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In vitro Study of the Microsporidian Parasite Loma morhua, Using Cod-derived Cells and Novel Culture Techniques

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By

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Abstract

The cod populations of the Canadian Atlantic were once highly productive, generating enormous annual harvests and attracting fishing fleets from many nations. However, through the late 1980s improved fishing technology, unprecedented capture rates, and poor fishery management brought wild stocks beyond the point of collapse. Dwindling harvests in the early 1990s resulted in cod fishing moratoriums, and an end to the productivity for which the fishery was once renowned. Atlantic cod remains a popular food worldwide and the collapse of cod fisheries has done little to abate market demands. Consequently, the cod is considered a prime candidate for aquaculture production, providing the impetus for commercial-scale farming operations. As aquaculture efforts continue to grow, disease management challenges have become a prominent concern. Many parasites are prevalent on cod farms, including intracellular pathogens such as viruses and microsporidians. Detailed research into diseases affecting farming operations is imperative if commercial-scale cod aquaculture is to develop.

Piscine cell culture techniques represent a valuable tool for studying the intracellular pathogens currently impeding cod aquaculture. To date however, few cell culture models have been made available for the Atlantic cod. This research details the establishment of a larval cod cell line, GML-5, investigations of infective processes in microsporidian parasites, and development of *in vitro* culture methods for a microsporidian parasite of the Atlantic cod.

GML-5 cells have been cultured for two years and survived more than 26 passages in L-15 media supplemented with 10% fetal bovine serum and incubated at 18°C. The cells have

tested positive for a marker of stem cell-like characteristics, had their origin identified as *Gadus morhua* by DNA barcoding, and been cryopreserved for long-term storage. The cells have been successfully used to support the growth of two microsporidian parasites. Infection-mediating effects of Mg^{2+} and EDTA have been confirmed in a previously-untested microsporidian species and novel pH treatments were successfully used to stimulate infection and development of *Loma morhua* in GML-5 cells.

The results of this research represent the foundations of an *in vitro* infection model for *Loma morhua*, and demonstrate shared responses to specific chemical conditions by microsporidian parasites with highly dissimilar host species.

Acknowledgements

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List of Abbreviations

AB Alamar blue

AIDS Acquired immune deficiency syndrome

ATCC American Type Culture Collection

CCAR Centre for Cooperative Aquaculture Research

DNA Deoxyribonucleic acid

DTT Dithiothretol

EDTA Ethylenediaminetetraacetic acid

FBS Fetal Bovine Serum

GAG Glycosoaminoglycan

GFSk-S1 Goldfish skin cell line

GML-5 *Gadus morhua* larvae trial 5

GS Germinated spore

IEC International Equipment Co.

IEF Isoelectric focusing

IPG Immobilized pH gradient

IPN Infectious pancreatic necrosis

IS Infective spore

ICS Intracellular spore

kDa Kilodaltons

L-15 Leibovitz's L-15 growth medium

L-15 ex Simple exposure media

M Meront

MDIBL Mount Desert Island Biological Laboratories

MEM Minimum essential medium

MS Mature spore

PBS Phosphate buffered saline

PF Polar filament

PI Post inoculation

PL Phagolysosome

PS Penicillin-streptomycin

PP Polaroplast

PSV Posterior vacuole

PV Parasitophorous vacuole

RCF Relative centrifugal force

RFU Relative fluorescence unit

RTgill-W1 Rainbow trout gill cell line

S Sporont

SB Sporoblast

SDS Sodium dodecyl sulphate

SP Sporoplasm

SSW Sterile seawater

VHS Viral hemorrhagic septicemia

VNN Viral nervous necrosis

Zeb 2J Zebrafish embryo cell line

Ch. 1: Introduction to Cod Fisheries, Aquaculture, and the Microsporidia

Fisheries and the Atlantic Cod

The human species has a long-standing history of utilizing fish as a reliable food source. Accessible with relatively little effort and abundant in most environments suitable for human habitation, fish have been exploited by people worldwide, with evidence of fishing activity dating back to the Stone Age (Rau, 1884). Due to small populations and limited technology, early harvesting pressures were kept relatively low and fish populations were able to remain stable.

As agriculture developed and human societies became increasingly settled, knowledge and equipment improved as well, begetting a feed-forward cycle of increasing populations and technological development. Greater numbers of people require greater quantities of food, and the tools for harvesting fish improved as civilizations developed. Better hooks, larger nets, and more sea-worthy vessels combined to allow larger catches and the exploitation of species formerly out of reach. Today, fishing takes place on an industrial scale, where catches of commercially-valuable species are measured in tonnes rather than individuals and many coastal economies are dependent on large annual harvests. Perhaps inevitably, some species have become more economically important and entire fishing industries have been built around them. Familiar examples of intensively-harvested fish species include a variety of scombrid and

salmonid species, but—in Europe and the Americas at least—none are better known than the Atlantic cod (*Gadus morhua*, Linnaeus, 1758).

A large Gadid species, *G. morhua* may be found along the continental shelf of all temperate and cool regions of the northern Atlantic ocean. Owing to the species' wide geographic distribution, tendency to form large schools, and the palatable flavour of its flesh, cod has become a favoured food fish worldwide. As a result, *G. morhua* is a highly sought-after species and many nations harvest the fish from northern Atlantic waters.

