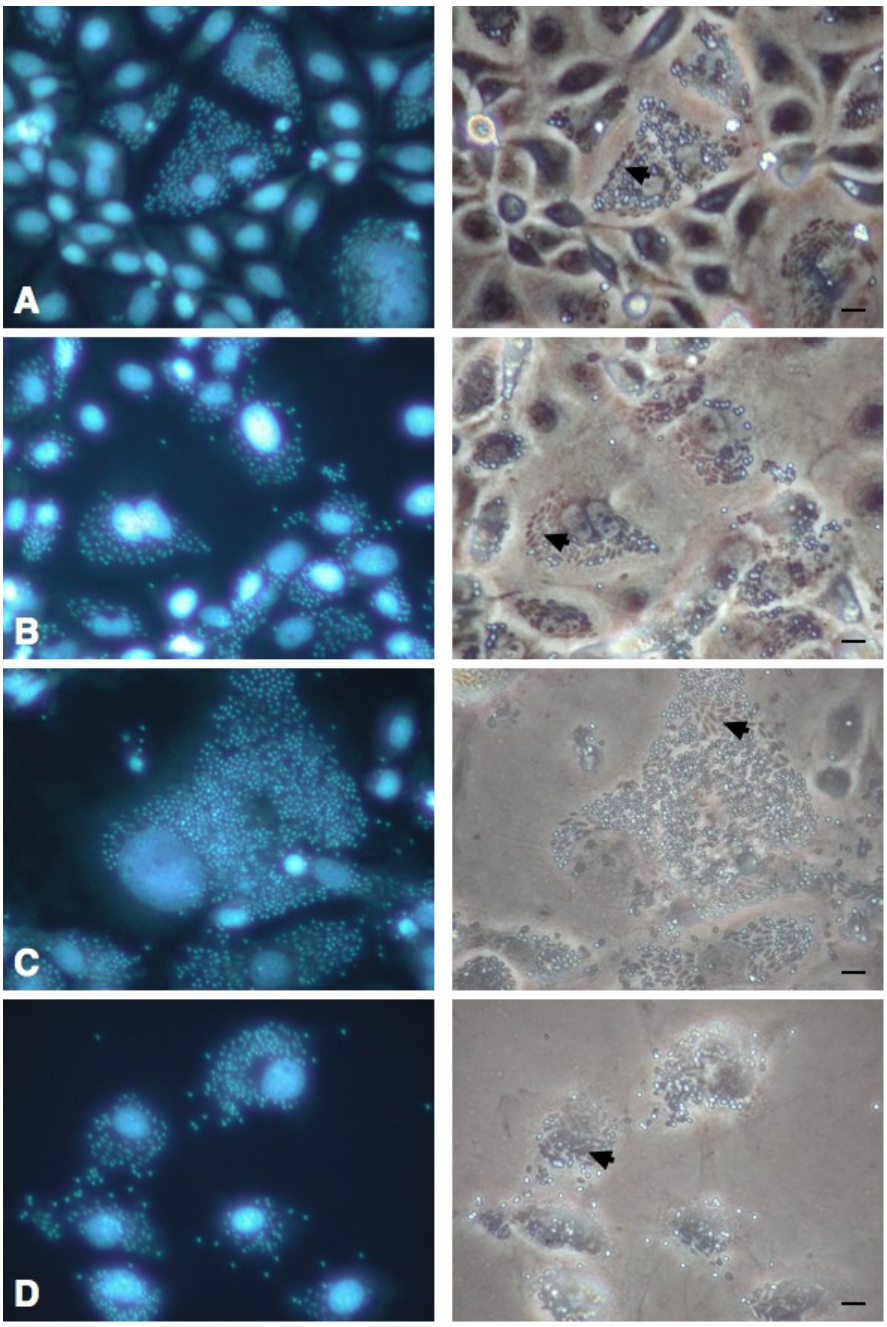

Figure 4.5 Effects of fluoroquinolones on the development of *A. algerae* in ZEB2J

DAPI stained *A. algerae* infected ZEB2J cultures 5 d after 20 µg/ml fluoroquinolone treatment (B, C, and D), with control (A), and corresponding phase contrast images (right). Treatment of *A. algerae* infected ZEB2J cultures with ciprofloxacin, norfloxacin and ofloxacin (B, C, D, respectively) did not alter the development of *A. algerae* compared to control (A). Normal sporont growth was observed and indicated with black arrows. Scale bar = 10 µm.

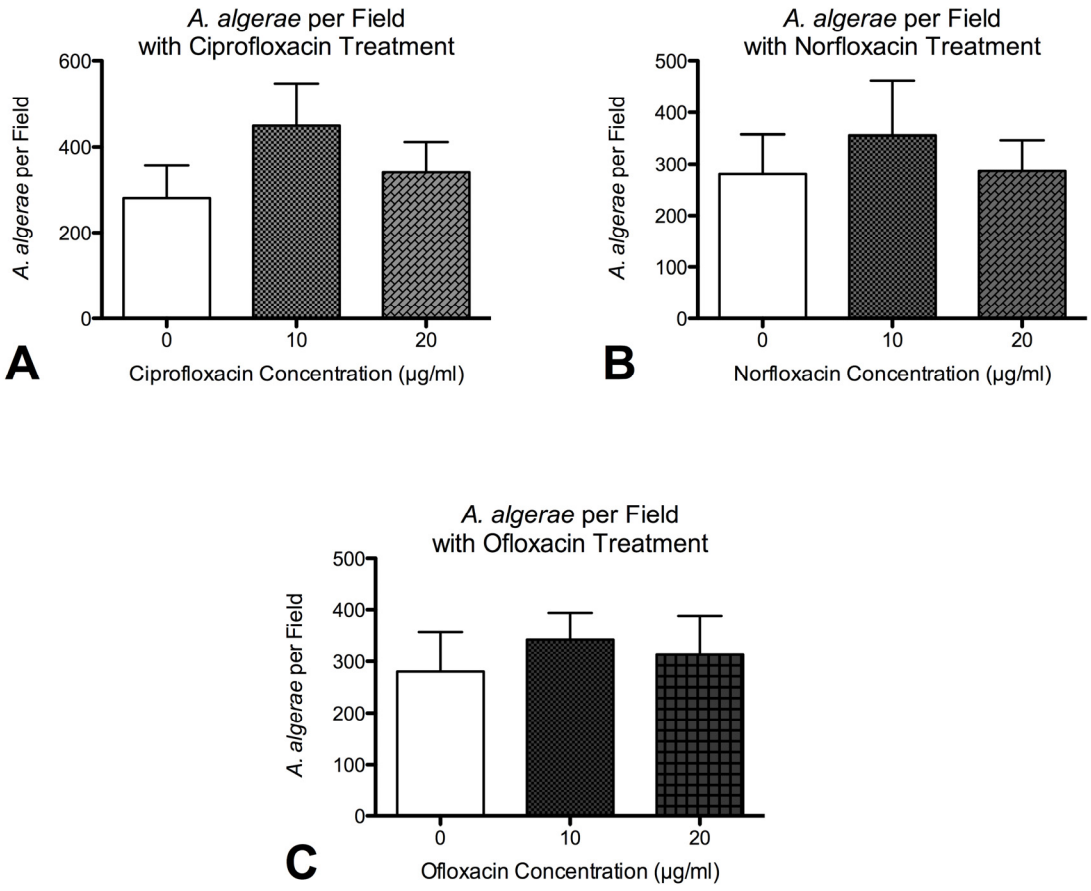

Figure 4.6 Effects of fluoroquinolones on the number of *A. algerae* developmental stages in ZEB2J cultures

Treatment of *A. algerae* infected ZEB2J cultures for 5 d at 27 °C with fluoroquinolones had no effect on the number of parasites per field of view among the concentrations tested. Treatment with 0, 10 and 20 µg/ml of (A) ciprofloxacin (p=0.11) , (B) norfloxacin (p=0.56), and (C) ofloxacin (p=0.51) had similar parasite numbers per microscopic field (n=3).

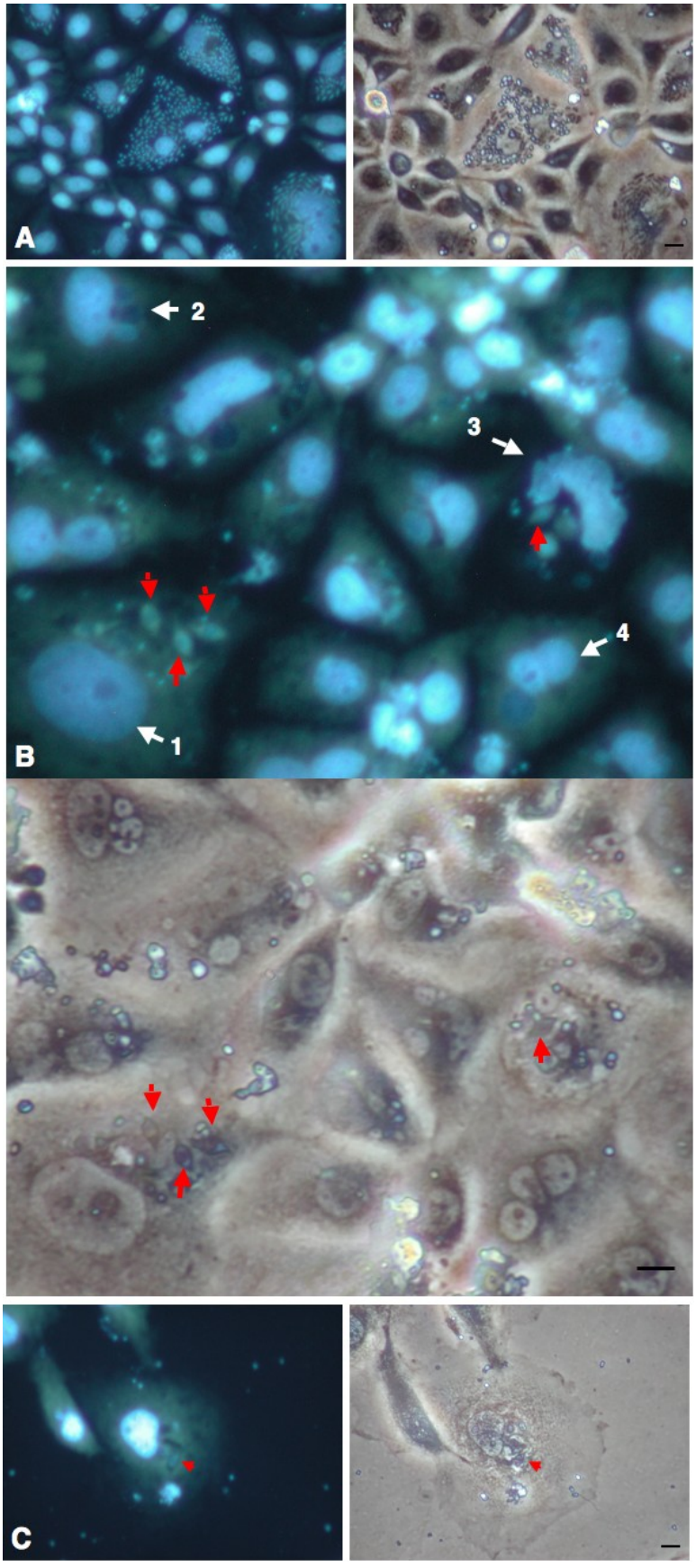
A
B
2
3
4
1
C

Figure 4.7 Effects of albendazole on the development of *A. algerae* in ZEB2J

On previous page: A) DAPI stained ZEB2J infected with *A. algerae* without treatment (control), and corresponding phase (right). B) DAPI stained *A. algerae* infected ZEB2J cultures after 5 d with 10 µg/ml albendazole. Host nuclear morphology is altered and white arrows indicate enlarged nuclei (1), micronuclei (2), irregular nuclear membranes (3), and multinucleation (4). Examples of enlarged *A. algerae* sporonts are indicated with red arrows. Corresponding phase (below right). C) DAPI stained *A. algerae* infected ZEB2J after 5 d with 20 µg/ml albendazole, and corresponding phase (right). Red arrow indicates enlarged *A. algerae* development. Scale bar = 10 µm.

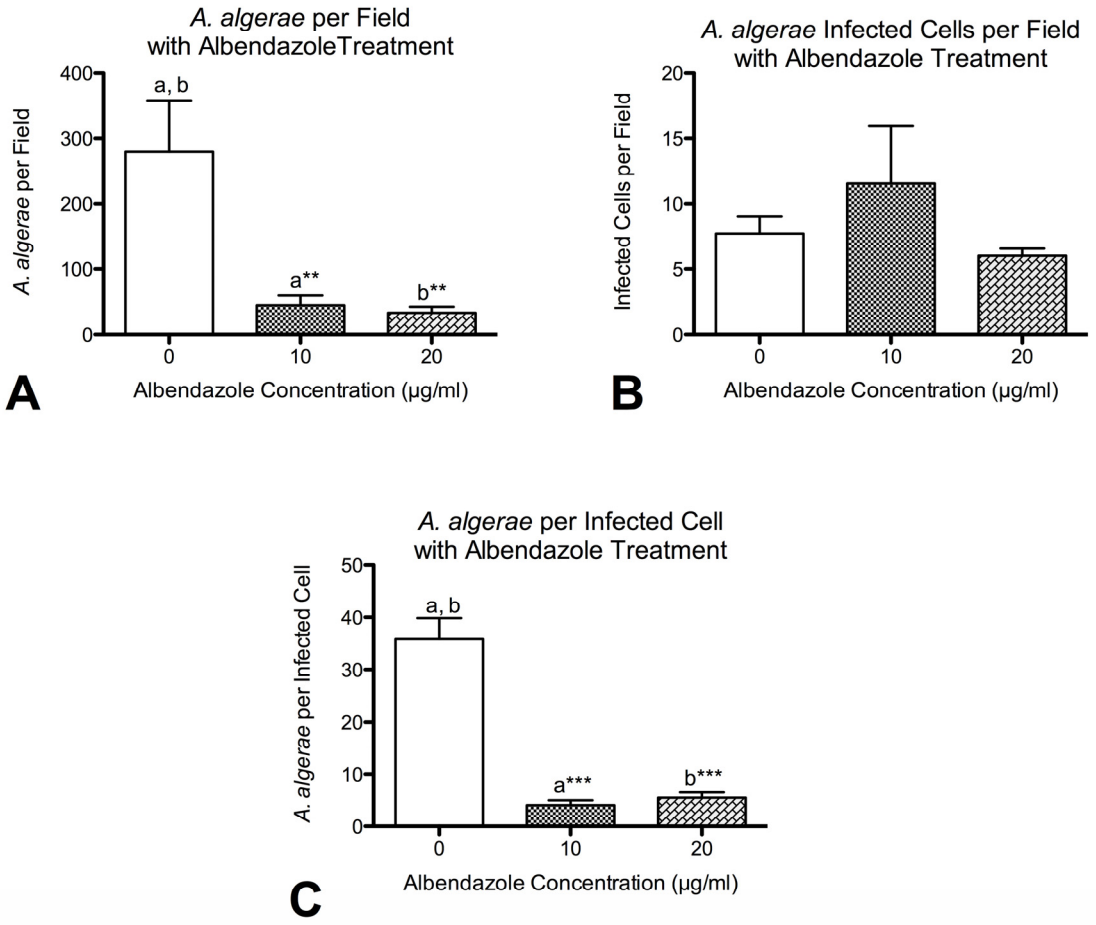

Figure 4.8 Effects of albendazole on the number of *A. algerae* developmental stages in ZEB2J cultures

Albendazole at concentrations of 10 and 20 µg/ml reduced the mean number of *A. algerae* per counted fields in ZEB2J infected cultures (A), but did not affect the mean number of infected cells per scored field (B) ($p>0.01$). The number of *A. algerae* spores per infected cell was reduced with albendazole treatment (C) ($p=0.0001$). Tukey-Kramer Multiple Comparisons test, $^{**}p<0.01$, $^{***}p<0.001$; n=3. Error bars = SD

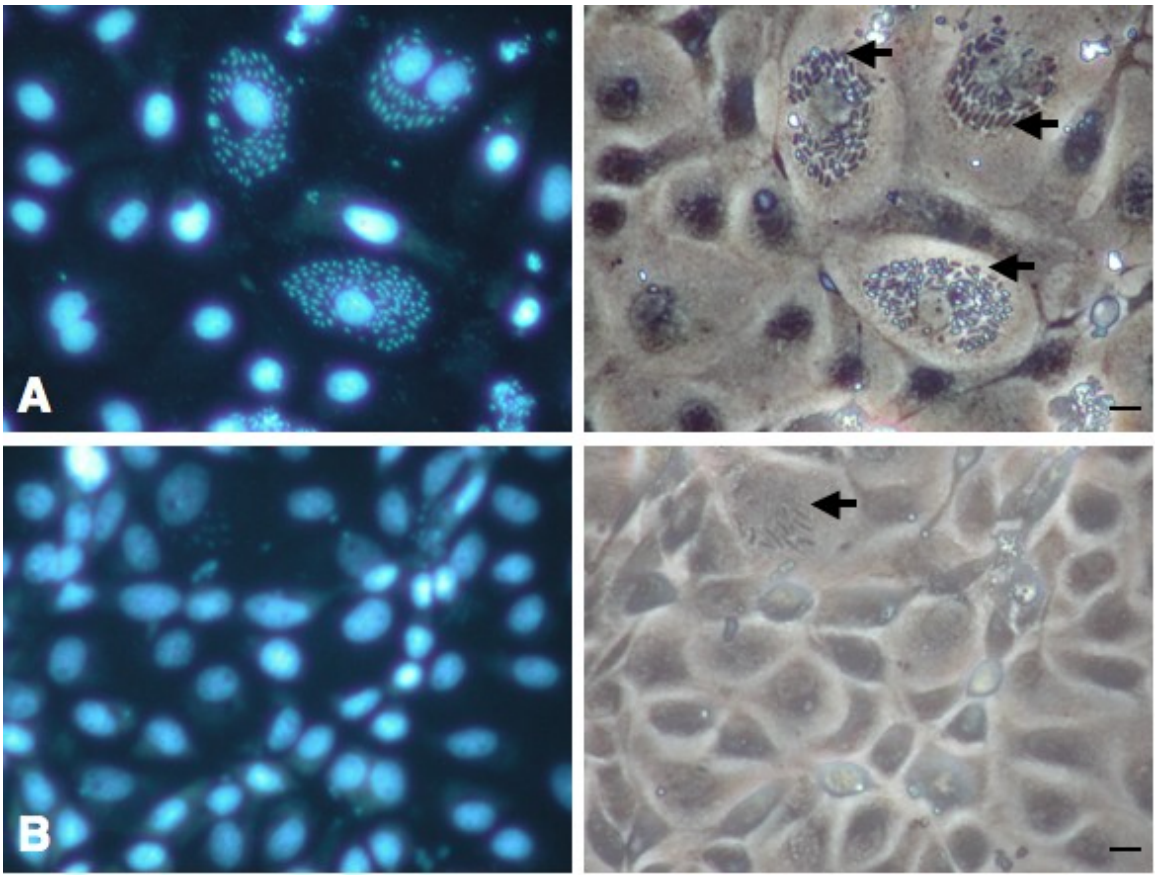

Figure 4.9 Effects of fumagillin on the development of *A. algerae* in ZEB2J

A) DAPI stained *A. algerae* infected ZEB2J cultures after 5 d without chemotherapeutic treatment (control), and corresponding phase (right). Several cells exhibit growth of the parasite with sporont development indicated with black arrows, and mature spores which appear as small phase bright ovid structures in the cytoplasm most visible in the bottom-most cell with sporont development. B) DAPI stained *A. algerae* infected ZEB2J cultures 5 d after 20 μg/ml fumagillin treatment, and corresponding phase (right). No mature spores are noted, but the morphology of sporont development (indicated with a black arrow) appears thinner than sporont morphology in control cultures.