The targeted fishing of cod populations has a long history. The discovery of enormous populations along the Canadian coast fuelled the formation of large fishing fleets, long-distance expeditions, and, in some cases, even international conflict. The fisheries of Newfoundland's Grand Banks have served as a particularly rich harvesting ground for Atlantic cod for several centuries, extending back to John Cabot's expedition to the new world in 1497. Many countries, including England, France, Spain, and Portugal came to fish these waters, placing a sustained burden on wild stocks. Due to limitations in seafaring technology, large travelling distances, and the high reproductive capacity of the cod itself, populations remained stable and a ready supply of fish was naturally maintained early on. However, with the advent of modern fishing technologies, harvesting pressures have surpassed the recuperative abilities of wild populations and formerly rich fisheries are in a state of crisis. In a pattern that has become familiar in coastal regions around the globe, cod stocks along Newfoundland's Grand Banks have exhibited a dramatic response to continual over-harvesting, providing a stark example of the fallout that can be caused by poor fisheries management.

Collapse

Once a highly productive and reliable fishery, cod stocks along Canada's Atlantic coast have been pushed past the point of collapse. Due to a combination of enhanced vessels, detection technology, and capture methods, North American cod harvests rose sharply during the 1970s, reaching historic highs of nearly 616,000 tonnes in 1982 (FAO, 2011) and continuing for the remainder of the decade. The rich fishing, as in centuries before, seemed boundless and many nations launched fleets to harvest the productive Canadian waters. Unfortunately, as is often the case with sharp rises in prosperity, the enormous catches of the 1980s could not be sustained. Cod landings in the early 1990s were marked by precipitous declines, falling to 187,963 tonnes in 1992 (Figure 1.1). The decline in annual harvests proved too dramatic to go unnoticed. In 1992 the Canadian government placed a moratorium on harvesting cod from northern populations, followed in 1994 by a moratorium on cod fishing in the Grand Banks.

It was hoped that the halt on cod fishing would bring about a rapid recovery and that normal fishing practices could soon be resumed (Lily, 2008). Unfortunately, this outlook proved to be too optimistic and cod populations have not rebounded as predicted. A similar—if less dramatic—pattern has been observed in cod capture rates worldwide (Figure 1.2; FAO, 2011).

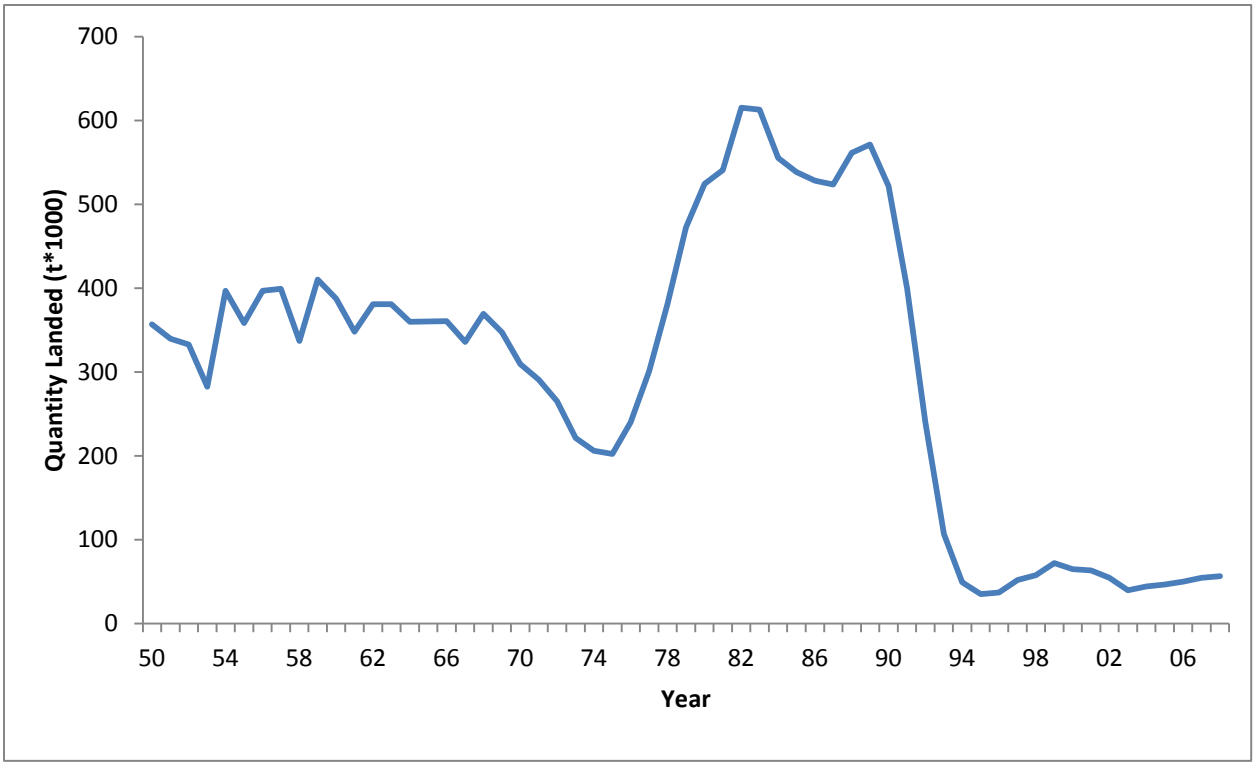

Figure 1.1: Reported annual North American landings of wild Atlantic cod (*Gadus morha*) from 1950 to 2008, showing severe decline in annual catches around 1990. Catches displayed in thousands of tonnes. Data from FAO Fishery Statistical Collections: http://www.fao.org/fishery/statistics/programme/3,1,1/en.

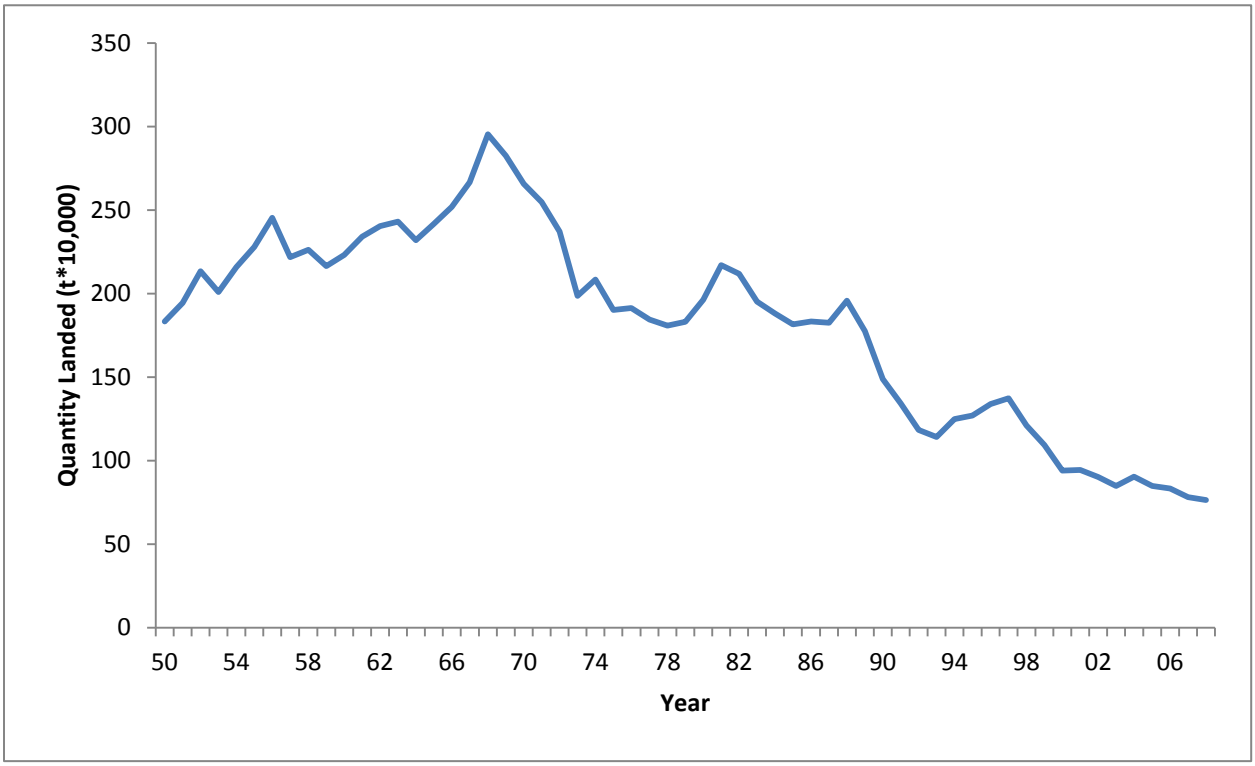

Figure 1.2: Reported cod landings worldwide from 1950 to 2008, in tonnes*10,000, showing a steady downward trend from the late 1960s onward. Data from FAO Fishery Statistical Collections: http://www.fao.org/fishery/statistics/programme/3,1,1/en.

Numerous explanations have been posed for the remaining cod population's apparent inability to recover. Possible causes include ecological changes, such as altered trophic arrangements due to the loss of an apex predator (the cod), and persistent predation pressure exerted on remaining cod populations by both wildlife and continued fishing. Populations are currently exhibiting reduced larval survival and recruitment rates (Brander, 2010) and a decline in phenotypic diversity in surviving stock (Olsen *et al*., 2009). Existing studies suggest that high predation intensity —natural or otherwise— can result in reduced age of sexual maturity and smaller adult sizes (Reznick and Endler 1982; Olsen and Moland, 2011), which is associated with the production of fewer eggs and lower average egg quality (reviewed in Merrett, 1994).

Environmental aspects may also be to blame, as changes in average ocean temperatures to those outside of the cod's thermal optimum (Brander, 2010) and increasing industrial activity (Balk *et al*., 2011) may have deleterious effects on any remaining fish.

As is often the case, many of the explanations offered for the current status of cod populations are not mutually exclusive, and the failure to recover may represent a combination of factors (reviewed in Hutchings and Rangely, 2011). For the time being, it would seem that the re-establishment of cod stocks along the Grand Banks and Atlantic shelf will require both tighter regulations and the use of management strategies that address the more subtle factors confounding cod recovery. Nevertheless, population changes will not occur rapidly and human demands for cod meat will likely outstrip the available supply for some time. To address this need, aquaculture represents one of the most viable methods of assuring a ready supply of cod for human consumption in the years to come.