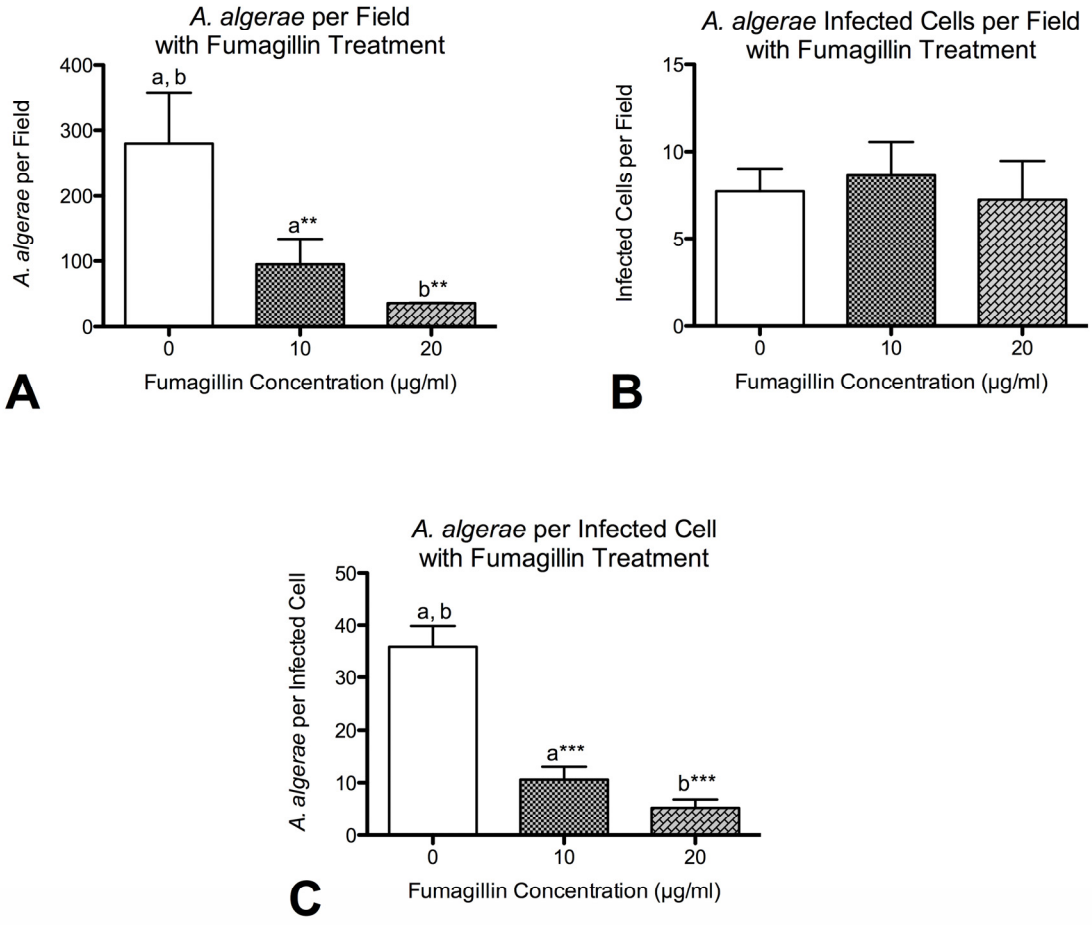

Figure 4.10 Effects of fumagillin on the number of *A. algerae* in ZEB2J cultures

After 5 d at 27 °C, fumagillin at 10 and 20 µg/ml reduced the mean number of *A. algerae* per counted fields in ZEB2J infected cultures (A) ($p=0.002$), but did not affect the mean number of infected cells per scored field (B) ($p=0.66$). The number of *A. algerae* per infected cells was reduced with fumagillin treatment (C) ($p<0.0001$) Tukey-Kramer Multiple Comparisons test, **$p<0.01$, ***$p<0.001$; n=3. Error bars = SD.

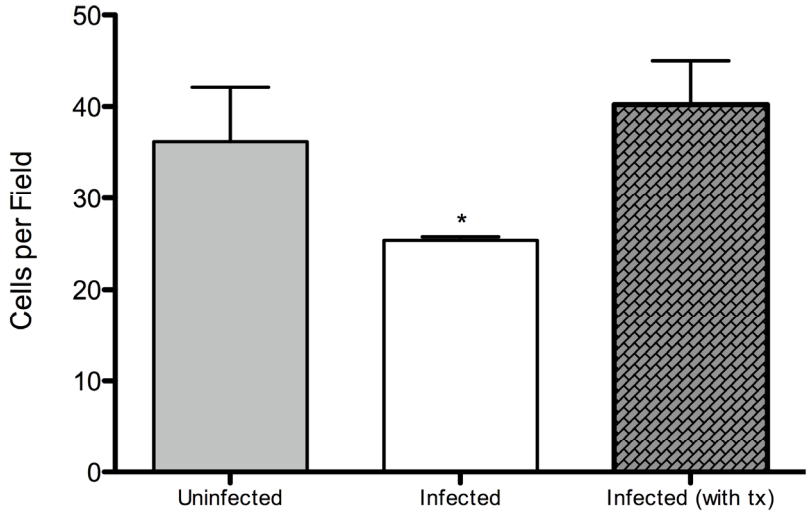

Figure 4.11 Effect of *A. algerae* and *A. algerae* with fumagillin treatment on ZEB2J numbers per field of view over 5 days

Cell numbers per scored field decrease with *A. algerae* infection, but the number of infected cells treated with 20 µg/ml fumagillin are similar to uninfected control. Tukey-Kramer Multiple Comparisons, *$p<0.05$; n=3. Error bars = SD.

4.4 Discussion

Three classes of antimicrosporidial drugs were evaluated for their ability to prevent *A. algerae* growth without impairing the host cells, the zebrafish cell line ZEB2J. Fluoroquinolones had little impact on *A. algerae* growth, and albendaozle impeded *A. algerae* development but at concentrations that caused sublethal damage to ZEB2J. By contrast, fumagillin held promise as a curative agent for this microsporidia/host cell combination: *A. algerae* growth was inhibited at concentrations that had little effect on ZEB2J. These treatments are discussed below.

Fluoroquinolones

Although causing little or no harm to the fish cells, the fluoroquinolones at up to 20 µg/ml appeared to be ineffective at blocking the growth of *A. algerae*. One of the three

fluoroquinolones, ciprofloxacin, also was reported to have little effect on *E. intestinalis* and *V. corneae* growth at concentrations as high as 3.3 µg/ml (Didier et al. 2005). By contrast, the growth of these two microsporidia in RK13 was inhibited significantly by the other two fluoroquinolones, norfloxacin and ofloxacin at 3.2 and 3.6 µg/ml respectively, with *E. intestinalis* being inhibited more profoundly than *V.corneae* (Didier et al. 2005). One possible target of fluoroquinolones in microsporidia is topoisomerase IV (Didier et al. 2005). The failure of *A algerae* to be inhibited by norfloxacin and ofloxacin at 20 µg/ml might due to the absence of topoisomerase IV or a different type of topoisomerase IV. As many fluoroquinoles are available and some of these might act slightly differently from each other (Didier et al., 2005), perhaps others are worth testing in the *A. algerae*/ZEB2J system. Any cytotoxic actions of the fluoroquinolones is likely similar in ZEB2J and RK13. Fluoroquinoles impaired cellular energy metabolism in ZEB2J as measured with alamar Blue at concentrations above 20 µg/ml after 5 days exposure and in RTK13 as measured with MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] at concentrations above approximately 3 µg/ml after 10 days exposure (Didier et al. 2005).

Albendazole

A. algerae, like several other species of microsporidia, appeared susceptible in vitro to albendazole. For *A. algerae*, albendazole lowered the number of the microsporidia in ZEB2J cultures. For *E. intestinalis* and *V. cornea* albendazole inhibited the increase in numbers that developed upon infection of mammalian cell cultures (Didier 1997, Didier et al. 1998). In cultures with *N. bombycis*, *V. corneae, T. hominis, E. cuniculi, E. hellem,* or *E. intestinalis* albendazole reduced the percentage of infected host cells (Beauvais et al. 1994, Ditrich et al. 1994, Haque et al. 1993, Lafranchi-Tristem et al. 2001, Silveira and Canning 1995). As in several of these studies, albendazole appeared in the current study to cause morphological abnormalities in the microsporidia. With *A. algerae* the spindle shaped sporonts appeared enlarged by albendazole treatment. For *N. bombycis*, the sporognic stages were disorganized (Haque et al. 1993); for *E. cuniculi* all developmental stages were swollen and misshapen (Colburn et al., 1994); and for *V. corneae* ultrastructural abnormalities were seen in all stages of the life cycle (Silveira and Canning 1995). Spore structural abnormalities were observed

for *E. cuniculi, E. hellmen, and T. hominis* (Beauvais et al. 1994, Lafranchi-Tristem et al. 2001).

Albendazole failed to reduce the percentage of cells infected with *A. algerae* but in other studies with different microsporidial species (*N. bombycis*, *T.hominis, E. cuniculi, E. hellem,* and *E. intestinalis*) albendazole did decrease the number of infected host cells (Beauvais et al. 1994, Ditrich et al. 1994, Haque et al. 1993, Lafranchi-Tristem et al. 2001). At least three technical differences in how experiments were carried out could account for these different outcomes, beginning with the method used to visualize microsporidia. For the evaluation of microsporidial numbers, *A. algerae* were scored as small DAPI-staining structures, whereas in other studies microsporidia were seen by several methods but most commonly Giemsa staining. DAPI likely allowed a better visualization of all life cycle stages than these methods, some of which required parasitic foci to be scored rather than individual parasites (Beauvais et al. 1994, Franssen et al. 1995, Lafranchi-Tristem et al. 2001). If DAPI were better at detecting a single *A. algerae*, this would make it harder to find cells completely free of microsporidia.

Along with DAPI staining, the short exposure time (5 days) and possibly the higher albendazole concentrations (10 and 20 µg/ml) might additionally have made the complete elimination of microsporidia from cells difficult to achieve. For a cell to be recorded as being free of microsporidia, the host cells must remove remnants of damaged or dead microsporidial nuclei so they are no longer detected by DAPI. This could occur by a type of autophagy, xenophagy (Levine 2005), and an exposure time of 5 days might not give the ZEB2J time to completely degrade the *A. algerae* nuclei. As autophagy can be inhibited or delayed by drugs interfering with microtubule polymerization (Monastyrska et al. 2009) and as albendazole interferes with microtubules in animal cells as well as in microsporidia (Chatterji et al. 2011), the high albendazole concentrations might slow down the digestion of *A. algerae* that have been damaged or killed by the high albendazole concentrations. Therefore, the completeness of microsporidial elimination from a cell could be influenced by the duration and concentration of albendazole treatments. For other microsporidial species, treatments have ranged from 3 days to 5 weeks (Beauvais et al. 1994, Lafranchi-Tristem et al. 2001) at concentrations from 0.008 µg/ml to 5.2 µg/ml (Ditrich et al. 1994, Haque et al. 1993), and in some studies a drop in the percent of infected cells was found to take time to

develop, not being evident at 7 days but becoming clear at times afterward, depending on the albendazole concentration (Colbourn et al. 1994, Lafranchi-Tristem et al. 2001).

A third and final technical consideration is the potential to generate false positives with the combination of DAPI and high albendazole. In mammalian cells the inhibition of microtubule polymerization by albendazole leads to interference with the mitotic spindle and formation of micronuclei (Ramirez et al. 2007). If this were to occur with ZEB2J, some micronuclei might be scored as microsporidia. Indeed albendazole-treated ZEB2J cultures had cells with large nuclei or abnormally shaped nuclei, suggesting that albendazole was interfering with the mitotic spindle in the fish cells. However, micronuclei were rare. With these technical considerations in mind, treating cultures with albendazole at lower concentrations for longer times might be interesting and could bring about a % reduction in the number of ZEB2J with *A. algerae*.

Biological differences between *A. algerae* and other tested microsporidia or between zebrafish and mammalian cells also might be advanced to explain why albendazole failed to eliminate *A. algerae* completely from individual ZEB2J cells, although no definitive conclusion can be drawn at this time. Albendazole is thought to target β-tubulin polymerization and differences in β-tubulin amino acid sequences can explain varying sensitivity to the drug (Chatterji et al. 2011). The sequence of the β-tubulin gene for *A. algerae* is known and has been used to show that *A. algerae* does not belong to the same clades as *N. bombycis, E. cuniculi, E. hellem,* and *E. intestinalis* (Lee et al. 2008). Whether a microsporidia species develops within the cytoplasm of the host cell or in a parasitophorous vaucole influences the effectiveness of albendazole on a species (Gross 2003), but in common with the other tested species *A. algerae* develops in the cytoplasm. Vertebrates metabolize albendazole and eventually inactivate the drug but the metabolism can differ between groups, even among fish species (Shaikh et al. 2006). If metabolic differences were to be maintained by cells in culture, this could make albendazole more or less effective in different in vitro systems.

Albendazole at above 20 µg/ml (75 µM) caused a loss of cell viability in ZEB2J cultures, whereas a sublethal action, the appearance of abnormal nuclear morphologies, was seen at 10 and 20 µg/ml. In mammalian cell cultures albendazole also was cytotoxic but at lower concentrations (~ 0.5 µM to 50 µM) (Baliharová et al. 2003, Whittaker and Faustman 1991),

with differences being observed between cell culture types (Baliharová et al. 2003). Piscine and mammalian cells might differ subtly in their metabolism of albendazole or in their β-tubulins to cause acute cytotoxicity to occur at lower concentrations in mammalian cells. As for a sublethal action, irregular nuclear outlines, multiple nuclei and enlarged nuclei were found in ZEB2J cultures with 20 µg/ml albendazole. These types of nuclear shapes have not been commented on specifically in studies with mammalian cells but might be expected as a general response of animal cells to a drug that can act as a mitotic spindle inhibitor.

Despite potential toxicity, albendazole has been used successfully to reduce the intensity of microsporidia infections in vivo. These include infections of *Encephalitozoon intestinalis* and *E. cuniculi* in humans (Gross 2003) and of *Glugea anomala* and *Loma salmonae* in fish (Schmahl and Benini 1998, Speare et al. 1999). Albendazole against *A. algerae* has been unsuccessfully used to treat clinical cases including a deep-tissue infection (Coyle et al. 2004), and infection of the vocal folds (Cali et al. 2010).

Fumagillin

This is the first report of fumagillin inhibiting the growth of *A. algerae*. Inhibition occurred at 10 and 20 µg/ml, but perhaps in the future concentrations below 10 µg/ml should be tested as well. Fumagillin has been reported to inhibit *E. cuniculi*, *E. intestinalis,* and *V. corneae* in vitro at lower concentrations, ranging from 0.001 to 5 µg/ml (Beauvais et al. 1994, Didier 1997, Didier et al. 2006, Franssen et al. 1995). For *E. cuniculi*, fumagillin inhibited the activity of MetAP-2, which is thought to be the site of its antimicrosporidial activity (Zhang et al. 2005). Possibly this is also the mechanism for the inhibition of *A. algerae* as the gene for MetAP-2 has been identified in this species (Zhang et al. 2005).

Over the last few decades, fumagillin has been explored as a drug to cure fish of microsporidian and myxosporean diseases (Molnár et al. 1986, Takahashi and Egusa 1976), although some toxicological consequences have been noted (Lauren et al. 1989). Like microsporidia, the myxozoa were once considered protozoa, but unlike microsporidia, their relocation has been to the metazoa (Smothers et al. 1994). For microsporidia, fumagillin has been found to reduce the severity of infections in ayu with *Glugea plecoglossi* (Takahashi and Egusa 1976) and in salmon with *Enterocytozoon salmonis, Loma salmonae*, and

Nucleospora salmonis (Hedrick et al. 1991, Kent and Dawe 1994, Speare et al. 1999). For myyxospora, fumagillin was promising as a treatment for several diseases, including proliferative kidney disease (PKD) (Hedrick et al. 1988) and whirling disease (El-Matbouli and Hoffmann 1991). Perhaps the most common toxicological consequence of fumagillin treatments has been a decline in haematopoietic cells (Hedrick et al. 1988, Lauren et al. 1989). The actions of fumagillin on fish cells in vitro are reported for the first time here.

As represented by ZEB2J, the sensitivity of fish cells to fumagillin in vitro was similar to that reported for mammalian cells. Impairment of ZEB2J cultures was observed only at concentrations above 20 µg/ml. Depending on the mammalian cell line, fumagillin has been reported to cause a 50 % reduction in neutral red uptake, a cytotoxic endpoint, at between 16.5 to 36.4 µg/ml (Bunger et al. 2004). MetAP-2 is the main biochemical target for the toxic actions of fumagillin and has been identified in zebrafish (Zhang et al. 2006). However, other fumagillin targets might exist. When zebrafish embryos were treated with the fumagillin analog, TNP-470, abnormalities were observed and these were attributed to TNP-470 acting as a selective inhibitor of noncanonical Wnt signaling (Zhang et al. 2006). The ability to use fish cell cultures to evaluate chemotherapeutic effects against *A. algerae* demonstrates that attempts to establish systems of various host-parasite combinations may be valuable to assess the differences among these effects on different microsporidian species and host cells.

Chapter 5

A microsporidian, *Nosema apis,* from honey bees can infect the zebrafish cell line, ZEB2J

Overview

A zebrafish cell line (ZEB2J) was exposed to *Nosema apis* spores from the western honey bee (*Apis mellifera*). Bees were collected from hives that had been naturally infected and confirmed by conventional polymerase chain reaction (PCR) to have *N. apis*. Frozen bees were crushed in water to yield a mixture of bee parts, pollen grains, yeast, and microsporidial spores. The mixture was filtered and then centrifuged through Percoll to produce a pellet of spores that was resuspended in L-15 with 10 % fetal bovine serum (FBS). Aliquots of this were added to ZEB2J cultures. Cultures were observed periodically for up to 24 days with a combination of phase contrast microscopy and of fluorescence microscopy, usually after staining with 4',6-diamidino-2-phenylindole (DAPI). Although earlier life cycle stages were not observed, structures that were concluded to be either sporonts, sporoblasts and/or spores were seen, but these were in less than 5 % of the fish cells. These *N. apis* life cycle stages had grown in ZEB2J because some appeared to be inside the cells and often they were arranged around the nucleus of the host cell rather than being randomly distributed in cultures. Despite repeated rinsing over a three week period, all cultures were ultimately lost due to yeast from the original spore preparations over growing the fish cell cultures. Although the infection frequency was low, these results suggest that cell lines from different vertebrates as well as different invertebrates should be explored as potential in vitro systems for growing *N. apis*.