Aquaculture

Aquaculture—the practice of farming marine and freshwater aquatic organisms—has a history dating as far back as 2000 B.C.E. Beginning in China with the common carp (*Cyprinus carpio*), early fish farming practices were likely the result of increasingly settled human populations and, consequently, heavy burdens on food production capacity (Costa-Pierce, 1987). The earliest written text on aquacultural practices was produced in 500 B.C.E. in a work detailing basic carp husbandry for commercial purposes (Rabanal, 1988). Efforts with carp continued for some time, expanding to different species, polyculture systems, and domestic varieties. As human populations expanded and trade became better developed, aquaculture

technology spread to other countries, producing the foundations for fish farming as we know it today.

Modern fish farming has expanded to meet the needs of a continually growing global population and now encompasses a wide variety of species. While the basic principles of rearing fish in controlled bodies of water remain largely intact, the fine details have advanced dramatically. Broodstock maturation, gamete production and out-of-season spawning are now induced by manipulations in photoperiod (Guerrero-Tortolero *et* al., 2008; Almeida *et al*., 2009), temperature (de Lapeyre *et al*., 2009), and nutritional status of livestock (Izquierdo *et al*., 2001). Larval and neonatal survival has been greatly enhanced by specialized maintenance regimes and improved feeding strategies (reviewed in Rosenlund and Halldórsson, 2007). Production capacity has been improved by advancements in water quality management (reviewed in Crab *et al*., 2007; Matos *et al*., 2009). The increase in farmed fish biomass is so great that approximately 50 per cent of seafood consumed today has been artificially raised. Aquaculture is now the fastest growing animal-producing agricultural practice in the world (FAO, 2010a), generating $801 million in revenue for Canada alone in 2010 (DFO, 2011).

Basic cod farming was initiated in the 1880s in the form of captive-hatched fry raised from fertilized eggs in artificial enclosures (Dannevig, 1884). In 1983, methods for rearing juvenile cod were established by Norwegian scientists using seawater enclosures (Øiestad, 1985). The advancements in juvenile rearing methods—often the limiting factor in production for a given species—spurred early attempts at commercial-scale operations. Unfortunately, these initial ventures did not prove profitable. Cod farming was left largely untouched until the

early 2000s, when commercial cod production was pursued again using lessons learned from the farming of other species such as sea bass (*Dicentrarchus labrax*). Norway saw the greatest investment in cod aquaculture, but programs were also established in the United Kingdom, United States of America, Canada, and Iceland. More recent efforts have proven both productive (Figure 1.3) and profitable. Current prospects for greater production are good, but the global financial crisis, coupled with disease management problems has limited the success achieved since 2008, and cod farms are currently being consolidated (FAO, 2010b).

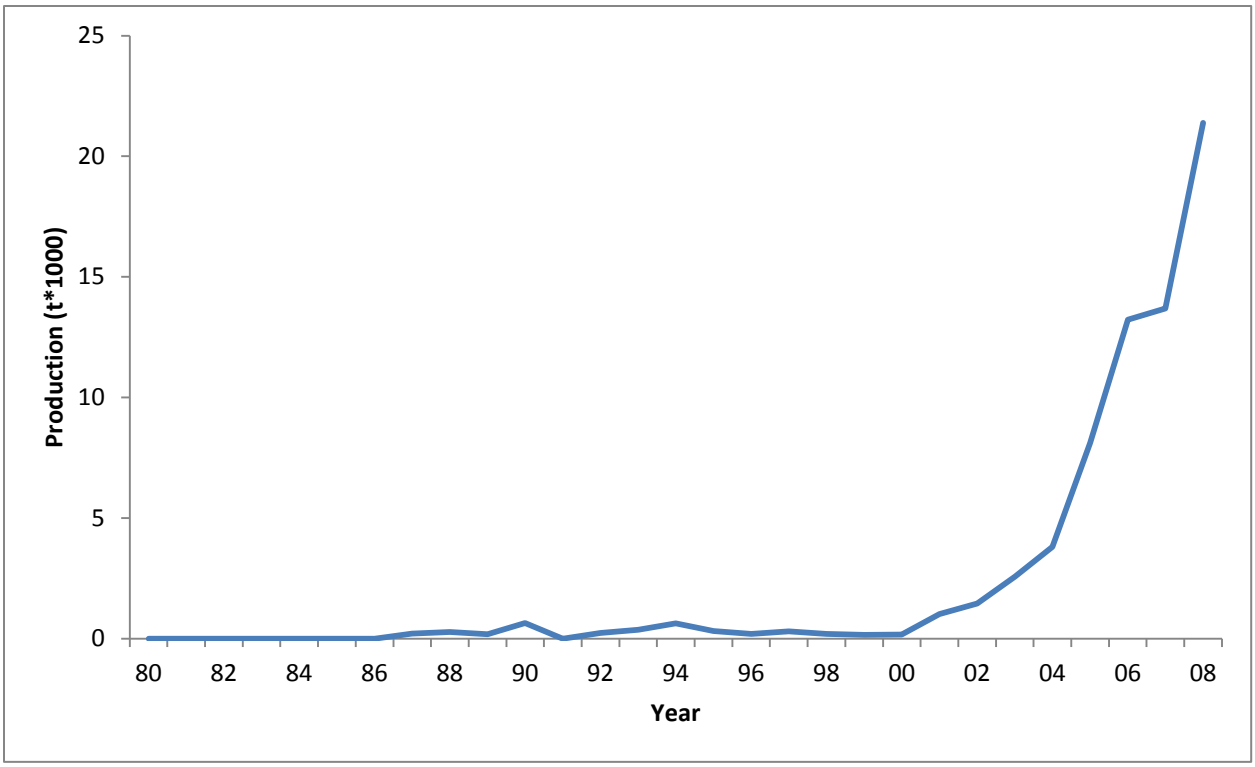

Figure 1.3: Reported worldwide aquaculture production of Atlantic cod (*Gadus morhua*) between 1980 and 2008 in thousands of tonnes. Data from FAO Fishery Statistical Collections: http://www.fao.org/fishery/statistics/programme/3,1,1/en.