5.1 Introduction

The western or European honey bee (*Apis mellifera*) is of significant importance to agriculture as it is the primary pollinator of crops accounting for 35% of the human diet (Klein et al. 2007). In 1992 it was estimated that consumers in the United States annually saved between $1.6 and $5.7 billion due to lower crop prices as a direct result from honey bee pollination (Southwick and Southwick 1992). Recent severe declines in honey bee populations, or Colony Collapse Disorder (CCD) are believed to be the result of a variety of factors including pathogen interactions and non-pathogenic issues such as climate induced stress (Bromenshenk et al. 2010, Le Conte and Navajas 2008, vanEngelsdorp and Meixner 2010). Despite the complexity of relationships among factors implicated in the causality of CCD, recent mass spectrometry-based proteomics (MSP) suggest that the probable pathogenic cause of CCD is the interaction between invertebrate iridescent virus (IIIV) infection, and parasitic infections from microsporidia and mites (Bromenshenk et al. 2010).

Microsporidia are a varied group of obligate intracellular fungal parasites with an exceptional host range and that infect most animal phyla. *Nosema apis* and *Nosema ceranae* are the two microsporidians known to infect the western honey bee. *N. apis* was identified in the western honey bee in 1909 (Zander) and although generally reported as affecting the epithelia of the midgut, has been described in thoracic, pharyngeal and mandibular glands, as well as ovaries and hemolymph (Gilliam and Shimanuki 1967). *N. ceranae* was originally a microsporidian pathogen of Asian honey bees (*Apis ceranae*) but is now an emerging pathogen of the western honeybee (Klee et al. 2007). It is believed that *N. ceranae* has been present in *A. mellifera* populations in parts of the United States since at least the 1990's (Chen et al. 2008). *N. ceranae* is now considered to be worldwide having been identified in North and South America, Asia and Europe (Chen and Huang 2010, Higes et al. 2006, Williams et al. 2008). The relatively recent identification of *N. ceranae* in the western honey bee, and reports of its broad tissue specificity and higher virulence compared to *N. apis,* have implicated the parasite as having more of a role in CCD (Martin-Hernandez et al. 2007). However, rates of *N. ceranae* infections are higher in normal colonies as well as CCD colonies, natural infections of *N. ceranae* are rarely without co-infection of *N. apis,* and regional differences may play a role (Cox-Foster et al. 2007, Gisder et al. 2010a, Read and Taylor 2001).

Historically, *Nosema* spp. have been of research interest because of the impact this genus has on insects and the silk industry. *N. bombycis* in the silkworm was the first microsporidian to be named (Nageli 1857), studied (Pasteur 1870), and grown in vitro using silkworm tissue cultures (Trager 1937). This initial development was followed by the growth of *N. bombycis* in several invertebrate cell culture systems (Ishihara 1969, Ishihara and Sohi 1966), and primary mammalian and chick embryo cultures (Ishihara 1968). After the successful growth of *N. bombycis* in culture, other *Nosema* spp. followed including: *N. mesnili* (Gupta 1964), *N. furnacalis* (Kurtti et al. 1994), and *N. disstriae* (Kurtti et al. 1983, Sohi and Wilson 1976). Other *Nosema* spp., including *N. algerae*, were grown in culture but subsequently reassigned to new genera (Khurad et al. 1991, Undeen 1975).

Despite the importance of establishing successful in vitro cultivation of *N. apis* and *N. ceranae*, this has proven difficult and over the years the difficulty has been attributed to the lack of cell lines from bees. However, recently the infection and growth of *N. apis* and *N. ceranae* has been observed in a lepdopteran cell line, although continuous propagation was not obtained (Gisder et al. 2010b). Inasmuch as the continuous in vitro of growth of these bee pathogens has yet to be achieved and as *Anncaliia algerae* grew in fish cell lines as well as in insect cell lines (Chapter 2), the possibility of growing *N. apis* and *N. ceranae* in a fish cell line was investigated.

5.2 Materials and Methods

Cell Lines and Their Growth

The ZEB2J fish cell line was developed at the University of Waterloo (Dr. Niels Bols Lab). ZEB2J is a predominantly epithelial-like, zebrafish blastula cell line (Xing et al. 2008). Cells were grown in 12.5 cm^2 non-vented flasks (Falcon), initially maintained at 27ºC but moved to 18°C (see Results: Yeast Contamination) in Leibovitz-15 (L-15; HyClone) with 2mM L-glutamine, 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) and 3% Antibiotic-Antimycotic (AB/AM; Gibco 15240).

Purification of N. apis and N. ceranae spores

Nosema spp. samples were received from Geoffrey R. Williams (Dr. David Shutler Lab, Acadia University) and had been PCR verified to be containing either *N. apis* or *N. ceranae*. Bees were euthanized from experimentally infected hives and macerated in water before shipping (Fig 5.1). Spores were obtained from the shipped samples by placing 5 ml of original sample in a 15 ml test tube with 5 ml 20% AB/AM for a final concentration of 10% AB/AM. The sample was left for 24 hours, centrifuged to aspirate supernatant, and resuspended in sterile water with 10% AB/AM. After 2 days, contents of the test tube were transferred to a new test tube and filtered using several 40 µm nylon filters (Falcon) to separate spores from larger particulate in the sample. Filtrate was centrifuged, supernatant removed and resuspended in 5 ml sterile water and 5 ml Percoll (Sigma P1644). The sample was then vortexed for approximately 5 s, and centrifuged at ~1,800 x *g* for 30 min. The spores pelleted to the bottom of the test tube, while cellular debris was in the Percoll/water supernatant, and removed by aspiration. The spore pellet was resuspended in sterile water with 5% AB/AM.

Infecting ZEB2J cultures with Nosema spp. spores

Infections were done in 12.5-cm^2 non-vented flasks (Falcon). ZEB2J was grown at 27 °C to near confluency. Spore suspensions in water were centrifuged at ~200x g, resuspended in L-15 media described above, and counted with a hemocytometer. For spore inoculation, 8 ZEB2J flasks had growth media removed and replaced with 3 ml of culture medium containing approximately 1.5 x 10^6 spores/ml of either *N. apis* or *N. ceranae* spores. Four ZEB2J flasks were maintained for uninfected controls.

Monitoring ZEB2J cultures infected with Nosema spp.

A Nikon TE300 inverted microscope with a TE-FM Epi-Fluorescence attachment and Nikon Cool Pix 5400 camera was used to monitor and photograph living and fixed cultures by phase and fluorescence microscopy, respectively. Phase contrast microscopy was used to

view living cultures daily and cultures were fixed and stained when the flask was overcome with yeast contamination. Cell cultures were fixed in 3:1 absolute methanol to glacial acetic acid, and stained for DNA with 4',6-diamidino-2-phenylindole (DAPI). For fixation, 1 ml of fixative (3:1, absolute methanol: glacial acetic acid) was added to the medium of a flask culture and left for 2 min. A bulb pipette was used to remove the fixative and another 1.5 ml of Carnoy's was added for a further 5 min before removal. Afterwards the culture flasks were rinsed in phosphate-buffered saline (PBS) and stained for at least 2 h in 10 μg/ml of DAPI in PBS. Some cultures were subsequently stained with calcofluor white (Sigma BioChemika18909) a fluorescent brightener, which binds to chitin and other structural β-linked polysaccharides (Hayashibe and Katohda 1973, Hughes and McCully 1975, Maeda and Ishida 1967). The microsporidian spore coat is comprised of chitin and brightly fluoresces when stained with calcofluor white.

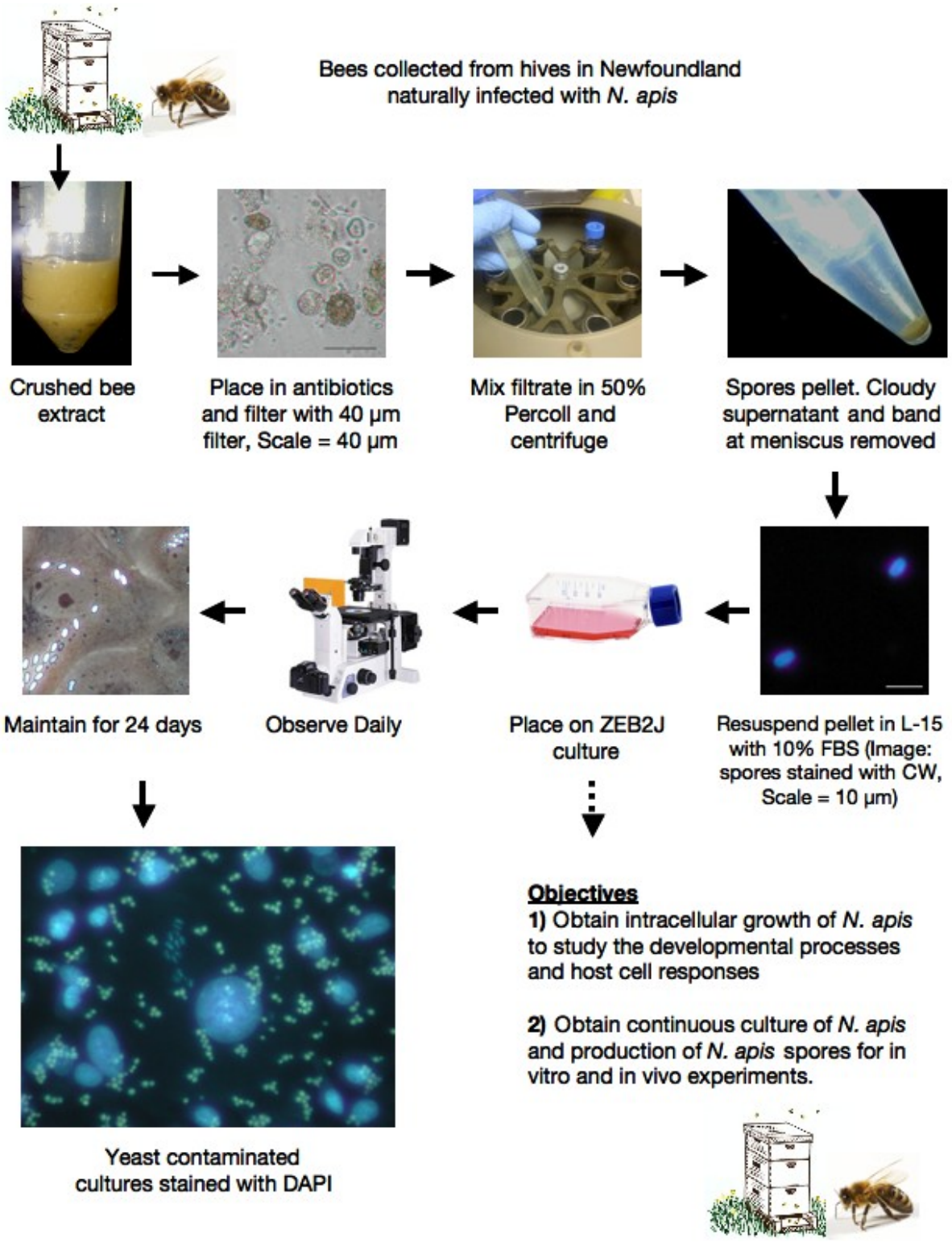

Figure 5.1 Experimental outline for testing whether *N. apis* would grow in ZEB2J cultures

5.3 Results

Preparation of N. apis *spores*

As outlined in Fig 5.1, spores were prepared from bees that had been naturally infected with *N. apis*. Hives that had the characteristic features of a microsporidia infection were the source of bees and the presence of *N. apis* was confirmed by PCR. After the bees had been crushed in distilled water, bee parts, pollen grains, yeast and spores were seen (Fig 5.1). This extract or mixture was filtered and centrifuged to yield a pellet that consisted primarily of spores, but some yeast and pollen grains were also present. The spores had the shape and size of *N. apis* spores as viewed by phase contrast microscopy and fluorescence microscopy after staining with DAPI (Fig 5.2).

Phase contrast microscopy of ZEB2J cultures after addition of N. apis *spores*

Fish cell cultures were examined thoroughly by phase contrast microscopy 48 h after the addition of the preparation of *N. apis* spores. Early life cycle stages, sporoplasms and meronts, of *N. apis* were not definitively seen, but late life cycle stages were observed clearly, although in less than 5 % of the cells. The late life cycle stages were characteristically localized around the nucleus of a ZEB2J cell, appearing as clusters of 10-30 oval phase bright objects (Fig 5.3). These could potentially be sporonts, sporoblasts or spores. Some were less phase bright than others suggesting that they were possibly sporonts or sporobalsts inside the ZEB2J cells (Fig 5.3A-D, red circles).

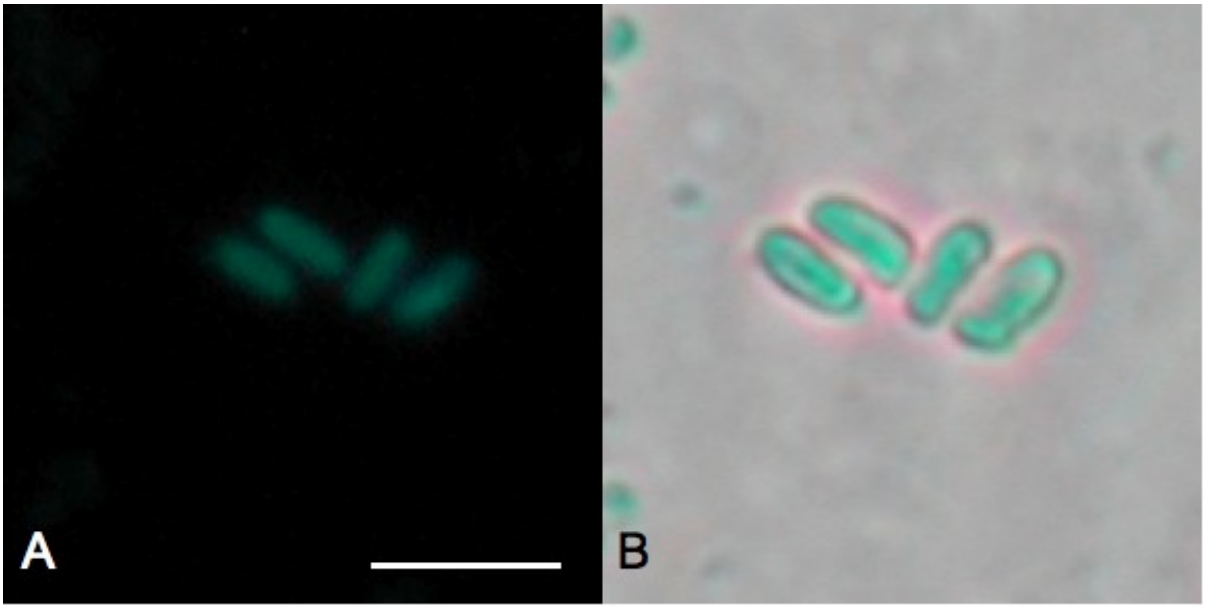

Figure 5.2 DAPI staining of a spore preparation from *N. apis* infected bees

Fluorescent micrograph of spore preparation stained with 10 µg/ml DAPI (A), and corresponding phase contrast (B) Scale bar = 10 µm.

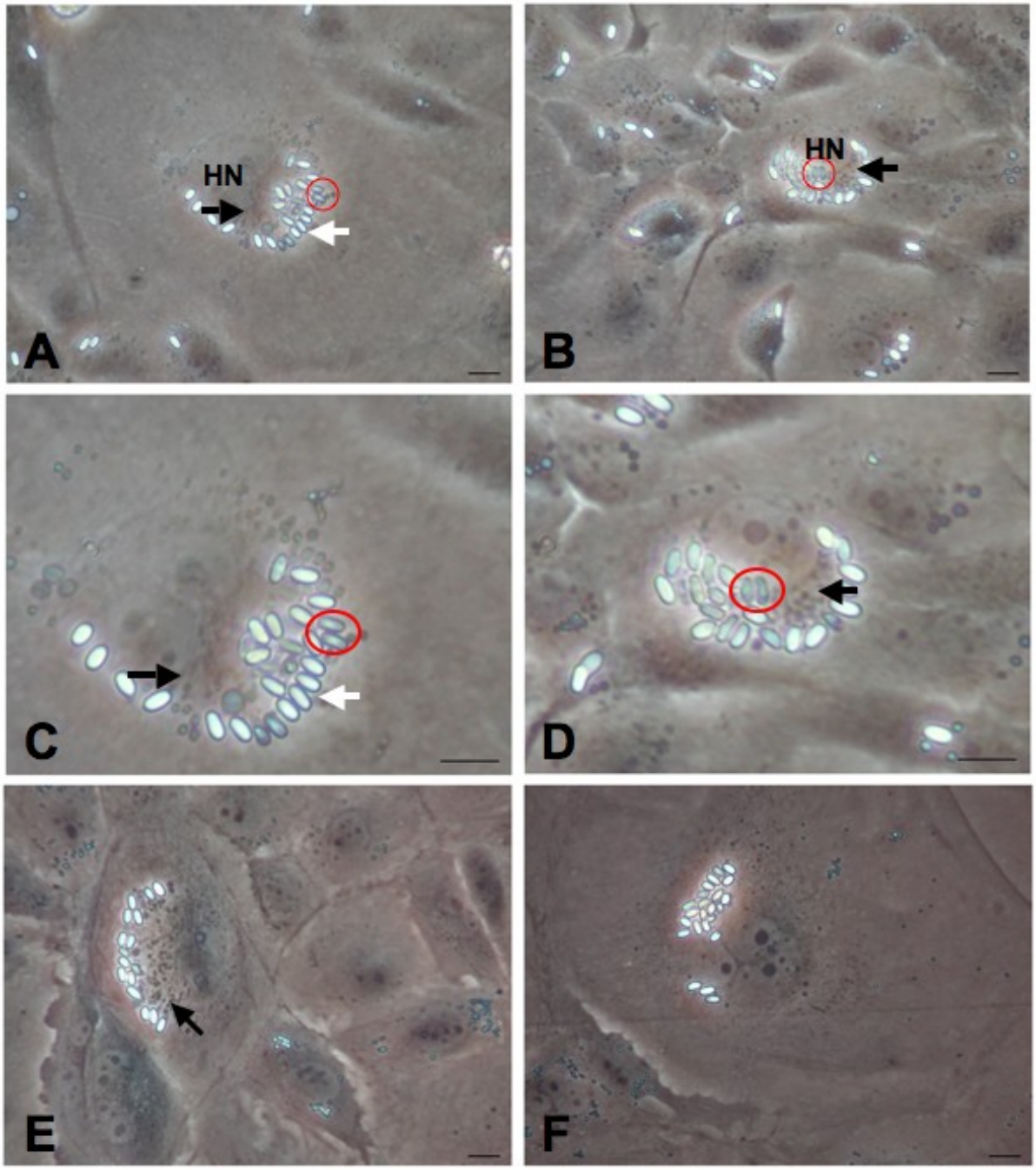

Figure 5.3 Phase contrast microscopy of ZEB2J cultures after addition of *N. apis* spores

N. apis life cycle stages were seen as oval, phase-bright structures in the cytoplasm of fish cells in cultures 48 h post infection (p.i.) following thorough washing of extracellular spores (A & B). Higher magnification of the images in A and B are shown in C & D. Red circles identify a possible intracellular proliferative stage with spore nuclei; mature spores (white arrows); and possible meronts (black arrows). Life cycle stages (black arrows) were often adjacent to host nuclei (HN). Cultures at 10 and 11 d p.i. also had ZEB2J cells with *N. apis* life cycle stages distributed around the host nucleus (E & F). Scale bar = 10 μm.

Fluorescence microscopy of ZEB2J cultures after addition of N. apis *spores*

Examining the fish cell cultures by fluorescence microscopy also suggested that *N. apis* spores infected ZEB2J cells and underwent development, but yeast in the cultures necessitated a cautious interpretation of the photographs. When cultures were examined after DAPI staining for DNA, small whitish blue structures were seen around the nucleus of at least one fish cell that possibly were meronts (Fig 5.4). The cultures also were seen to contain yeast but these were outside the cells and had a greenish fluorescence (Fig 5.4). Cultures also were stained with DAPI followed by Calcofluor white (CW), which has been used to stain chitin in the cell walls of microsporidia and yeast (Harrington and Hageage 2003, Vávra et al. 1993). With this combination of dyes, fish cells were seen with structures that faintly stained, in contrast to the bright staining of *N. apis* spores not associated with fish cells (Fig 5.5). These faint staining structures are interpreted to represent *N. apis* life cycle stage(s) inside rather than outside the fish cells. Even after cultures had been rinsed numerous times over several weeks some ZEB2J cells had *N. apis* life cycle stages as viewed by phase and fluorescence microscopy (Fig. 5.6), but yeast were beginning to overwhelm the cultures and so they were terminated.

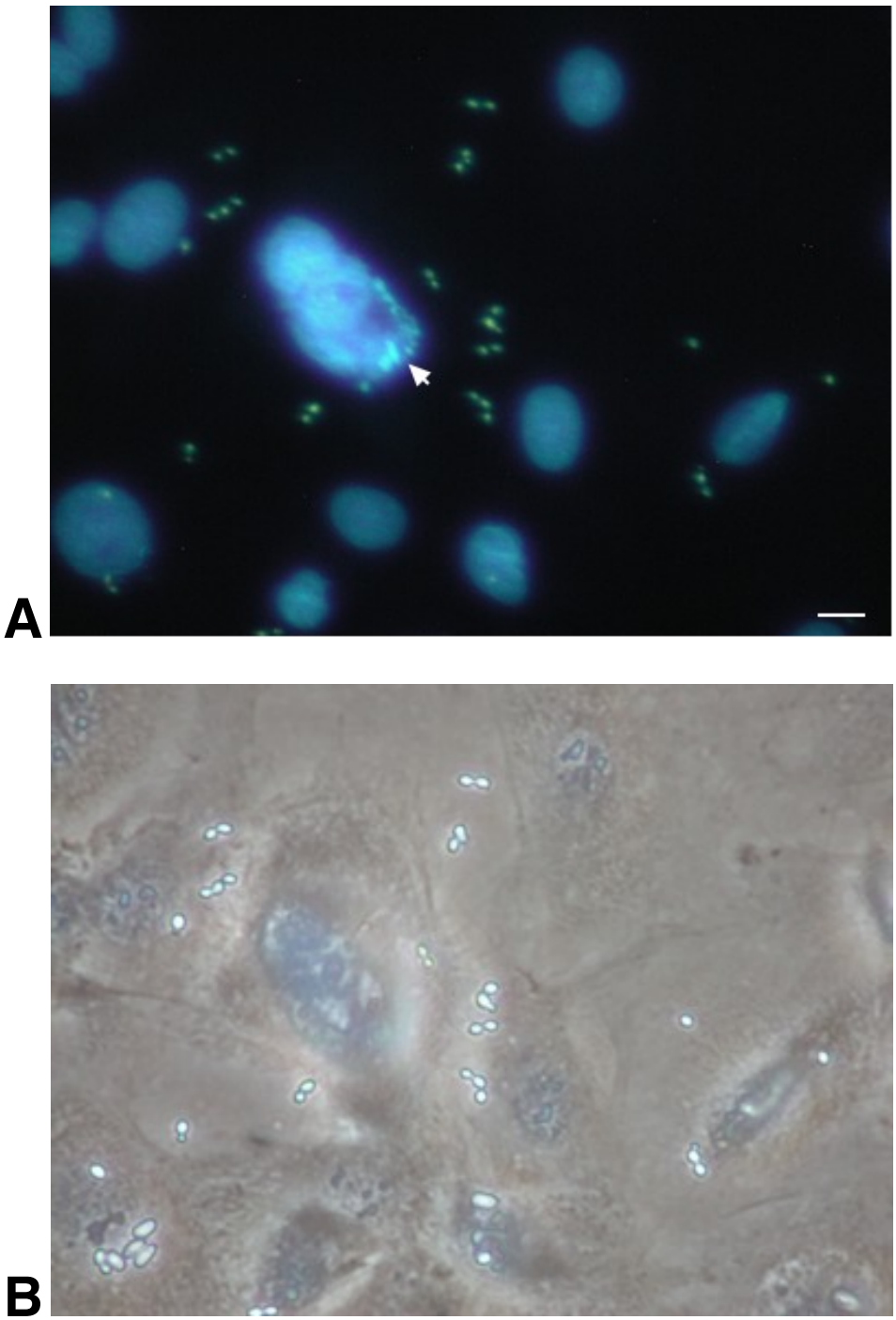

Figure 5.4 ZEB2J cultures 16 days after addition of *N. apis* spores

(A) Examination of a DAPI stained culture (16 d p.i.) revealed next to a ZEB2J nucleus small whitish blue structures (white arrow), which are possibly *N. apis* meronts or sporonts. Yeast fluoresced greenish in the extracellular millieu; ZEB2J nuclei, whitish blue. Corresponding phase contrast image depicting intracellular micarosporidian spores and extracellular yeast is depicted in (B). Scale bar = 10 µm.

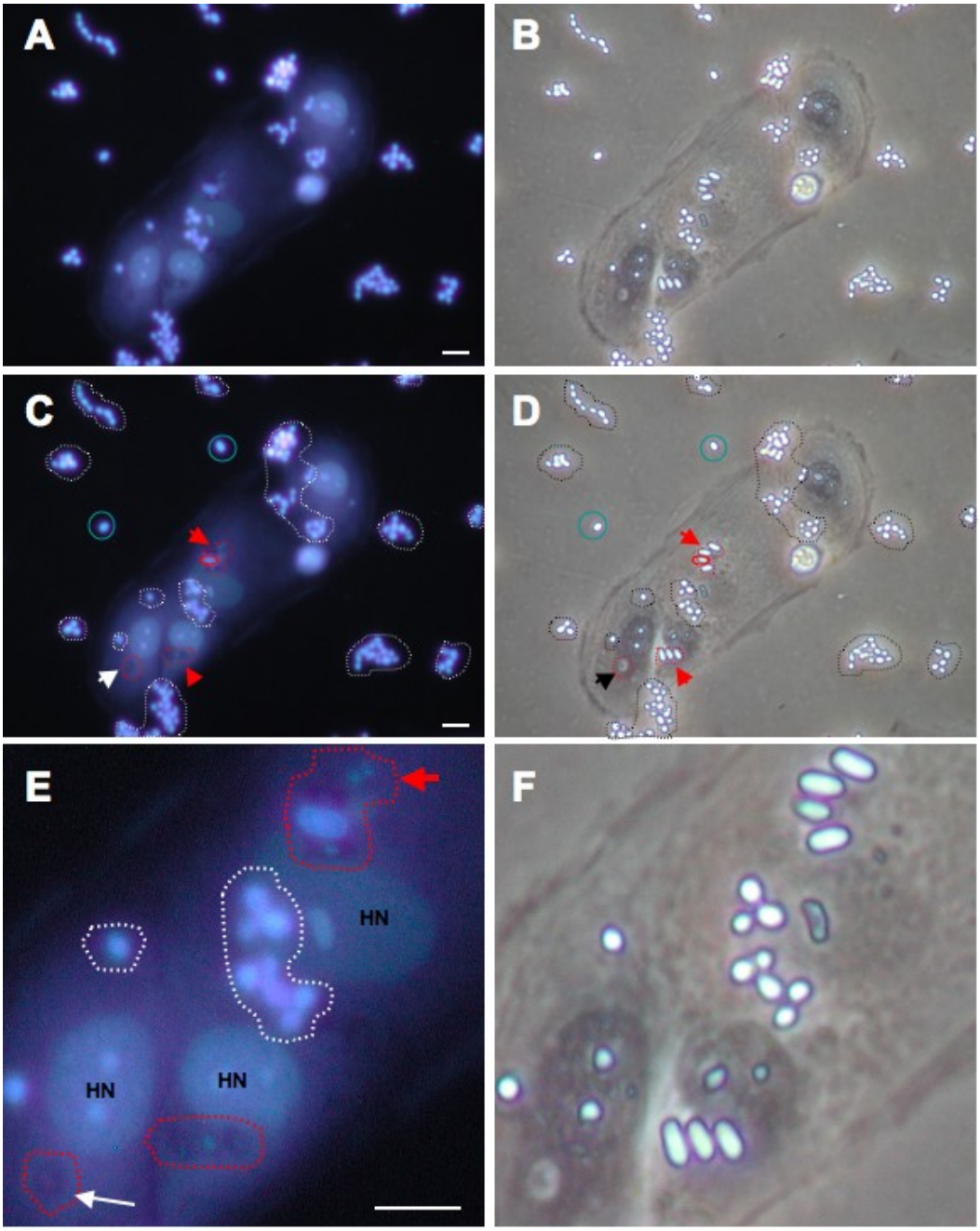

Figure 5.5 ZEB2J cultures stained first with DAPI followed by Calcofluor White (CW)

The same field of view was examined by fluorescence (A, C & E) and by phase contrast (B, D & F) microscopy. The photos in A and B are repeated in C and D but with interpretative markings and repeated again in E and F at a higher magnification. The ZEB2J nuclei (HN) are whitish blue with one or two brighter structures, which are interpreted to be nucleoli. In C and D the white and black dotted lines delineate yeast contamination, whereas the red arrows point to weakly fluorescent structures that appear to be *N. apis* life cycle stages. Two ambiguous structures, which could be either yeast or microsporidia, are delineated within teal circles (C & D). In E the white dotted line outlines yeast contamination and the red circles demarcate possible *N. apis* sporont stages with two nuclei. Scale bar = 10 μm.

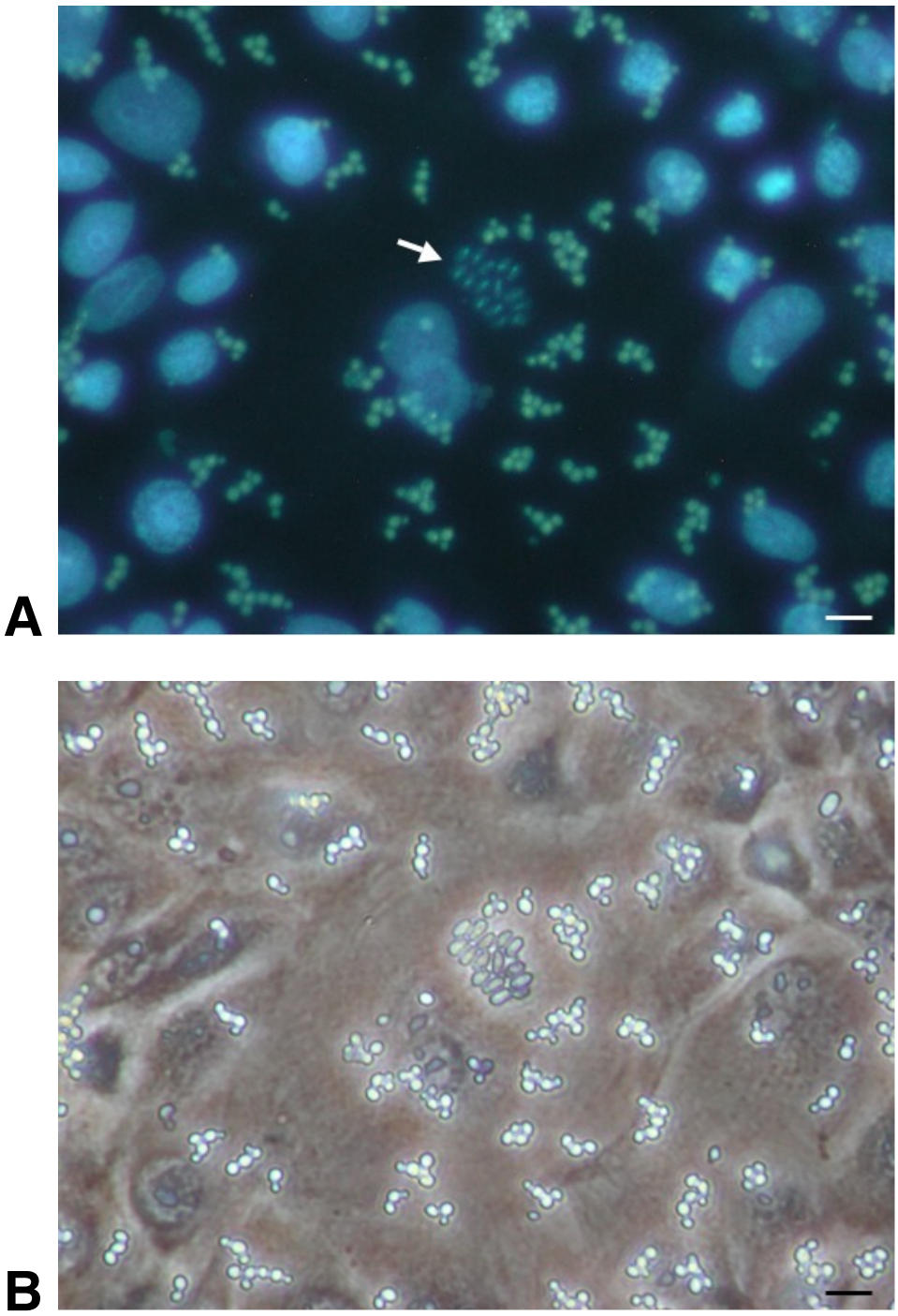

Figure 5.6 ZEB2J monolayers 26 days after the addition of *N. apis* spores

Fluorescence (A) and phase contrast (B) examination of a DAPI-stained ZEB2J culture reveals many green fluorescent, phase-bright structure that are interpreted to be yeast but at least one ZEB2J cell has *N. apis* sporoblast and/or spores (white arrow). DAPI stained ZEB2J nuclei fluoresced whitish blue. Scale bar = 10 µm.

Efforts to reduce or eliminate yeast contamination

Several different strategies were tried without success to get cultures to persist longer than 26 days with little or no yeast. One method was to rinse cultures several times every 8 h with medium supplemented with 4% AB/AM, and replaced with 3% AB/AM. Another strategy was to move cultures to 18°C in the hope that yeast but not microsporidia growth would be inhibited. Other measures to remove yeast included disrupting yeast attachment to cells with a 20 s agitated rinse of TrypLE and striking the flask on the lab bench prior to rinsing. All methods resulted in a decrease of yeast contamination, but also a decrease in both infected and non-infected ZEB2J cells. Passaging of *N. apis* infected flasks to new flasks after rinsing also resulted in a reduction of adherent cells, and a temporary reduction of yeast contamination. However, with all methods the number of yeast ultimately increased to high levels.

5.4 Discussion

Evidence of infection and propagation

The current work supports a recent study that *N.apis* can infect animal cells in culture (Gisder et al. 2010b) but different cell lines and visualization techniques have been used. The zebrafish cell line, ZEB2J, was used in this thesis, whereas the cell line, IPL-LD-657, from *Bombyx mori* (silkmoth) was used by Gisder et al. (2010b). Although working with both *N. ceranae* and *N. apis*, Gisder et al. (2010b) presented pictures mainly for *N.ceranae* but made similar observations for *N.apis*. An infection frequency of 15-30% was estimated for *N.ceranae* and 5-10% for *N.apis*. In this thesis infections were initiated with both *N.ceranae* and *N.apis* but only cultures with *N.apis* were followed closely and photographed and the infection frequency was less than 5 %.

Gisder et al (2010b) visualized the microsporidial life cycle stages generally by staining with Giemsa and specifically by fluorescence in situ hybridization (FISH) with a Nosema-specific 16S rRNA-targeted oligonucleotide probe. With both techniques they were able to see within host cells small spherical bodies that were thought to be sporoplasms and spindle-shaped bodies that were interpreted as meronts. Sporoplasms were seen as early as

16 h post infection. Sporonts, sporoblasts and spores began to be seen between 48 and 72 h and by 96 h the spores were thought to be either primary or environmental spores. However the differences between sporonts, sporoblasts, and spore types were subtle. Both sporonts and sporoblasts were described as round to oval. Differential interfering contrast microscopy was used to tell apart primary spores, which were described as spherical, from environmental spores, which were described as oval.

In this thesis a combination of phase contrast and fluorescence microscopy after staining with DAPI, sometimes followed by calcofluor white (CW), has been used to identifying *N.apis* life cycle stages. Cells with sporoplasms and meronts could not be identified in the current study. Certainly the low infection frequency would have made detection difficult but other factors likely contributed to them not been seen. In retrospect, for seeing sporoplasts cultures should have been examined more thoroughly within the first 16 h post infection. Possibly the contrast between meronts with the host cytoplasm is too small to make easy phase contrast visualization of these stages. As well, the chromatin of these stages could be too diffuse for strong DAPI staining. However sporonts, sporoblasts, and spores were seen but distinguishing between these life cycle stages was difficult.

Several observations support the case that sporonts, sporoblasts, and spores developed in ZEB2J cultures, although the presence of yeast in the starting spore preparation complicated observations. Firstly as judged by phase contrast microscopy and by DAPI staining, host cells were seen with structures that had the shape and size of *N. apis* spores. These stages were distinguished from yeast by being slightly larger, having smooth oval outlines with no indication of budding, and appearing whitish blue rather than green after DAPI staining. Secondly, some of the structures appeared to be inside rather outside the host cells based on their weak staining with CW and being slightly phase darker relative to spores outside cells. Thirdly, these stages appear to have developed in the culture, rather than just representing spores that had been applied at the start of the infection and persisting unchanged over the period of observation. This conclusion is reached because many host cells were seen with the putative *N. apis* stages arranged around the host cell nucleus rather than being randomly distributed in the cell or in the culture. Additionally this arrangement with host cells was maintained despite repetitive rinsing of the cultures over a 24 day period. On the other hand, other explanations for these structures can be advanced. Possibly *N apis*

spores attach on the outside of just a few cells in a characteristic pattern around the nucleus and persist in this arrangement. Possibly the structures are another type of microbe in the bee extract and not microsporidia. Despite these reservations, the simplest explanation for these observations is that a few ZEB2J were infected with *N. apis* and supported development to at least the sporont stage and possibly sporoblast and spore stages as well.