Parasites and Disease

Disease and pathogen management is one of the major problems currently facing cod farms (Sung *et al*., 2011), and fish pathogens are responsible for the majority of mortalities incurred beyond larval stages. Piscine veterinary medicine is largely undeveloped and many aquatic diseases have no specific treatment (Burka. In the event of an outbreak at an aquaculture facility, the recommended approach typically involves formalin baths, isolation or culling, and improving water quality. Intensive aquaculture programs typically rely on extremely high stocking densities to maintain suitable yields, which can compound the difficulties inherent to disease management in fish and result in sub-optimal production (Glasser and Oswald, 2001; reviewed in Lawrence, 2007). Several factors play a role in enhancing the susceptibility to, and damage caused by, diseases in fish farming operations. First, the high stocking densities typical of aquaculture farms results in extremely close proximity between fish, greatly enhancing the physical spread of pathogens between livestock (Pulkkinen *et al*., 2010) and decreasing immune function (Suomalainen *et al*., 2005). Second, livestock stress levels, even in social species, are known to elevate beyond normal levels in fish maintained at higher-than-natural schooling densities (Vijayan and Leatherland, 1990; Santos *et al*., 2010). Lastly, more fish produce more waste, which, combined with the low water volume of most systems, can result in poor water quality and increased vulnerability to disease.

A variety of pathogens are responsible for ongoing problems maintaining livestock health and profitability in cod farms. In the majority of cases, comprehensive treatments do not exist and risk management is the only approach available to reduce the incidence and spread of disease. Bacterial infection is responsible for a large proportion of the difficulties facing large-scale production of adult cod, but viral, fungal, and macroparasite pathogens are commonly encountered as well.

As cod farming practices have developed and expanded, several pathogens have proven particularly problematic. Viral infections such as infectious pancreatic necrosis (IPN), viral nervous necrosis (VNN), and viral hemorrhagic septicemia (VHS) have caused considerable losses in all cod-producing countries (reviewed in Samuelsen *et al*., 2006). Norway and Iceland have ongoing battles with a bacterial disease caused by *Listonella anguillarum* (Samuelsen *et al*., 2006), while *Aeromonas salmonicida* ssp. *archomogenes* (Cornick *et al*., 1984) and *Francisella noatunensis* (Ellingsen *et al*., 2011) are emerging concerns. Among animal parasites, sea lice such as the gadoid-specific *Caligus curtis* (Hamre *et al*., 2011), and nematodes (MacKenzie *et al*., 2009) have been identified as potential threats. Very few specific treatments exist for cod-infecting pathogens, and continuing efforts will be needed to ensure the success of cod farming operations in the future. To date the only vaccinations available are for listonellosis (Caipang *et al*., 2008) and furunculosis (Lund *et al*., 2008). Additionally, as each threat is identified and dealt with, new pathogens will emerge, such as the increasingly problematic microsporidian parasite *Loma morhua*.

Microsporidia

The microsporidia are a unique group of uni-cellular obligate intracellular parasites. They are observed as free-living, mature spores of approximately 1-4µm in length and characterized by a protective chitinous exospore containing a polaroplast, posterior vacuole, and coiled polar filament that transfers the spore contents (sporoplasm) into the host-cell cytoplasm (reviewed in Texier *et al*., 2010). Existing freely in a variety of environments, spores exhibit high resistance to extremely low temperatures (Koudela *et al*., 1999), dessication (Waller, 1979), and a range of pH values (Shadduck and Polley, 1978). Consequently, spores can remain viable for time periods ranging from months to years in various environments (Kramer, 1970) and may be considered ubiquitous.

Microsporidia infect host cells by a germination process in which the polar filament is everted from inside the spore, and penetrates the host's cell membrane. The spore subsequently extrudes the sporoplasm into the host cytoplasm. The sporoplasm then undergoes repeated division, creating multiple meronts. This is followed by sporogony, producing sporonts, which further divide into sporoblasts. Sporoblasts subsequently develop into mature spores that are released into the surrounding environment by host cell rupture (Figure 1.4). Entry to host cells may be initiated from outside the host membrane or by phagocytic uptake (Franzen *et al*., 2005), but ultimately it appears to depend on specific triggers preceding germination. The germination process seems to be mediated by chemical conditions specific to suitable hosts (reviewed in Keohane and Weiss, 1998), such as pH shifts between the stomach and intestine or chemical changes in the phagosome (Franzen, 2004). Stimulation of

spore activation and germination by such specific conditions likely serves an adaptive function by ensuring that infective processes are initiated only when in close proximity to a suitable host. Given the 50-100µm length of a spore's polar filament (Xu and Wiess, 2005), host cell-detection prior to germination would greatly increase the probability of successful infection.