Although illustrating the importance of exploring cell lines from a variety of organisms for growing *N. apis*, this thesis with a fish cell line and the work of Gisder et al (2010b) with the lepidopteran cell line stress the need to get better in vitro systems. The infection frequency was low with the lepidopteran cells and very low with the fish cells. Neither system allowed the continuous culturing of *N. apis*. Perhaps cell lines from other organisms, especially bees, might allow continuous propagation. On the other hand, the nature of the *N. apis* life cycle might prevent continuous in vitro growth. This is illustrated by the behavior of other *Nosema* spp in vitro. *N. bombycis* and *N. furnacalis* can be grown continuously in culture but *N. pyrausta* cannot (Sagers et al. 1996). This difference is attributed to the failure of *N. pyrausta*, unlike the other two species, to continuously produce primary spores, which germinate intracellularly to infect neighboring cells and maintain an infection (Sagers et al. 1996).

Regardless of the animal cell culture system chosen, culturing *N. apis* has additional technical difficulties. In this work, a problem was obtaining spores from infected bees free of yeast. In the future this might be overcome by using bee midguts as the starting material and to rinse them well as done by Gisder et al. (Gisder et al. 2010a). Other ways of reducing yeast also could be considered. These could include trying to separate microsporidia from yeast with different Percoll concentrations and centrifugation speeds. Additionally, some new antifungal drugs might be explored to see if they might preferentially kill yeast over microsporidia (Lorand and Kocsis 2007). Finally the low infection frequency is another difficulty and might be partially technical in the current study because the bees were stored at -20 °C prior to *N. apis* spore isolation. Recent studies suggest that *Nosema* spores might loose viability with freezing and long-term storage (Fenoy et al. 2009, Gisder et al. 2010a).

Chapter 6

Future research and broader implications of this thesis

6.1 Future Research

In this study, fish cell lines were evaluated for their ability to support microsporidial growth, and for their value in studying the host-parasite relationship. The establishment of unique in vitro systems of *A. algerae* and fish cell lines outlined in this thesis allow for new and future avenues of research. For example, there are several possible areas of investigation that could use this system to evaluate general antimicrosporidial defense mechanisms in fish cells, or the movement of spores using various cell types. Several specific areas to investigate are outlined below:

Firstly, the *A. algerae* and fish cell system could be used to assess the effects of antimicrobial peptides on fish cells and levels of infection. Methods to evaluate the effects of antimicrobial agents in fish cell culture, as outlined in Chapter 4, could be used to assess a variety of compounds or conditions on microsporidial growth. Pleurocidin, for example, is a antimicrobial peptide known to be produced by winter flounder in the gut (Syvitski et al. 2005) and has demonstrated antifungal properties (Sung and Lee 2008). Winter flounder are often host to *Glugea stephani* microsporidia infection of the gut (Cali and Takvorian 1991, Takvorian and Cali 1984). Using the *A. algerae*/fish cell culture system can be used to evaluate the effects of pleurocidin on *A. algerae* growth. Ultimately, developing a similar system with *G. stephani* in a winter flounder cell line would be of interest.

Secondly, the cell culture system could be used to assess if phagocytosis and respiratory burst impede infection. Shaw et al. (2001) investigated phagocytosis of *Loma salmonae* spores by susceptible Chinook salmon derived macrophages, and resistant Atlantic salmon macrophages have been evaluated. It was determined that the resistant Atlantic salmon macrophages were more effective at phagocytosis of the *Loma* spores than resistant Chinook macrophages. These differences may play an important role in antimicrosporidial defense mechanisms. It also appears that immunemodulation may occur through phagocytosis and the prevention of the respiratory burst. Weidner and Sibley (1985) observed that aggregates of phagocytized spores were destroyed by macrophages, but single spores prevented phagosome-lysosome fusion. Germination has be observed to occur with changes in pH, including with lower pH shifts (Hashimoto et al. 1976, Undeen 1983, Undeen and Avery 1988). Therefore, there is the possibility that the respiratory burst could promote infection by stimulating germination of phagocytized spores through an acidification pH shift and allow

for autoinfection of the cell, or infection of adjacent cells. Evaluation of the effects of phagocytosis by macrophages in co-cultures with other fish cells, may be a way to evaluate the role of macrophages in the promotion or inhibition of infection.

Thirdly, the microsporidia/fish cell culture system could be used to evaluate if *A. algerae* induces specific modes of cell death in fish cells. Generally speaking parasites do not benefit from killing their hosts and in the case of microsporidia, proliferation of the parasite can only occur in a living cell. However, once a cell has been used by microsporidia to grow mature spores, there may be mechanisms of programmed cell death in apoptotic, autophagic or necrotic cell death that could promote transmission to surrounding cells. Therefore, evaluating mechanisms of cell death may elucidate factors that promote infection.

Fourthly, *A. algerae*/fish cell culture could be used to evaluate the induction of various immune genes. Does infection regulate the expression of antimicrobial, cytokine, or Major Histocompatibility Complex (MHC) genes? When Chinook salmon leukocytes were challenged with *Nucleospora salmonis*, factors were released which promoted leukocyte proliferation (Wongtavatchai et al. 1994, 1995). The leukocytes were primary cell cultures isolated from the peripheral blood, which may be and important feature of the culture in order to elicit the upregulation of various cytokines. Future work using monocyte/macrophage cell lines or primary cultures may assist in the identification of what role cytokines play in immune responses to microsporidia infection.

Lastly, the microsporidia/fish cell culture systems could be used to study the movement of spores across a gill or gut epithelium, and the transport of the parasite by host macrophages. In vivo, the life cycle of *Loma salmonae*, for example, is transmitted to the gut mucosa (lamina propria), and macrophages carry the parasite to the heart (Rodriguez-Tovar et al. 2002, Sanchez et al. 2000). In the heart the parasite undergoes merogony and is again transported by macrophages to the pillar cells of the gills where sporogony and xenoma formation occurs (Rodriguez-Tovar et al. 2002). Understanding the way in which microsporidia can enter and exit cells during various stages of the life cycle may assist in understanding mechanisms of control. Various in vitro protocols could allow for these observations such as the co-culturing of macrophages with various cell lines/types, isolating and various life cycle stages, and using meronts, sporonts, sporoplasts or mature spores to observe the mechanism whereby meronts can leave a cell during aspects of the life cycle.

6.2 Broader Implications

The experience and techniques gained by working with the *A. algerae*/fish cell culture system can be used to develop other in vitro systems. The goal of this work was to be able to use fish cell cultures to study microsporidia, and ultimately to use fish cell cultures cultivate microsporidia known to infect economically important fish. *Loma salmonae*, for example, would be an important fish microsporidian to grow in culture. It could allow for a greater understanding of mechanisms of immunemodulation, allow for evaluation of control measures, and potentially be the source from which spores are harvested for the *L. salmonae* whole spore vaccine (Rodriguez-Tovar et al. 2011, Speare et al. 2007).

Lastly, my research suggests that the restriction of a microsporidial species to a particular animal host is not accomplished at the cellular level but through physiological systems expressed at the organismal level. Questions then arise from this work, since *A. algerae* can infect various fish cell lines, can *A. algerae* also infect fish (such as goldfish or zebrafish) in vivo? If microsporidia is considered an emerging pathogen of humans due to what are believed to be zoonotically transferred infections (Didier 2005, Didier et al. 2004, Didier and Weiss 2006), is it possible that various microsporidial infections previously only known in insects and mammals have to possibility to emerge in fish? This could include various microspordia including *Paranosema locustae* (previously Nosema) (Sokolova et al. 2003), a microsporidian that is known to infect grasshoppers and crickets and has recently been approved for conditional use of as a microbial pesticide to be sprayed on crops in Canada (Health Canada 2010). Certainly this thesis suggests that further research is required to fully understand mechanisms that allow for transmission between different hosts, and to gain a greater understanding of host specificity.

References

Chapter 1 References

Ackermann G and Paw BH (2003). Zebrafish: a genetic model for vertebrate organogenesis and human disorders. *Front Biosci* 8: 1227-1253

Ashton N, Wirasinha P and Wirasinha A (1973). Encephalitozoonosis (nosematosis) of the cornea. *Br J Ophthalmol* 60: 618-631

Beauvis B, Sarfati C, Challier S and Derouin F (1994). *In vitro* model to assess the effect of antimicrobial agents on *Encephalitozoon cuniculi*. *Antimicrob Agents Chemother* 38: 2440-2448

Becker JA and Speare DJ (2007). Transmission of the microsporidian gill parasite *Loma salmonae*. *Anim Health Res Rev* 8: (1)59-68

Becnel JJ (2006). Expanding frontiers for microsporidia: A tribute to Professor Elizabeth U. Canning. *J Invert Pathol* 92: 116-124

Bedrnik P and Vávra J (1972). Further observations on the maintenance of *Encephalitozoon cuniculi* in tissue culture. *J Protozool* 19: 75S

Belkorchia A, Biderre C, Militon C, Polonais V, Wincker P, Jubin C, Delbac F, Peyretaillade E and Peyret P (2008). *In vitro* propagation of the microsporidian pathogen *Brachiola algerae* and studies of its chromosome and ribosomal DNA organization in the context of the complete genome sequencing project. *Parasitol Inter* 57: 62-71

Bigliardi E, Riparbelli MG, Selmi MG, Bini L, Liberatorii S, Pallini V, Bernuzzi A, Gatti S, Scaglia M and Sacchi L (1999). Evidence of actin in the cytoskeleton of microsporidia. *J Eukaryot Microbiol* 46: 410-415

Bols NC, Dayeh VR, Lee LEJ and Schirmer K (2005) Use of fish cell lines in the toxicology and ecotoxicology of fish. Piscine cell lines in environmental toxicology. In Biochemistry and Molecular Biology of Fishes (Mommsen, TP and Moon, TW, eds): Elsevier Science, pp 60-84.

Bols NC and Lee LEJ (1991). Technology and uses of cell-cultures from the tissues and organs of bony fish. *Cytotechnol* 6: (3)163-187

Bromenshenk JJ, Henderson CB, Wick CH, Stanford MF, Zulich AW, Jabbour RE, Deshpande SV, Mccubbin PE, Seccomb RA, Welch PM, Williams T, Firth DR, Skowronski E, Lehmann MM, Billimoria SL, Gress J, Wanner KW and Cramer Jr. RA (2010). Iridovirus and microsporidian linked to honey bee colony decline. *PLoS ONE* 5: (10)e13181

Cali A and Takvorian PM (1991). The incidence of *Glugea stephani* (Protozoa: Microsporida) in winter flounder, *Pseudopleuronectes americanus*, from the New York - New Jersey Lower Bay Complex and factors influencing it. *Can J Zool* 69: 317-321

Cali A and Takvorian PM (2003). Ultrastructure and development of *Pleistophora ronneafiei* n. sp., a microsporidium (Protista) in the skeletal muscle of an immune-compromised individual. *J Eukaryot Microbiol* 50: 77-85

Cali A and Takvorian PM (1999) Developmental morphology and life cycles of the microsporidia. In The Microsporidia and Microsporidiosis (Wittner, M and Weiss, LM, eds), Washington, D.C.: ASM Press, pp 85-128.

Canning EU (1953). A new microsporidian, *Nosema locustae* n. sp., from the fat body of the African migratory locust, *Locusta migratoria migratorioides* R. and F. *Parasitol* 43: 287-291

Canning EU and Lom J (1986) The Microsporidia of Vertebrates. Toronto: Academic Press, pp.289.

Chen Y, Evans JD, Smith B and Pettis JS (2008). *Nosema ceranae* is a long-present and wide-spred microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. *J Invert Pathol* 97: 186-188

Couzinet S, Cejas E, Schittny J, Deplazes P, Weber R and Zimmerli S (2000). Phagocytic uptake of *Encephalitozoon cunculi* by nonprofessional phagocytes. *Infect Immun* 68: (12)6939-6945

Coyle CM, Weiss LM, Rhodes III LV, Cali A, Takvorian PM, Brown DF, Visvesvara GS, Xiao L, Naktin J, Young E, Gareca M, Colasante G and Wittner M (2004). Fatal myositis due to the microsporidian *Brachiola algerae,* a mosquito pathogen. *N Engl J Med* 351: (1)42-47

del Aguila C, Izquierdo F, Granja AG, Hurtado C, Fenoy S, Fresno M and Revilla Y (2006). *Encephalitozoon* microsporidia modulates p53-mediated apoptosis in infected cells. *Int J Parasitol* 36: 869-876

Desportes I, Le Charpentier Y, Galian A, Bernard F, Cochand-Priolett B, Lavergne A, Ravisse P and Modigliani R (1985). Occurrence of a new microsporidian: *Enterocytozoon bieneusi* n.g., n. sp., in the enterocytes of a human patient with AIDS. *J Protozool* 32: 250-254

Desportes-Livage I, Chilmonczyk S, Hedrick R, Ombrouck C, Monge D, Maiga I and Gentilini M (1996). Comparative development of two microsporidian species: *Enterocytozoon bieneusi* and *Enterocytozoon salmonis*, reported in AIDS patients and salmonid fish, respectively. *J Eukaryot Microbiol* 43: (1)49-60

Didier ES (2005). Microsporidiosis: An emerging and opportunisitc infection in humans and animals. *Acta Tropica* 94: 61-76

Didier ES, Bowers L, Stovall ME, Kuebler D, Mittleider D, Brindley PJ and Didier PJ (2005). Antimicrosporidial activity of (fluoro)quinolones *in vitro* and *in vivo*. *Folia Parasitol* 52: 173-181

Didier ES, Didier PJ, Friedberg DN, Stenson SM, Orenstein JM, Yee RW, Tio FO, Davis RM, Vossbrinck C and Millichamp N (1991). Isolation and characterization of a new human microsporidian, Encephalitozoon hellem (n. sp.), from three patients with keratoconjunctivitis. *J Infect Dis* 163: 617-621

Didier ES and Weiss LM (2006). Microsporidiosis: current status. *Cur Op Infect Dis* 19: 485-492

Didier PJ, Phillips JN, Kuebler D, Nasr M, Brindley PJ, Stovall ME, Bowers LC and Didier ES (2006). Antimicrosporidial activities of fumagillin, TNP-470, ovalicin, and ovalicin derivatives in vitro and in vivo. *Antimicrob Agents Chemother* 50: (6)2146-2155

Dobbins WOI and Weinstein W (1985). Electron microscopy of the intestine and rectum in acquired immunodeficiency syndrome. *Gastroenterol* 88: (3)738-749

Ellis JD and Munn PA (2005). The worldwide health status of honey bees. *Bee World* 86: 88-101

Enders JF, Weller TH and Robbins FC (1949). Cultivation of the Lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. *Science* 109: 85-87

Fasshauer V, Gross U and Bohne W (2005). The parasitophorous vacuole membrane of *Encephalitozoon cuniculi* lacks host cell membrane proteins immediately after invasion. *Eukaryot Cell* 4: (1)221-224

Fischer J, Tran D, Juneau R and Hale-Donze H (2008). Kinetics of *Encephalitozoon* spp. infection of human macrophages. *J Parasitol* 94: (1)169-175

Fischer J, West J, Agochukwu N, Suire C and Hale-Donze H (2007). Induction of host chemotactic response by *Encephalitozoon* spp. *Infect Immun* 75: (4)1619-1625

Franzen C, Fischer S, Schroeder J, Bleiss W, Schneuwly S, Scholmerich J and Salzberger B (2005a). *In vitro* cultivation of an insect microsporidian *Tubulinosema ratisbonensis* in mammalian cells. *J Eukaryot Microbiol* 52: (4)349-355

Franzen C, Hosl M, Salzberger B and Hartmann P (2005b). Uptake of *Encephalitozoon* spp. and *Vittaforma corneae* (Microsporidia) by different cells. *J Parasitol* 91: (4)745-749

Franzen C and Muller A (2001). Microsporidiosis: human diseases and diagnosis. *Microb Infect* 3: (5)389-400

Fryer JL and Lannan CN (1994). Three decades of fish cell culture: A current listing of cell lines derived from fishes. *J Tiss Cult Meth* 16: 87-94

Ganassin RC and Bols NC (1996). Development of long-term rainbow trout spleen cultures that are haemopoietic and produce dendritic cells. *Fish Shellfish Immunol* 6: 17-34

Ganassin RC and Bols NC (1998). Development of a monocyte/macrophage-like cell line, RTS11, from rainbow trout spleen. *Fish Shellfish Immunol* 8: (457-476)

(2005) Great Lakes Panel Priority Invasive Species List, 2005. http://www.glc.org/ans/pdf/priority-species.pdf Cited 26 Aug 2008. Available online (accessed Aug 26, 2008.

Hayman JR, Southern TR and Nash TE (2005). Role of sulfated glycans in adherence of the microsporidian *Encephalitozoon intestinalis* to host cells *in vitro*. *Infect Immun* 73: 841-848

Henry JE and Onsager JA (1982). Large-scale test of control grasshoppers on rangland with *Nosema locustae*. *J Econ Entomol* 75: 31-35

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lucking R, Lumbsch HT, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai Y-C, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Koljalg U, Maidlikowska J, Miller A, Moncalvo J-M, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schubler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao Y-J and Zhang N (2007). A higher-level phylogenetic classification of the Fungi. *Mycolog* 111: 509-547

Hirt RP, Logsdon JM, Healy B, Dorey MW, Doolittle WF and Embley TM (1999). Microsporidia are related to Fungi: Evidence from the largest subunit of RNA polymerase II and other proteins. *Proc Natl Acad Sci USA* 96: (580-585)

Hollister WS, Canning EU and Anderson CL (1996). Identification of microsporidia causing human disease. *J Eukaryot Microbiol* 43: (5)S104-S105

Hsiung GD (1989). The impact of cell culture sensitivity on rapid viral diagnosis: a historical perspective. *Yale J Biol Med* 62: 79-88

Ishihara R (1968). Growth of Nosema bombycis in primary cell cultures of mammalian and chick embryos. *J Invert Pathol* 11: (2)328

Iwano H and Kurtti TJ (1995). Identification and isolation of dimorphic spores from *Nosema furnacalis* (Microspora: Nosematidae). *J Invert Pathol* 65: 230-236

Jaronski ST (1984). Microsporidia in Cell Culture. *Adv Cell Cult* 18: 183-229

Jelinek J, Salat J, Sak B and Kopecky J (2007). Effects of interferon gamma and specific polyclonal antibody on the infection of murine peritoneal macrophages and murine macrophage cell line PMJ2-R with *Encephalitozoon cuniculi*. *Folia Parasitol* 54: (3)172-176

John DE, Nwachuku N, Pepper IL and Gerba CP (2003). Development and optimization of a quantitative cell culture infectivity assay for the microsporidium *Encephalitozoon intestinalis* and application to ultraviolet light inactivation. *J Microbiol Meth* 52: 183-196

Keeling PJ and Fast NM (2002). Microsporidia: Biology and evolution of highly reduced intracellular parasites. *Annu Rev Microbiol* 56: 93-116

Keeling PJ, Luker MA and Palmer JD (2000). Evidence from Beta-tubulin phylogeny that microsporidia evolved from within the Fungi. *Mol Biol Evol* 17: (1)23-31

Kent ML and Fournie JW (2007) Parasites of Fishes. In Flynn's Parasites of Laboratory Animals (Baker, DG, ed: Blackwell Publ., pp 69-117.

Khurad AM, Raina SK and Pandharipande TN (1991). *In vitro* propagation of *Nosema locustae* using fat body cell line derived from *Mythimna convecta* (Lepidoptera: Noctuidae). *J Protozool* 38: 91S-93S

Kim JH, Ogawa K and Wakabayashi H (1999). Lectin-reactive components of the microsporidian *Glugea plecoglossi* and their relation to spore phagocytosis by head kidney macrophages of ayu *Plecoglossus altivelis*. *Dis Aquat Org* 39: (1)59-63

Kotler DP and Orenstein JM (1999) Clinical syndromes associated with microsporidiosis. In The Microsporidia and Microsporidiosis, Washington, D.C.: ASM Press.

Kou G-H, Wang C-H, Hung H-W, Jang Y-S, Chou C-M and Lo C-F (1995). A cell line (EP-1 cell line) derived from "Beko disease" affected Japanese eel elver (*Anguilla japonica*) persistently infected with *Pleistophora anguillarum*. *Aquaculture* 132: 161-173

Kurtti TJ, Ross SE, Liu Y and Munderloh UG (1994). *In vitro* developmental biology and spore production in *Nosema furnacalis* (Microspora: Nosematidae). *J Invert Path* 63: 188-196

Ledford DK, Overman MD, Gonzalvo A, Cali A, Mester SW and Lockey RF (1985). Microsporidiosis myositis in a patient with the acquired immunodeficiency syndrome. *Ann Intern Med* 102: (5)628-629

Lee S-J, Yokoyama H and Ogawa K (2004). Modes of transmission of *Glugea plecoglossi* (Microspora) via the skin and digestive tract in an experimental infection model using rainbow trout, Oncorhynchus mykiss (Walbum). *J Fish Dis* 27: 435-444

Leiro J, Iglesias R, Parama A, Sanmartin ML and Ubeira FM (2001). Effect of *Tetramicra brevifilum* (Microspora) infection on respiratory-burst responses of turbot (*Scophthalmus maximus* L.) phagocytes. *Fish Shellfish Immunol* 11: 639-652

Leitch GJ, Shaw AP, Colden-Stanfield M, Scanlon M and Visvesvara GS (2005). Multinucleate host cells induced by *Vittaforma corneae* (Microsporidia). *Folia Parasitol* 52: 103-110

Lom J (2002). A catalogue of described genera and species of microsporidians parasitic in fish. *Syst Parasitol* 53: 81-99

Lom J and Dyková I (2005). Microsporidian xenomas in fish seen in wider perspective. *Folia Parasitol* 52: 69-81

Lom J, Dyková I, Wang C-H, Lo C-F and Kou G-H (2000). Ultrastructural justification for the transfer of *Pleistophora anguillarum* Hoshina, 1959 to the genus *Heterosporis* Schubert, 1969. *Dis Aquat Org* 43: 225-231

Lores B, Rosales MJ, Mascaro C and Osuna A (2003). *In vitro* culture of *Glugea* sp. *Vet Parasitol* 112: 185-196

Lowman PM, Takvorian PM and Cali A (2000). The effects of elevated temperatures and various time-temperature combinations on the development of *Brachiola* (*Nosema*) *algerae* N. Comb. in mammalian cell culture. *J Eukaryot Microbiol* 47: (3)221-234

Marcus PB, van der Watt JJ and Burger PJ (1973). Human tumor microsporidiosis. *Arch Pathol* 95: 341-343

Margileth AM, Strano AJ, Chandra R, Neafie R, Blum M and McCully RM (1973). Disseminated nosematosis in an immunogically compromised infant. *Arch Pathol* 95: 145-150

Mathis A, Weber R and Deplazes P (2005). Zoonotic potential of the microsporidia. *Clin Microbiol Rev* 18: 423-445

Matsubayashi H, Koike T, Mikata I, Takei H and Hagiwara S (1959). A case of *Encephalitozoon*-like body in man. *Arch Pathol* 67: 181-187

Mertens RB, Didier ES, Fishbein MC, Bertucci DC, Rogers LB and Orenstein JM (1997). Encephalitozoon cuniculi microsporidiosis: infection of the brain, heart, kidneys, trachea, adrenal glands and urinary bladder in a patient with AIDS. *Mod Pathol* 10: 68-77

Miller NW, Rycyzyn MA, Wilson MR, Warr GW, Naftel JP and Clem LW (1994). Development and characterization of channel catfish long term B cell lines. *J Immunol* 152: 2180-2189

Modigliani R, Bories C, Le Charpentier Y, Salmeron M, Messing b, Galian A, Rambaud JC, Lavergne A, Cochand-Priolett B and Desportes I (1985). Diarrhoea and malabsorption in acquired immunodeficiency syndrome: a study of four cases with special emphasis on opportunistic protozoan infestation. *Gut* 26: (2)179-187

Moniez R (1887). Observations pour la revision des microsporidies. *CR Acad Sci Paris* 104: 1312-1314

Morris JA, McCown JM and Blount RE (1956). Acites and hepatosplenomegaly in mice associated with protozoan-like cytoplasmic structures. *J Infect Dis* 306-311

Nageli K (1857). Ueber die neue Krankheit die Seidenraupe und vervandte Organismen. *Bot Zietung* 15: 760-761

Olmstead A and Woolen DB (1987). Bee pollination and productivity growth: the case of alfalfa. *Amer J Agr Econ* 69: 56-63

Pasteur L (1870) Etude sur la maladie des vers a soie. Paris, France.

Rinder H, Thomschke A, Dengjel B, Gothe R, Loscher T and Zahler M (2000). Close genotypic relationship between Enterocytozoon bieneusi from humans and pigs and first detection in cattle. *J Parasitol* 86: 185-188

Ronnebaumer K, Gross U and Bohne W (2008). The nascent parasitophorous vacuole membrane of *Encephalitozoon cuniculi* is formed by host cell lipids and contains pores which allow nutrient uptake. *Eukaryot Cell* 7: (6)1001-1008

Schaeffer WI (1990). Terminology associated with cell, tissue and organ culture, molecular biology and molecular genetics. *In Vitro Cell Dev Biol - Animal* 26: (1)97-101

Schottelius J, Schmetz C, Kock NP, Schuler T, Sobottka I and Fleischer B (2000). Presentation by scanning electron microscopy of the life cycle of microsporidia of the genus Encephalitozoon. *Microb Infect* 2: 1401-1406

Shadduck JA, Meccoli RA, Davis R and Font RL (1990). Isolation of a microsporidian from a human patient. *J Infect Dis* 162: 773-776

Sharma S, Das S, Joseph J, Vemuganti GK and Murthy S (2011). Microsporidial keratitis: Need for increased awareness. *J Surv Ophthalmol* 56: (1)

Shaw RW and Kent ML (1999) Fish Microsporidia. In The Microsporidia and Microsporidiosis (Wittner, M and Weiss, LM, eds), Washington, D.C.: ASM Press.

Shaw RW, Kent ML and Adamson ML (2001). Phagocytosis of *Loma salmonae* (Microsporidia) spores in Atlantic salmon (*Salmo salar)*, a resistant host, and chinook salmon (*Oncorhynchus tshawytscha*), a susceptible host. *Fish Shellfish Immunol* 11: 91-100

Smith JE and Sinden RE (1980). A technique for the culture of *Nosema algerae* in primary cultures of rat brain. *J Protozool* 27: A59-A59

Snowden KF and Shadduck JA (1999) Microsporidia in higher vertebrates. In The Microsporidia and Microsporidiosis (Wittner, M and Weiss, LM, eds), Washington, D.C.: ASM Press, pp 393-417.

Sokolova YY, Dolgikh VV, Morzhina EV, Nassonova ES, Issi IV, Terry RS, Ironside JE, Smith JE and Vossbrinck CR (2003). Establishment of the new genus *Paranosema* based on the ultrastructure and molecular phylogeny of the type species *Paranosema grylli* Gen. Nov. comb. Nov. (Sokolova, Selezniov, Dolgikh, Issi 1994), from the cricket *Gryllus bimaculatus* Deg. *J Invert Pathol* 84: 159-172

Southern TR, Jolly CE, Lester ME and Hayman JR (2007). EnP1, a microsporidian spore wall protein that enables spores to adhere to and infect host cells *in vitro*. *Eukaryot Cell* 6: 1354-1362

Sprague V (1974). Nosema connori n. sp.: a microsporidian parasite of man. *Trans Am Microsc Soc* 93: (3)400-403

T'sui W-H and Wang C-H (1988). On the *Plistophora* infection in eel II. The development of *Plistophora anguillarum* in experimentally infected elvers, *Anguilla japonica*. *Bull Instit Zool* 27: (4)249-258

Takvorian PM, Weiss LM and Cali A (2005). The early events of *Brachiola algerae* (Microsporidia) infection: spore germination, sporoplasm structure, and development within host cells. *Folia Parasitol* 52: 118-129

Taupin V, Metenier G, Delbac F, Vivares CP and Prensier G (2006a). Expression of two cell wall proteins during the intracellular development of *Encephalitozoon cuniculi*: an immunocytochemical and *in situ* hybridization study with ultrathin frozen sections. *Parasitol* 132: 815-825

Taupin V, Metenier G, Vivares CP and Prensier G (2006b). An improved procedure for Percoll gradient separation of sporogonial stages in *Encephalitozoon cuniculi* (Microsporidia). *Parasitol Res* 99: (6)708-714

Tosoni A, Nebuloni M, Ferri A, Bonetto S, Antinori S, Scaglia M, Xiao L, Moura H, Visvesvara GS, Vago L and Costanzi G (2002). Disseminated microsporidiosis caused by *Encephalitozoon cuniculi* III (Dog Type) in an Italian AIDS patient: a retrospective study. *Mod Pathol* 15: (5)577-583

Trager W (1937). The hatching of spores of *Nosema bombycis* Nageli and the partial development of the organism in tissue culture. *J Parasitol* 23: 226-227

Trammer T, Chioralia G, Maier WA and Seitz HM (1999). *In vitro* replication of *Nosema algerae* (Microsporidia), a parasite of anopheline mosquitoes, in human cells above 36 ºC. *J Eukaryot Microbiol* 46: 464-468

(2000) Fisheries and Aquaculture. http://www.oceansatlas.org Cited 26 Aug 2008. Available online (accessed 26 Aug 2008.

Undeen AH (1975). Growth of *Nosema algerae* in pig kidney cell cultures. *J Protozool* 22: (1)107-110

Visvesvara GS (2002). In vitro cultivation of microsporidia of clinical importance. *Clin Microbiol Rev* 15: (3)401-413

Visvesvara GS, Da Silva AJ, Croppo GP, Pieniazek NJ, Leitch GJ, Ferguson D, De Moura H, Wallace S, Slemenda SB, Tyrrel I, Moore DF and Meador J (1995a). *In vitro* culture and serologic and molecular identification of *Septata intestinalis* isolated from urine of a patient with AIDS. *J Clin Microbiol* 33: 930-936

Visvesvara GS, Leitch GJ, Pieniazek NJ, Da Silva AJ, Wallace S, Slemenda SB, Weber R, Schwartz DA, Gorelkin L, Wilcox CM and Bryan RT (1995b). Short-term *in vitro* culture and molecular analysis of the microsporidian, *Enterocytozoon bieneusi*. *J Eukaryot Microbiol* 42: (5)506-510

Wasson K and Barry P (2003). Molecular characterization of *Encephalitozoon intestinalis* (Microspora) replication kinetics in a murine intestinal cell line. *J Eukaryot Microbiol* 50: (3)169-174

Weidner E (1972). Ultrastructural study of microsporidian invasion into cells. *Z Parasitenkd* 40: 227-242

Williams GR, Shafer ABA, Rogers REL, Shutler D and Stewart DT (2008). First detection of *Nosema ceranae*, a microsporidian parasite of European honey bees (*Apis mellifera*), in Canada and Central USA. *J Invert Pathol* 97: 189-192

Wittner M (1999) Historic Perspective on the Microsporidia: Expanding Horizons. In The Microsporidia and Microsporidiosis (Wittner, M and Weiss, LM, eds), Washington, D.C.: ASM Press, pp 1-6.

Wolk DM, Johnson CH, Rice EW, Marshall MM, Grahn KF, Plummer CB and Sterling CR (2000). A spore counting method and cell culture model for chlorine disinfection studies of *Encephalitozoon* syn. *Septata intestinalis*. *Appl Environ Microbiol* 66: (4)1266-1273

Wongtavatchai J, Conrad PA and Hedrick RP (1995). *In vitro* characteristics of the microsporidian: *Enterocytozoon salmonis*. *J Eukaryot Microbiol* 42: (4)401-405

Wongtavatchai J, Conrad PA and Hedrick RP (1994). *In vitro* cultivation of the microsporidian: *Enterocytozoon salmonis* using a newly developed medium for salmonid lymphocytes. *J Tiss Cult Meth* 16: 125-131

Chapter 2 References

Avery SW and Undeen AH (1987). The isolation of microsporidia and other pathogens from concentrated ditch water. *J Am Mosq Control Assoc* 3: (1)54-58

Becker JA and Speare DJ (2007). Transmission of the microsporidian gill parasite *Loma salmonae*. *Anim Health Res Rev* 8: (1)59-68

Belkorchia A, Biderre C, Militon C, Polonais V, Wincker P, Jubin C, Delbac F, Peyretaillade E and Peyret P (2008). In vitro propagation of the microsporidian pathogen Brachiola algerae and studies of its chromosome and ribosomal DNA organization in the context of the complete genome sequencing project. *Parasitol Int* 57: 62-71

Bols NC and Lee LEJ (1994) Cell lines: availability, propagation and isolation. In Biochemistry and Molecular Biology of Fishes (Hochachka, PW and Mommsen, TP, eds), Amsterdam: Elsevier Science, pp 145-159.

Bols NC, Mosser DD and Steels GB (1992). Temperature studies and recent advances with fish cells in vitro. *Comp Biochem Physiol* 103A: 1-14

Cali A, Weiss LM and Takvorian PM (2004). An analysis of the microsporidian genus *Brachiola*, with comparisons of human and insect isolates of *Brachiola algerae*. *J Eukaryot Microbiol* 51: (6)678-685

Coyle CM, Weiss LM, Rhodes III LV, Cali A, Takvorian PM, Brown DF, Visvesvara GS, Xiao L, Naktin J, Young E, Gareca M, Colasante G and Wittner M (2004). Fatal myositis due to the microsporidian *Brachiola algerae,* a mosquito pathogen. *N Engl J Med* 351: (1)42-47

Didier ES (2005). Microsporidiosis: An emerging and opportunisitc infection in humans and animals. *Acta Tropica* 94: 61-76

Franzen C, Nassonova ES, Scholmerich J and Issi IV (2006). Transfer of the members of the genus *Brachiola* (Microsporidia) to the genus *Anncaliia* based on ultrastructural and molecular data. *J Eukaryot Microbiol* 53: (1)26-35

Hester JD, Varma M, Bobst AM, Ware MW, Lindquist HDA and Schaefer III FW (2002). Species-specific detectin of three human-pathogenic microsporidial species from genus *Encephalitozoon* via fluorogenic 5' nuclease PCR assays. *Mol Cell Probes* 16: 435-444

Keeling PJ and Fast NM (2002). Microsporidia: Biology and evolution of highly reduced intracellular parasites. *Annu Rev Microbiol* 56: 93-116

Kiryu I and Wakabayashi H (1999). Adherence of suspended particles to the body surface of rainbow trout. *Fish Pathol* 34: 177-182

Koudela B, Visvesvara GS, Moura H and Vávra J (2001). The human isolate of *Brachiola algerae* (Phylum Microspora): development in SCID mice and description of its fine structure features. *Parasitol* 123: 153-162

Kucerova Z, Moura H, Visvesvara GS and Leitch GJ (2004). Differences between Brachiola (Nosema) algerae isolates of human and insect origin when tested using and in vitro spore germination assay and a cultured cell infection assay. *J Eukaryot Microbiol* 51: (3)339-343

Lee LEJ, Caldwell SJ and Gibbons J (1997). Development of a cell line from skin of goldfish, *Carassius auratus*, and effects of ascorbic acid on collagen deposition. *Histochem* 29: 31-43

Leitch GJ and Ceballos C (2008). Effects of host temperature and gastric and duodenal environments on microsporidia spore germination and infectivity of intestinal epithelial cells. *Parasitol Res* 104: 35-42

Lowman PM, Takvorian PM and Cali A (2000). The effects of elevated temperatures and various time-temperature combinations on the development of *Brachiola* (*Nosema*) *algerae* N. Comb. in mammalian cell culture. *J Eukaryot Microbiol* 47: (3)221-234

Monaghan SR, Kent ML, Watral VG, Kaufman RJ, Lee LEJ and Bols NC (2009). Animal cell cultures in microsporidial research: their general roles and their specific use for fish microsporidia. *In Vitro Cell Dev Biol - Animal* 45: 135-147

Moura H, da Silva AJ, Moura INS, Schwartz DA, Leitch G, Wallace S, Pieniazek NJ, Wirtz RA and Visvesvara GS (1999). Characterization of *Nosema algerae* isolates after continuous cultivation in mammalian cells at 37°C. *J Eukaryot Microbiol* 46: (5)14S-16S

Scanlon M, Leitch GJ, Shaw AP, Moura H and Visvesvara GS (1999). Susceptibility to apoptosis is reduced in the microsporidia-infected host. *J Eukaryot Microbiol* 46: (5)34S-35S

Servili A, Bufalino MR, Nishikawa R, de Melo IS, Muñoz-Cueto JA and Lee LEJ (2009). Establishment of long term cultures of neural stem cells from adult sea bass, *Dicentrarchus labrax*. *Comp Biochem Physiol* Part A 152: 245-254

Shaw RW and Kent ML (1999) Fish Microsporidia. In The Microsporidia and Microsporidiosis (Wittner, M and Weiss, LM, eds), Washington, D.C.: ASM Press.