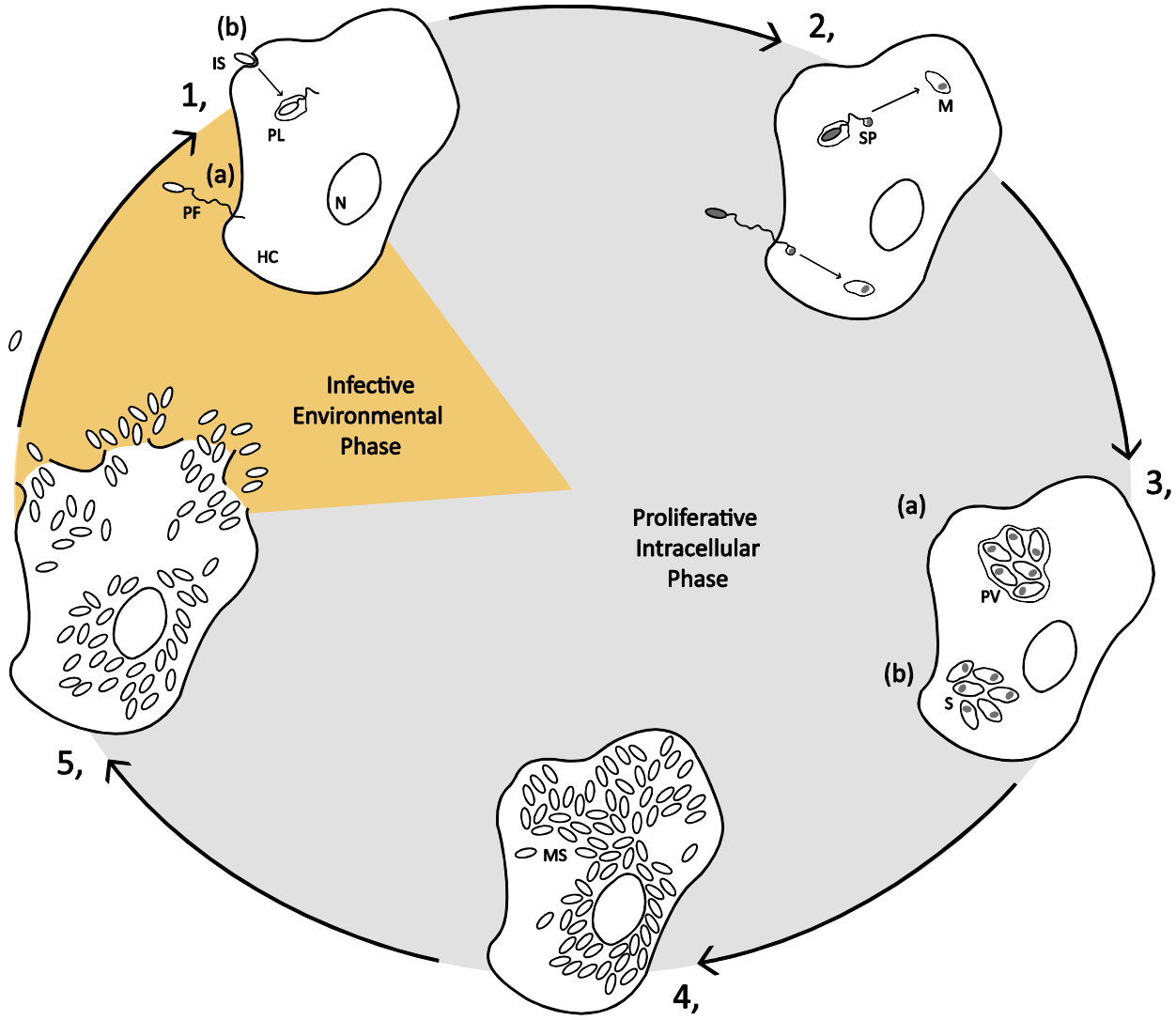

Figure 1.4: Generalized life cycle schematic for microsporidian infection. 1) Infective spores (IS) detect host cells (HC) and pierce the host membrane with a polar filament (PF). This may occur from outside the cell (a), or from within a phagolysosome following phagocytic uptake (b). 2) Spore contents (sporoplasm, SP) are extruded into host cell cytoplasm where merogeny occurs, producing meronts (M). 3) Spore proliferative development into sporonts (S) may occur within (a) a parasitophorous vacuole (PV) derived from host membrane or (b) in direct contact with host cell cytoplasm. 4) Proliferation continues and sporonts metamorphose into mature spores (MS) that eventually fill host cytoplasm. 5) Subsequent death of host cell releases mature infective spores into the environment. N – host cell nucleus. Image © Mike MacLeod, 2012.

A large body of scientific efforts have contributed to our understanding of the biochemistry and mechanics of spore germination, yielding insights into specific events preceding infection, the activation of the polar filament, and the infection-modulating effects of various chemical conditions. The foundation for our current understanding is the culmination of more than three decades of research, establishing the polymeric protein construction of the polar filament (Weidner, 1976) and the importance of specific environmental triggers in activating the discharge process (Pleshinger and Weidner, 1985). The initiation of germination has been found to rely on increases in osmotic pressure and subsequent polaroplast swelling, which may be achieved by a variety of means, such as the uptake of calcium ions (Weidner and Byrd, 1982) or breakdown of internal trehalose sugars to glucose (Undeen and Vander Meer, 1999). More recent efforts have uncovered the role of spore adhesion to host cells prior to activation, involving cell surface sulfated proteoglycans (Hayman *et al*., 2005) and modulation by divalent cations such as Mg^{2+} and Mn^{2+} (Southern *et al*., 2006). As the basic understanding of the microsporida and microsporidosis was established, attention was directed towards more specific questions of microsporidian genetics and proteomics.