Smith JE, Barker RJ and Lai PF (1982). Culture of microsporidia from invertebrates in vertebrate cells. *Parasitol* 85: 427-435

Smith JE and Sinden RE (1980). A technique for the culture of *Nosema algerae* in primary cultures of rat brain. *J Protozool* 27: A59-A59

Streett DA, Ralph D and Hink WF (1980). Replication of *Nosema algerae* in three insect cell lines. *J Protozool* 27: 113-117

Takvorian PM, Weiss LM and Cali A (2005). The early events of Brachiola algerae (Microsporidia) infection: spore germination, sporoplasm structure, and development within host cells. *Folia Parasitol* 52: 118-129

Trammer T, Chioralia G, Maier WA and Seitz HM (1999). *In vitro* replication of *Nosema algerae* (Microsporidia), a parasite of anopheline mosquitoes, in human cells above 36 ºC. *J Eukaryot Microbiol* 46: 464-468

Trammer T, Dombrowski F, Doehring M, Maier WA and Hanns MS (1997). Opportunistic properties of *Nosema algerae* (Microspora), a mosquito parasite, in immunocompromised mice. *J Eukaryot Microbiol* 44: (3)258-262

Undeen AH (1975). Growth of *Nosema algerae* in pig kidney cell cultures. *J Protozool* 22: (1)107-110

Undeen AH and Maddox JV (1973). The infection of nonmosquito hosts by injection with spores of the microsporidian *Nosema algerae*. *J Invert Path* 22: 258-265

Undeen AH and Vander Meer RK (1999). Microsporidian intrasporal sugars and their role in germination. *J Invert Path* 73: 294-302

Vávra J and Undeen AH (1970). *Nosema algerae* n. sp. (Cnidospora, Microsporidia) a pathogen in a laboratory colony of *Anopheles stephensi* Liston (Diptera, Culicuidae). *J Protozool* 17: 240-249

Visvesvara GS (2002). In vitro cultivation of microsporidia of clinical importance. *Clin Microbiol Rev* 15: (3)401-413

Visvesvara GS, Belloso M, Moura H, Da Silva A, Moura I, Leitch G, Schwartz DA, Chevez-Barrios P, Wallace S, Pieniazek NJ and Goosey J (1999). Isolation of *Nosema algerae* from the cornea of an immunocompetent patient. *J Eukaryot Microbiol* 46: 10S

Visvesvara GS, Moura H, Leitch GJ, Schwartz DA and Xiao LX (2005). Public health importance of *Brachiola algerae* (Microsporidia) - an emerging pathogen of humans. *Folia Parasitol* 52: 83-94

Xing JG, Lee LEJ, Fan L, Collodi P, Holt SE and Bols NC (2008). Initiation of a zebrafish blastula cell line on rainbow trout stromal cells and subsequent development under feeder-free conditions into a cell line, ZEB2J. *Zebrafish* 5: 49-63

Chapter 3 References

National Institutes of Health (NIH). ImageJ: Image processing and analysis in Java. Available online at: http://rsbweb.nih.gov/ij/.

Federal Energy Regulatory Commission (FERC) (2007). Aquatic Resources Amended Preliminary Draft Environmental Assessment. Attachment I Trout temperature requirements. FERC Project Nos. 2085, 2175, 67 & 120. *Southern California Edison Company*

Andreadis TG (2007). Microsporidian parasites of mowquitoes. *Am Mos Con Assoc Bull* 23: 6-29

Avery SW and Anthony DW (1983). Ultrastructural study of early development of *Nosema algerae* in *Anopheles albimanus*. *J Invert Path* 42: (1)87-95

Avery SW and Undeen AH (1987). The isolation of microsporidia and other pathogens from concentrated ditch water. *J Am Mosq Control Assoc* 3: (1)54-58

Bader JA, Shotts Jr EB, Steffens WL and Lom J (1998). Occurrence of *Loma* cf. *salmonae* in brook, brown and rainbow trout from Buford Trout Hatchery Georgia, USA. *Dis Aquat Org* 34: 211-216

Beaman HJ, Speare DJ and Brimacombe M (1999). Regulatory effects of water temperatue on Loma salmonae (Microspora) development in rainbow trout. *J Aquat Anim Health* 11: 237-245

Becker JA and Speare DJ (2004). Impact of water temperature shift on xenoma clearance and recovery time during a *Loma salmonae* (Microsporidia) infection in rainbow trout *Oncorhynchus mykiss*. *Dis Aquat Org* 58: 185-191

Becker JA, Speare DJ and Dohoo IR (2006). Interaction of water temperature and challenge model on xenoma development rates for *Loma salmonae* (Microspora) in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Fish Dis* 29: 139-145

Becnel JJ, White SE and Shapiro AM (2005). Review of microsporidia-mosquito relationships: from the simple to the complex. *Folia Parasitol* 52: 41-50

Belkorchia A, Biderre C, Militon C, Polonais V, Wincker P, Jubin C, Delbac F, Peyretaillade E and Peyret P (2008). *In vitro* propagation of the microsporidian pathogen *Brachiola algerae* and studies of its chromosome and ribosomal DNA organization in the context of the complete genome sequencing project. *Parasitol Inter* 57: 62-71

Bols NC, Barlian A, Chirino-Trejo M, Caldwell SJ, Goegan P and Lee LEJ (1994). Development of a cell line from primary cultures of rainbow trout, *Oncorhynchus mykiss* (Walbaum), gills. *J Fish Dis* 17: 602-611

Cali A, Neafie R, Weiss LM, Ghosh K, Vergara R, Gupta R and Takvorian PM (2010). Human vocal cord infection with the microsporidium *Anncalii algerae*. *J Eukaryot Microbiol* 57: (6)562-567

Cali A, Weiss LM and Takvorian PM (2002). *Brachiola algerae* spore membrane systems, their activity during extrusion, and a new structural entity, the multilayered interlaced network, associated with the polar tube and the sporoplasm. *J Eukaryot Microbiol* 49: (2)164-174

Cali A, Weiss LM and Takvorian PM (2004). An analysis of the microsporidian genus *Brachiola*, with comparisons of human and insect isolates of *Brachiola algerae*. *J Eukaryot Microbiol* 51: (6)678-685

Chioralia G, Trammer T, Maier WA and Seitz HM (1998). Morphologic changes in *Nosema algerae* (Microspora) during extrusion. *Parasitol Res* 84: 123-131

Coyle CM, Weiss LM, Rhodes III LV, Cali A, Takvorian PM, Brown DF, Visvesvara GS, Xiao L, Naktin J, Young E, Gareca M, Colasante G and Wittner M (2004). Fatal myositis due to the microsporidian *Brachiola algerae,* a mosquito pathogen. *N Engl J Med* 351: (1)42-47

Dall DJ (1983). A theory for the mechanism of polar filament extrusion in the Microspora. *J Theor Biol* 105: 647-659

Findley AM, Weidner EH, Carman KR, Xu Z and Godbar JS (2005). Role of the posterior vacuole in Spraguea lophii (Microsporidia) spore hatching. *Folia Parasitol* 52: 111-117

Fries I, Granados RR and Morse RA (1992). Intracellular germination of sporees of *Nosema apis* Z. *Apidologie* 23: 61-70

Frixione E, Ruiz L, Santillan M, de Vargas LV, Tejero JM and Undeen AH (1992). Dynamics of polar filament discharge and sporoplasm by microsporidian spores. *Cell Motil Cytoskeleton* 22: 38-50

Hayman JR, Southern TR and Nash TE (2005). Role of sulfated glycans in adherence of the microsporidian *Encephalitozoon intestinalis* to host cells *in vitro*. *Infect Immun* 73: 841-848

Ishihara R (1969). The life cycle of *Nosema bombycis* as revealed in tissue culture cells of *Bombyx mori*. *J Invert Pathol* 14: (3)316-320

Iwano H and Ishihara R (1989). Intracellular germination of spores of a *Nosema* sp. immediately after their formation in cultrued cell. *J Invert Path* 54: 125-127

Kawarabata T and Ishihara R (1984). Infection and development of *Nosema bombycis* (Microsporida: Protozoa) in a cell line of *Antheraea eucalypti*. *J Invert Pathol* 44: (1)52-62

Kucerova Z, Moura H, Visvesvara GS and Leitch GJ (2004). Differences between *Brachiola* (*Nosema*) *algerae* isolates of human and insect origin when tested using and in vitro spore germination assay and a cultured cell infection assay. *J Eukaryot Microbiol* 51: (3)339-343

Kurtti TJ, Tsang KR and Brooks MA (1983). The spread of infection by the microsporidian, *Nosema disstriae*, in insect cell lines. *J Protozool* 30: 652-657

Lom J and Vávra J (1963). The mode of sporoplasm extrusion in microsporidian spores. *Acta Protozool* 1: 81-92

Lovy J, Speare DJ, Stryhn H and Wright GM (2008). Effects of dexamethasone on host innate and adaptive immune responses and parasite development in rainbow trout *Oncorhynchus mykiss* infected with *Loma salmonae*. *Fish Shellfish Immunol* 24: 649-658

Lowman PM, Takvorian PM and Cali A (2000). The effects of elevated temperatures and various time-temperature combinations on the development of *Brachiola* (*Nosema*) *algerae* N. Comb. in mammalian cell culture. *J Eukaryot Microbiol* 47: (3)221-234

Monaghan SR (2011). The use of fish cell cultures for the study and cultivation of microsporidia. Ph.D. Thesis; Department of Biology, University of Waterloo: 1-136

Monaghan SR, Rumney RL, Vo NTK, Bols NC and Lee LEJ (2011). In vitro growth of microsporidia *Anncaliia algerae* in cell lines from warm water fish. *In Vitro Cell Dev Biol - Animal* 47: (2)104-113

Morrison CM and Sprague V (1983). *Loma salmonae* (Putz, Hoffman and Dunbar, 1965) in the rainbow trout, *Salmo gairdneri* Richardson, and *L. fontinalis* sp. nov. (Microsporidia) in the brook trout, *Salvelinus fontinalis* (Mitchell). *J Fish Dis* 6: 345-353

Moura H, da Silva AJ, Moura INS, Schwartz DA, Leitch G, Wallace S, Pieniazek NJ, Wirtz RA and Visvesvara GS (1999). Characterization of *Nosema algerae* isolates after continuous cultivation in mammalian cells at 37°C. *J Eukaryot Microbiol* 46: (5)14S-16S

Olson RE (1981). Effects of low temperature on the development of the microsporidan *Glugea stephani* in English sole (*Parophrys vetulus*). *J Wildlife Dis* 17: (4)559-562

Ramsay JM, Speare DJ, Becker JA and Daley J (2003). *Loma salmonae*-associated xenoma onset and clearance in rainbow trout, *Oncorhynchus mykiss* (Walbaum): comparisons of per os and cohabitation exposure using survival analysis. *Aquacult Res* 34: (1329-1335)

Rodriguez-Tovar LE, Speare DJ and Markham RJF (2011). Fish microsporidia: Immune response, immunomodulation and vaccination. *Fish & Shellfish Immunol* 30: 999-1006

Scanlon M, Leitch GJ, Shaw AP, Moura H and Visvesvara GS (1999). Susceptibility to apoptosis is reduced in the microsporidia-infected host. *J Eukaryot Microbiol* 46: (5)34S-35S

Sinden RE and Canning EU (1974). The ultrastructure of the spore of *Nosema algerae* (Protozoa, Microsporida), in relation to the hatching mechanism of microsporidian spores. *J Gen Microbiol* 85: 350-357

Staiber W (1994). Effects of *Nosema algerae* infections on the gene activity of the salivary gland chromosomes of *Acricotopus lucidus* (Diptera, Chironomidae). *Parasitol Res* 80: 108-111

Takvorian PM and Cali A (1984). Seasonal prevalence of the microsporidan, *Glugea stephani* (Hagenmuller), in winter flounder, *Pseudopleuronectes americanus* (Walbaum), from the New York-New Jersey Lower Bay Complex. *J Fish Biol* 24: 655-663

Takvorian PM, Weiss LM and Cali A (2005). The early events of Brachiola algerae (Microsporidia) infection: spore germination, sporoplasm structure, and development within host cells. *Folia Parasitol* 52: 118-129

Trammer T, Chioralia G, Maier WA and Seitz HM (1999). *In vitro* replication of *Nosema algerae* (Microsporidia), a parasite of anopheline mosquitoes, in human cells above 36 ºC. *J Eukaryot Microbiol* 46: 464-468

Trammer T, Dombrowski F, Doehring M, Maier WA and Hanns MS (1997). Opportunistic properties of *Nosema algerae* (Microspora), a mosquito parasite, in immunocompromised mice. *J Eukaryot Microbiol* 44: (3)258-262

Undeen AH (1975). Growth of *Nosema algerae* in pig kidney cell cultures. *J Protozool* 22: (1)107-110

Undeen AH and Frixione E (1990). The role of osmotic pressure in the germination of *Nosema algerae* spores. *J Protozool* 37: 561-567

Undeen AH and Maddox JV (1973). The infection of nonmosquito hosts by injection with spores of the microsporidian *Nosema algerae*. *J Invert Path* 22: 258-265

Vávra J and Undeen AH (1970). *Nosema algerae* n. sp. (Cnidospora, Microsporidia) a pathogen in a laboratory colony of *Anopheles stephensi* Liston (Diptera, Culicuidae). *J Protozool* 17: 240-249

Visvesvara GS, Belloso M, Moura H, Da Silva A, Moura I, Leitch G, Schwartz DA, Chevez-Barrios P, Wallace S, Pieniazek NJ and Goosey J (1999). Isolation of *Nosema algerae* from the cornea of an immunocompetent patient. *J Eukaryot Microbiol* 46: 10S

Visvesvara GS, Moura H, Leitch GJ, Schwartz DA and Xiao LX (2005). Public health importance of *Brachiola algerae* (Microsporidia) - an emerging pathogen of humans. *Folia Parasitol* 52: 83-94

Williams BAP (2009). Unique physiology of host-parasite interactions in microsporidia infections. *Cell Microbiol* 11: 1551-1560

Xing JG, Lee LEJ, Fan L, Collodi P, Holt SE and Bols NC (2008). Initiation of a zebrafish blastula cell line on rainbow trout stromal cells and subsequent development under feeder-free conditions into a cell line, ZEB2J. *Zebrafish* 5: 49-63

Xu Y and Weiss LM (2005). The microsporidian polar tube: A highly specialised invasion organelle. *Int J Parasitol* 35: 941-953

Chapter 4 References

Baliharová V, Skálová L, Maas RFM, De Vrieze G, Bull S and Fink-Gremmels J (2003). The effects of benzimidazole anthelmintics on P4501A in rat hepatocytes and HepG2 cells. *Res Vet Sci* 75: 61-69

Beauvais B, Sarfati C, Challier S and Derouin F (1994). In vitro model to assess effect of antimicrobial agents on *Encephalitozoon cuniculi*. *Antimicrob Agents & Chemo* 38: (10)2440-2448

Bunger J, westphal G, Monnich A, Hinnendahl B, Haller E and Muller M (2004). Cytoxicity of occupationally and environmentally relevant mycotoxins. *Toxicology* 202: 199-211

Cali A, Neafie R, Weiss LM, Ghosh K, Vergara R, Gupta R and Takvorian PM (2010). Human vocal cord infection with the microsporidium *Anncalii algerae*. *J Eukaryot Microbiol* 57: (6)562-567

Chatterji BP, Jindal B, Srivastava S and Panda D (2011). Microtubules as antifungal and antiparasitic drug targets. *Expert Opin Ther Pat* 21: 167-186

Colbourn NI, Hollister WW, Curry A and Canning EU (1994). Activity of albendazole against *Encephalitozoon-cuniculi in-vitro*. *Eur J Protistol* 30: 211-220

Costa SF and Weiss LM (2000). Drug treatment of microsporidiosis. *Drug Resistance Updates* 3: 384-399

Coyle CM, Weiss LM, Rhodes III LV, Cali A, Takvorian PM, Brown DF, Visvesvara GS, Xiao L, Naktin J, Young E, Gareca M, Colasante G and Wittner M (2004). Fatal myositis due to the microsporidian *Brachiola algerae,* a mosquito pathogen. *N Engl J Med* 351: (1)42-47

Dayeh VR, Schirmer K, Lee LEJ and Bols NC (2005) Evaluating the toxicity of water samples with the rainbow trout gill cell line microplate cytotoxicity test. In Small-Scale Fresh Water Toxicity Investigations (Blaise, C and Ferard, JF, eds): Springer, pp 473-504.

Didier ES (1997). Effects of albendazole, fumagillin, and TNP-470 on microsporidial replication in vitro. *Antimicrob Agents Chemother* 41: (7)1541-1546

Didier ES, Bowers L, Stovall ME, Kuebler D, Mittleider D, Brindley PJ and Didier PJ (2005). Antimicrosporidial activity of (fluoro)quinolones *in vitro* and *in vivo*. *Folia Parasitol* 52: 173-181

Didier ES, Maddry JA, Kwong CD, Green LC, Snowden KF and Shadduck JA (1998). Screening of compounds for antimicrosporidial activity in vitro. *Folia Parasitol* 45: 129-139

Didier PJ, Phillips JN, Kuebler D, Nasr M, Brindley PJ, Stovall ME, Bowers LC and Didier ES (2006). Antimicrosporidial activities of fumagillin, TNP-470, ovalicin, and ovalicin derivatives in vitro and in vivo. *Antimicrob Agents Chemother* 50: (6)2146-2155

Ditrich O, Kucerova Z and Koudela B (1994). In vitro sensitivity of *Encephalitozoon cuniculi* and *E. hellem* to albendazole. *J Eukaryot Microbiol* 41: 37S

El-Matbouli M and Hoffmann RW (1991). Prevention of experimentally induced whirling disease in rainbow trout *Oncorhyncus mykiss* by fumagillin. *Dis Aquat Org* 10: 109-113

Franssen FFJ, Lumeij TJ and van Knapen F (1995). Susceptibility of *Encephalitozoon cuniculi* to several drugs in vitro. *Antimicrob Agents & Chemo* 39: (6)1265-1268

Gross U (2003). Treatment of microsporidiosis including albendazole. *Parasitol Res* 90: S14-S18

Haque MA, Hollister WS, Wilcox A and Canning EU (1993). The antimicrosporidial activity of albendazole. *J Invert Path* 62: 171-177

Hedrick RP, Groff JM and Baxa DV (1991). Experimental infections with *Enterocytozoon salmonis* Chilmonczyk, Cox, Hedrick (Microsporea) - an intranuclear microsporidium from chinook salmon *Oncorhynchus tshawytscha*. *Dis Aquat Org* 10: (2)103-108

Hedrick RP, Groff JM, Foley P and McDowell T (1988). Oral administration of fumagillin DCH protects chinook salmon *Oncorhynchus tshawytscha* from experimentally-induced proliferative kidney disease. *Dis Aquat Org* 4: 165-168

Katznelson H and Jamieson CA (1952). Control of nosema disease of honeybees with fumagillin. *Science* 414: 70-71

Kent ML and Dawe SC (1994). Efficacy of fumigillin DCH against experimentally induced *Loma salmonae* (Microsporea) infections in Chinook salmon *Oncorhynchus tshawytscha*. *Dis Aquat Org* 20: 231-233

Kurtti TJ and Brooks MA (1977). The rate of development of a microsporidian in moth cell culture. *J Invert Path* 29: 126-132

Lafranchi-Tristem NJ, Curry A, Cheney SA and Canning EU (2001). Growth of *Trachipleistophora hominis* (Microsporidia: Pleistophoridae) in C2, C12 mouse myoblast cells and response to treatment with albendazole. *Folia Parasitol* 48: 192-200

Lauren DJ, Wishkovsky A, Groff JM, Hedrick RP and Hinton DE (1989). Toxicity and pharmacokinetics of the antibiotic fumagillin in yearling rainbow trout (*Salmo gairdneri*). *Toxicol Appl Pharmacol* 98: 444-453