Molecular work on microsporidian parasites is still in its early stages, but great strides are being made in this area, supporting earlier molecular work and laying the foundations for continuing efforts. As intracellular parasites, the microsporidia have extremely limited genomes, including some of the smallest known for eukaryotes at 2.3 mega base pairs (Peyretaillade *et al*., 2011). The extremely limited size of microsporidian genomes has been achieved by not only the reduction of gene-coding regions, but also compaction—the microsporidian genome possesses the greatest gene density of any sequenced eukaryotic

organism (reviewed in Keeling and Slamovits, 2004). Consequently, the parasites possess extremely limited proteomes (reviewed in Texier *et al*., 2005) and, interestingly, shortened protein sequences (Katinka *et al*., 2001). The restricted genome and proteome of the microsporidia has the potential benefit of reducing the complexity of identifying proteins with probable roles in the infection process. Early efforts have seen the construction of a whole-spore protein reference map for *Encephalitozoon cuniculi* (Brosson *et al*., 2006), as well as the identification of several spore wall proteins (Li *et al*., 2012) and an improved understanding of phylogenetic relationships through genetic research (Lom and Nilson, 2003). Despite great strides in our understanding of the parasite, many details remain unknown and microsporidosis continues to cause health complications in humans and livestock.

Microsporidosis in Aquaculture

Nearly all invertebrate and most vertebrate species are susceptible to infection by one or more microsporidian species (reviewed in Wasson and Pepper, 2000; Didier *et* al., 2004; Williams, 2009). Fish seem to be particularly prone to microsporidian infection and are the definitive host for a large number of known vertebrate-infecting microsporidia (reviewed in Rodriquez-Tovar *et al*., 2011). Host-parasite pairs can be found in a wide variety of aquatic habitats, many of which have only recently been identified (Abdel-Ghaffar *et al*., 2009; Brown *et al*., 2010). Despite considerable variability in the virulence and host-specificity exhibited by different microsporidian species, most commercially valuable fish species seem to be susceptible to infection and microsporidosis has proven to be problematic for the reliable production of many species of farmed fish.

Microsporidosis typically causes chronic health problems rather than acute illness and rapid mortality. As a result, microsporidia were not identified as being particularly problematic prior to the advent of intensive aquaculture practices. Microsporidian parasites have now been identified in all major branches of aquaculture, affecting species used for ornamental trade, scientific research, and food production. In most cases outbreaks are persistent and difficult to contain, leading to slow growth, low but chronic death rates, and sub-optimal production levels. Consequently, initial infections may not be identified as a critical threat, but substantial losses can be incurred if the outbreak is left unchecked.

A range of microsporidian species have caused difficulties in maintaining the good health of livestock on fish farms. In the case of ornamental fish, *Pleistophora hyphessobryconis* infects characid species popular in home aquariums (Michel *et al*., 2002; Hongslo and Jansson, 2009). *Pseudoloma neurophilia* infects the scientifically valuable zebrafish (*Danio rerio*) (Ramsay *et al*., 2009). Fish farms producing food for human populations are affected by a multitude of microsporidia species, with many more being discovered as aquaculture practices expand to new fish species. Among commercially valuable food species, microsporidia of the genus *Loma* are particularly problematic, with many identified host-parasite pairs. Affected species include the Atlantic haddock (host to *L. branchialis,* Brown *et al*., 2010), the pollock (*L. wallae*, Brown *et al*., 2010), salmonids of the genus *Oncorhynchus* (*L. salmonae*, Bruno *et al*., 1995), and the Atlantic cod (*L. morhua*, Morrison, 1983).

An attribute shared amongst infections caused by different species of microsporidia is the difficulty in effectively treating afflicted animals. Treatments are often ineffective or fatal to

hosts. Despite research into vaccinations for lomosis (Speare *et al*., 2007) and the antimycotic fumagillin (Higes *et al*., 2011), the most comprehensive approach currently available is a program of minimizing potential exposure by vigilant monitoring and culling of infected fish. Given the value and relative scarcity of farmed livestock, *in vivo* investigations into improved treatment methods—though desirable—are not always feasible given the number of animals that would need to be sacrificed. *In vitro* methodologies represent a potential avenue for research into new approaches for managing microsporidosis on fish farms while simultaneously limiting the unnecessary use of livestock for purposes other than food production.

Cell Culture

In vitro cell culture—the practice of growing eukaryotic cells under controlled laboratory conditions—is a relatively new technology with a short but productive history. The basic principles of tissue culture were first established in the early 1900s in an elegant set of experiments that allowed observations of living nerve fibres derived from frogs (Harrison, 1907), which were used to evaluate then-current questions regarding the origins of nerve fibres (Harrison, 1910). The culture system developed by Harrison utilized tissues dissected from frogs in frog lymph that were suspended on a sterile cover slip sealed over a glass depression slide, essentially creating a hanging drop culture. While somewhat less advanced than the methods currently employed, Harrison's work has much in common with cell culture today: the ability to make real time observations of living cells using specialized growth media, culture vessels, and aseptic technique.