Lee BCH, Williams BAP, Brown AMV, Adamson ML, Keeling PJ and Fast NM (2008). α- and β-tubulin phylogenies support a close relationship between the microsporidia *Brachiola algerae* and *Antonospora locustae*. *J Eukaryot Microbiol* 55: 388-392

Levine B (2005). Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. *Cell* 120: 159-162

Mittleider D, Green LC, Mann VH, Michael SF, Didier ES and Brindley PJ (2002). Survey of genome sequences from the opportunistic microsporidian pathogen, *Vittaforma corneae*. *J Eukaryot Microbiol* 49: (393-401)

Molnár K, Baska F and Székely CS (1986). Fumagillin, an efficacious drug against renal sphaerosporosis of the common carp *Cyprinus carpio*. *Dis Aquat Org* 2: 187-190

Monastyrska I, Rieter E, Klionsky DJ and Reggiori F (2009). Multiple roles of the cytoskeleton in autophagy. *Biol Rev* 84: 431-448

Ramirez T, Eastmond DA and Herrara LA (2007). Non-disjunction events induced by albendazole in human cells. *Mutation Res* 626: 191-195

Schmahl G and Benini J (1998). Treatment of fish parasites. 11. Effects of different benzimidazole derivatives (albendazole, mebendazole, fenbendazole) on Glugea anomala, Moniez, 1887 (Microsporidia): ultrastructural aspects and efficacy studies. *Parasitol Res* 84: 41-49

Shadduck JA (1980). Effect of fumagillin on *in vitro* multiplication of *Encephalitozoon cuniculi*. *J Protozool* 27: 202-208

Shaikh B, Rummel N, Giesker G and Reimschuessel R (2006). Metabolism and depletion of albendazole in the muscle tissue of channel catfish following oral treatment. *J Vet Pharmacol Therap* 29: 525-530

Sichtova M, Haque MA, Vávra J, Canning EU and Robert-Gero M (1993). Evaluation of the antibiotic sinefungin as an antimicrosporidial drug. *Folia Parasitol* 40: 85-91

Silveira H and Canning EU (1995). In vitro cultivation of the human microsporidium *Vittaforma corneae:* development and effect of albendazole. *Folia Parasitol* 42: 241-250

Sin N, Meng L, Wang MQW, Wen JJ, Bornmann WG and Crews CM (1997). The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase MetAP-2. *Proc Natl Acad Sci* 94: 6099-6103

Smothers JF, Vondohlen CD, Smith LH and Spall RD (1994). Molecular evidence that the myxozoan protists are metazoans. *Science* 265: 1719-1721

Sobottka I, Bartscht K, Schafer P, Weitzel T, Schottelius J, Kock N and Laufs R (2002). In vitro activity of polyoxin D and nikkomycin Z against *Encephalitozoon cuniculi*. *Parasitol Res* 88: (451-453)

Speare DJ, Athanassopoulou F, Daley J and Sanchez JG (1999). A preliminary investigation of alternatives to fumagillin for the treatment of Loma salmonae infection in rainbow trout. *J Comp Path* 121: 241-248

Takahashi S and Egusa S (1976). Studies on *Glugea* infection of the ayu, *Plecoglossus altivelis* II. On the prevention and treatment. I. Fumagillin as a treatment. *Japanese J Fish Biol* 11: 83-88

Weiss LM, Zhou GC and Zhang H (2003). Characterization of recombinant microsporidian methionine aminopiptidase type 2. *J Eukaryot Microbiol* 50: 597-599

Whittaker SG and Faustman EM (1991). Effects of albendazole and albendazole sulfoxide on cultures of differentiating rodent embryonic cells. *Toxicol Appl Pharmacol* 109: 73-84

Xing JG, Lee LEJ, Fan L, Collodi P, Holt SE and Bols NC (2008). Initiation of a zebrafish blastula cell line on rainbow trout stromal cells and subsequent development under feeder-free conditions into a cell line, ZEB2J. *Zebrafish* 5: 49-63

Zhang H, Huan H, Cali A, Takvorian PM, Feng X, Zhou G and Weiss LM (2005). Investigations into microsporidian methionine aminopeptidase type 2: a therapeutic target for microsporidiosis. *Folia Parasitol* 52: (1-2)189-192

Zhang Y, Yeh JR, Mara A, Ju R, Hines JF, Cirone P, Griesbach HL, Schneider I, Slusarski DC, Holley SA and Crews CM (2006). A chemical and genetic approach to the mode of action of fumagillin. *Chem Biol* 13: 1001-1009

Chapter 5 References

Bromenshenk JJ, Henderson CB, Wick CH, Stanford MF, Zulich AW, Jabbour RE, Deshpande SV, Mccubbin PE, Seccomb RA, Welch PM, Williams T, Firth DR, Skowronski E, Lehmann MM, Billimoria SL, Gress J, Wanner KW and Cramer Jr. RA (2010). Iridovirus and microsporidian linked to honey bee colony decline. *PLoS ONE* 5: (10)e13181

Chen Y, Evans JD, Smith B and Pettis JS (2008). *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. *J Invert Pathol* 97: 186-188

Chen YP and Huang ZY (2010). Nosema ceranae, a newly identified pathogen of *Apis mellifera* in the USA and Asia. *Apidologie* 41: 364-374

Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, Quan P-L, Briese T, Hornig M, Geiser DM, Martinson V, vanEngelsdorp D, Kalkstein AL, Drysdale A, Hui J, Zhai J, Cui L, Hutchision SK, Simons JF, Egholm M, Pettis JS and Lipkin WI (2007). A metagenomic survey of microbes in honey bee colony collapse disorder. *Science* 318: 283-287

Fenoy Z, Rueda C, Higes M, Martin-Hernandez R and Aguila C (2009). High-level resistance of *Nosema ceranae*, a parasite of the honeybee, to temperature and dessication *Appl Environ Microbiol* 75: 6886-6889

Gilliam M and Shimanuki H (1967). In vitro phagocytosis of *Nosema apis* spores by honey-bee hemocytes. *J Invert Pathol* 9: 387-389

Gisder S, Hedtke K, Mockel N, Frielitz M-C, Linde A and Grenersch E (2010a). Five-year cohort study of *Nosema* spp. in Germany: Does climate shape virulence and assertiveness of *Nosema ceranae*. *App Environ Microbiol* 76: (9)3032-3038

Gisder S, Mockel N, Linde A and Genersch E (2010b). A cell culture model for *Nosema ceranae* and *Nosema apis* allows new insights into the life cycle of these important honey bee-pathogenic microsporidia. *Environ Microbiol* doi: 10.1111/j.1462_2920.2010.02346.x

Gupta KS (1964). Cultivation of *Nosema mesnili* Paillot (Microsporidia) *in vitro*. *Curr Sci* 33: 407-408

Hayashibe M and Katohda S (1973). Initiation of budding and chitin-ring. *J Gen Appl Microbiol* 19: 23-39

Higes M, Martin R and Meana A (2006). *Nosema ceranae*, a new microsporidian parasite in honey bees in Europe. *J Invert Pathol* 92: 93-95

Hughes J and McCully ME (1975). The use of an optical brightener in the study of plant structure. *Stain Technol* 30: 319-319

Ishihara R (1969). The life cycle of *Nosema bombycis* as revealed in tissue culture cells of *Bombyx mori*. *J Invert Pathol* 14: (3)316-320

Ishihara R (1968). Growth of Nosema bombycis in primary cell cultures of mammalian and chick embryos. *J Invert Pathol* 11: (2)328

Ishihara R and Sohi SS (1966). Infection of ovarian tissue culture of *Bombyx mori* by *Nosema bombycis* spores. *J Invert Pathol* 8: (4)538-540

Khurad AM, Raina SK and Pandharipande TN (1991). *In vitro* propagation of *Nosema locustae* using fat body cell line derived from *Mythimna convecta* (Lepidoptera: Noctuidae). *J Protozool* 38: 91S-93S

Klee J, Besana AM, Genersch E, Gisder S, Nanetti A, Tam DQ, Chinh TX, Puerta F, Ruz JM, Kryger P, Message D, Hatjina F, Korpela S, Fries I and Paxton RJ (2007). Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. *J Invert Pathol* 96: 1-10

Klein A-M, Vaissière BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C and Tscharntke T (2007). Importance of pollinators in chaging landscapes for world crops. *Proc R Soc B* 274: 303-313

Kurtti TJ, Ross SE, Liu Y and Munderloh UG (1994). *In vitro* developmental biology and spore production in *Nosema furnacalis* (Microspora: Nosematidae). *J Invert Path* 63: 188-196

Kurtti TJ, Tsang KR and Brooks MA (1983). The spread of infection by the microsporidian, *Nosema disstriae*, in insect cell lines. *J Protozool* 30: 652-657

Le Conte Y and Navajas M (2008). Climate change: impact on honey bee populations and diseases. *Rev Sci Tech Off Int Epiz* 27: (2)499-510

Lorand T and Kocsis B (2007). Recent advances in antifungal agents. *Mini Rev Med Chem* 7: 900-911

Maeda H and Ishida N (1967). Specificity of binding of hexopyranosyl polysaccharides with fluorescent brightener. *J Biochem* 62: (276-278)

Martin-Hernandez R, Meana A, Prieto L, Salvador AM, Garrido-Bailon E and Higes M (2007). Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. *Appl Environ Microbiol* 73: 6331-6338

Nageli K (1857). Ueber die neue Krankheit die Seidenraupe und vervandte Organismen. *Bot Zietung* 15: 760-761

Pasteur L (1870) Etude sur la maladie des vers a soie. Paris, France.

Read AF and Taylor LH (2001). The ecology of genetically diverse infections. *Science* 292: 1099-1102

Sagers JB, Munderloh UG and Kurtti TJ (1996). Early events in infection of a *Helicoverpa zea* cell line by *Nosema furnacalis* and *Nosema pyrausta* (Microspora: Nosematidae). *J Invert Pathol* 67: 28-34

Sohi SS and Wilson GG (1976). Persistent infection of Malacosoma disstria (Lepidoptera: Lasiocampidae) cell cultures with Nosema (Glugea) disstrae (Microsporida: Nosematidae). *Can J Zool* 54: 336-342

Trager W (1937). The hatching of spores of *Nosema bombycis* Nageli and the partial development of the organism in tissue culture. *J Parasitol* 23: 226-227

Undeen AH (1975). Growth of *Nosema algerae* in pig kidney cell cultures. *J Protozool* 22: (1)107-110

vanEngelsdorp D and Meixner MD (2010). A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *J Invert Path* 103: S80-S95

Williams GR, Shafer ABA, Rogers REL, Shutler D and Stewart DT (2008). First detection of Nosema ceranae, a microsporidian parasite of European honey bees (*Apis mellifera*), in Canada and Central USA. *J Invert Pathol* 97: 189-192

Xing JG, Lee LEJ, Fan L, Collodi P, Holt SE and Bols NC (2008). Initiation of a zebrafish blastula cell line on rainbow trout stromal cells and subsequent development under feeder-free conditions into a cell line, ZEB2J. *Zebrafish* 5: 49-63

Zander E (1909). Tierische parasiten als krankenheitserreger bei der biene. *Münchener Bienenzeitung* 31: 196-204

Chapter 6 References

Cali A and Takvorian PM (1991). The incidence of *Glugea stephani* (Protozoa: Microsporida) in winter flounder, *Pseudopleuronectes americanus*, from the New York - New Jersey Lower Bay Complex and factors influencing it. *Can J Zool* 69: 317-321

Didier ES (2005). Microsporidiosis: An emerging and opportunisitc infection in humans and animals. *Acta Tropica* 94: 61-76

Didier ES, Stovall ME, Green LC, Brindley PJ, Sestak K and Didier PJ (2004). Epidemiology of microsporidiosis: sources and modes of transmission. *Vet Parasitol* 126: 145-166

Didier ES and Weiss LM (2006). Microsporidiosis: current status. *Cur Op Infect Dis* 19: 485-492

Hashimoto K, Sasaki Y and Takinami K (1976). Conditions for extrusion of the polar filament of the spore of *Plistophora anguillarum,* a microsporidian parasite in *Anguilla japonica*. *Bull Jap Soc Sci Fish* 42: (8)837-845

Health_Canada (2010). Evaluation Report ERC2010-06, *Nosema* (*Paranosema*) *locustae* Canning. *Consumer Product Safety* www.hc-sc.gc.ca/cps-spc/pubs/pest/_decisions/erc2010-2006/index-eng.php

Rodriguez-Tovar LE, Speare DJ and Markham RJF (2011). Fish microsporidia: Immune response, immunomodulation and vaccination. *Fish & Shellfish Immunol* 30: 999-1006

Rodriguez-Tovar LE, Wright GM, Wadowski D, Speare D and Markham R (2002). Ultrastructural study of the early development and localization of *Loma salmonae* in the gills of experimentally infected rainbow trout. *J Parasitol* 88: 244-253

Sanchez JG, Speare DJ and Markham RJF (2000). Normal and aberrant tissue distribution of *Loma salmonae* (Microspora) within rainbow trout, *Oncorhynchus mykiss* (Walbaum), following experimental infection at water temperatures within and outside of the xenoma-expression temperature boundaries. *J Fish Dis* 23: 235-242

Shaw RW, Kent ML and Adamson ML (2001). Phagocytosis of *Loma salmonae* (Microsporidia) spores in Atlantic salmon (*Salmo salar)*, a resistant host, and chinook salmon (*Oncorhynchus tshawytscha*), a susceptible host. *Fish Shellfish Immunol* 11: 91-100

Sokolova YY, Dolgikh VV, Morzhina EV, Nassonova ES, Issi IV, Terry RS, Ironside JE, Smith JE and Vossbrinck CR (2003). Establishment of the new genus *Paranosema* based on the ultrastructure and molecular phylogeny of the type species *Paranosema grylli* Gen. Nov. comb. Nov. (Sokolova, Selezniov, Dolgikh, Issi 1994), from the cricket *Gryllus bimaculatus* Deg. *J Invert Pathol* 84: 159-172

Speare DJ, Markham RJF and Guselle NJ (2007). Development of an effective whole-spore vaccine to protect against microsporidial gill disease in rainbow trout (*Oncorhynchus mykiss*) by using a low-virulence strain of *Loma salmonae*. *Clin Vac Immunol* 14: (12)1652-1654

Sung W and Lee D (2008). Pleurocidin-derived antifungal peptides with selective membrane-disruption effect. *Biochem Biophys Res Com* 369: (3)858-861

Syvitski R, Burton I, Mattatall N, Douglas S and Jakeman D (2005). Structural characterization of the antimicrobial peptide pleurocidin from winter flounder. *Biochem* 44: (19)7282-7293

Takvorian PM and Cali A (1984). Seasonal prevalence of the microsporidan, *Glugea stephani* (Hagenmuller), in winter flounder, *Pseudopleuronectes americanus* (Walbaum), from the New York-New Jersey Lower Bay Complex. *J Fish Biol* 24: 655-663

Undeen AH (1983). The germination of *Vávraia culicis*. *J Protozool* 30: 274-277

Undeen AH and Avery SW (1988). Effect of anions on the germination of *Nosema algerae* (Microspora: Nosematidae) spores. *J Invert Path* 52: 84-89

Weidner E and Sibley LD (1985). Phagocytized intracellular microsporidian block phagosome acidification and phagosome-lysosome fusion. *J Protozool* 32: 311-317

Wongtavatchai J, Conrad PA and Hedrick RP (1994). *In vitro* cultivation of the microsporidian: *Enterocytozoon salmonis* using a newly developed medium for salmonid lymphocytes. *J Tiss Cult Meth* 16: 125-131

Wongtavatchai J, Conrad PA and Hedrick RP (1995). *In vitro* characteristics of the microsporidian: *Enterocytozoon salmonis*. *J Eukaryot Microbiol* 42: (4)401-405

Appendices

Appendix A: Table of Microsporidia Genera in Fish

(Based on Lom 2002)

	Genus		Examples of Reported Infection Sites
Xenoma Formation	*Glugea*	Thelohan, 1891	Connective tissue of body organs, intestinal wall, gall bladder, mesentery, ovary, testes, subcutaneous tissue
	Ichthyosporidium	Caullery & Mesnil, 1905	Liver, adipose, subcutaneous connective tissues
	Loma	Sprague, 1981	Gill, digestive tract, organs
	Microfilum	Faye, Toguebay & Bouix, 1991	Vessels of gill secondary lamellae
	Microgemma	Ralphs & Mathews, 1986	Liver
	Nosemoides	Faye, Toguebay & Bouix, 1996	Gills, digestive tract
	Pseudoloma	Mathews, Brown, Larison, Bishop-Stewart & Kent, 2001	Central nervous system
	Spraguae	Sprague and Vavra, 1976	Ganglion cells of the central nervous system
	Tetramicra	Mathews and Mathews, 1980	Skeletal muscle tissue
Absence of Xenoma Formation	*Heterosporis*	Schubert, 1969	Skeletal muscle tissue
	Kabatana	Lom, Dykova & Tonguthae, 2000	Trunk muscles, heart and other muscles in body
	Ovipleistophora	Pekkarinnen, Lom & Nilsen, 2002	Oocytes
	Nucleospora	Hedrick, Groff & Baxa, 1991	Haematopetic cells, kidney, enterocytes
	Pleistophora	Gurley, 1893	Skeletal muscles, muscle of swim bladder and stomach, gill operula, intestinal walls, mesentery

Reference

Lom J (2002). A catalogue of described genera and species of microsporidians parasitic in fish. *Syst Parasitol* 53: 81-99

Appendix B: Video of *Anncaliia algerae* growth and germination in a rainbow trout cell line, RTgill

This appendix is comprised of two QuickTime™ movie files of *Anncaliia algerae* development and germination within a single cell from the RTgill cell line. During one video, *A. algerae* can be observed to divide within the cell, and 2 mature spores germinate. The second video is a short section of the first video in which intracellular germination is highlighted.

Riveal microscopy from Quorum Technologies Inc. was used for this video. For more information on Riveal microscopy, see Chapter 3 of this thesis or visit www.quorumtechnologies.com .

The file is available on the supplementary DVD that is included in the pocket inside the back cover. The file name of full movie file is "*Anncaliia algerae* growth and germination in RTgill.mov" and the file name for the section of video that highlights intracellular germination is "*Anncaliia algerae* germination in RTgill.mov"

If you accessed this thesis from a source other than the University of Waterloo, you may not have access to this file. You may access it by searching for this thesis at http://uwspace.uwaterloo.ca .

Appendix C: Video of *Anncaliia algerae* spore misfires

This appendix is a QuickTime™ movie file of incomplete germination of *Anncaliia algerae* spores, or spore misfires. Figure 3.7 images are taken from this video, but the video provide clearer evidence of germinated spores that did not discharge their nuclei.

Riveal microscopy from Quorum Technologies Inc. was used for this video. For more information on Riveal microscopy, see Chapter 3 of this thesis or visit www.quorumtechnologies.com .

The file name of this movie file is “Anncaliia algerae spore misfires.mov”.

If you accessed this thesis from a source other than the University of Waterloo, you may not have access to this file. You may access it by searching for this thesis at http://uwspace.uwaterloo.ca .