As is frequently the case with the development of novel research technologies, the establishment of tissue culture methods proved useful for many lines of research, spurring continued advancements and enhanced reliability. The new technology was employed by researchers in a variety of fields throughout the early 1900s, but it wasn't until the 1940s that it grew to resemble cell culture as it is today. Virology research conducted at the time provided the driving force for enhancing cell culture practices, as well as one of its most widely-known products: the polio vaccine (Robbins *et al*., 1950). Modern cell culture practices have expanded considerably from these early efforts and existing cell lines now represent a diverse array of animal groups, including insects, mammals, amphibians, and fish.

Piscine-specific cell culture did not develop until well after the principles of *in vitro* tissue cultivation were established, with the first cell line derived from teleost tissues reported in the 1960s (Wolf and Quimby, 1962). Subsequent efforts have yielded cell lines from many different species of fish, representing most taxa and a variety of aquatic environments. The production of cultures derived from aquatic organisms has allowed *in vitro* methods to be utilized for research that traditional mammalian cell models would be poorly-suited to address, including aquatic toxicology (Saito *et al*., 2002), fish physiology (Hightower and Renfro, 1988), and piscine parasites (Woo and Li, 1990). Due to the increasing burden placed on fish farm production, as well as dwindling wild stocks, research using otherwise marketable live fish can be impractical and costly. Consequently, cell culture techniques represent valuable tools for researching the maintenance of good health in aquatic livestock at lower expense, with the added benefit of simultaneously reducing the number of animals needed for experimentation.

Microsporidia Culture

A unique benefit of cell culture technology is the ability to view living cells singly and in real time, permitting specific observations that would be difficult or impossible *in vivo*. This is particularly beneficial for the study of intracellular parasites, a wide variety of which have been studied using *in vitro* techniques. Initially, cell culture was utilized solely for virology research, but this has expanded into research on clinically and commercially important parasites including *Plasmodium* (Moneriz *et al*., 2011), *Chlamydia* (Amirshahi *et al*., 2011), and the microsporidia (Monaghan *et al*., 2009; Gisder *et al*., 2011).

The greatest benefit of *in vitro* culture of microsporidian parasites is the ability to produce spores in large numbers without the difficulties associated with obtaining spores from living animals (such as capture, dissection, and removal of xenomas). The challenges involved in obtaining sufficient quantities of spores from non-cultured species often make scientific experimentation prohibitively difficult. By producing the parasites in a laboratory setting, numerous avenues of research are opened, facilitating a greater understanding of the disease and insights into its treatment.

The utility of cell culture technologies for microsporidian investigations has provided the impetus for research into culture methods for a variety of species. The first infection model using microsporidian spores co-cultured with host-cell cultures was established in 1937, through the use of a silkworm derived cell culture to support the growth of the insect-infecting microsporidian *Nosema bombycis* (Trager, 1937). Since this early effort, additional infection models have been produced, representing a growing number of microsporidian parasites

(reviewed in Monaghan *et al*., 2009). It appears that the majority of research conducted to date has focused on arthropod- and mammal-infecting microsporidia (Chen *et al.*, 2009; reviewed in Visvesvara, 2002). This can be explained in part by the relatively limited number of available piscine cell lines. A more critical factor, however, is the larger importance of microsporidosis in clinical settings and the losses incurred by well-established farming industries such as apiculture and sericulture. Of well-studied microsporidian parasites, the mammal-infecting microsporidia of the genus *Encephalitozoon* are common opportunistic pathogens in AIDS patients, while *Nosema bombycis* and *N. apis* infect silkworms and honeybees, respectively (Bhat *et al*., 2009; Klee *et al*., 2005).

Ultimately, a great deal remains unknown about the nature of microsporidia and microsporidosis. The development of culture methods for new species has the potential to yield new insights into microsporidian parasites, including their basic biology, host parasite interactions, proteomics, and potential treatments—all without the effort and expense associated with securing parasites from living hosts, and difficulties associated with rearing pathogens in tightly controlled quarantine facilities.

Building an Infection Model

As aquaculture programs continue to develop, basic research into fish pathogens, including the microsporidia, will become increasingly important. This can be facilitated in part by the development of *in vitro* infection models for commercially important parasites such as the microsporidian parasite *Loma morhua*. Of specific concern for cod aquaculture, the *Loma* parasite has been identified in both North American and Icelandic cod farms. Due to the

widespread incidence of microsporidosis, the difficulty in managing outbreaks, and considerable losses that can be incurred, continuing research into the disease is urgently needed.

As culture methods had not been established for *Loma morhua* prior to the initiation of this project, the formation of an *in vitro* cod-*Loma* infection model required several areas of focus. This included the creation of a stable cell line derived from the tissues of the Atlantic cod, and the development of methods to stimulate infection and growth of the *Loma* parasite in laboratory culture.

For a representative *in vitro* host, primary cell cultures were created from the tissues of 14-day old larval cod following methods successfully employed in the establishment of cell lines from other cold-water marine species such as the Pacific herring (*Clupea pallasii*) by Ganassin *et al*. (1999) and haddock (*Melanogrammus aeglefinus*) by Bryson *et al*. (2006). Once continuous, stable growth was achieved, the cell line was characterized for a stem cell marker, physiochemical requirements, optimal growth conditions, and susceptibility to microsporidian infection.

Novel culture methods were developed for *L. morhua*, using cod-derived cells as a representative host and manipulations of culture conditions to trigger spore activation. Building on methods used for previously cultured species such as *A. algerae* (Monaghan *et al*., 2011), *in vitro* culture of *L. morhua* was attempted using a variety of piscine cell lines, and modified culture conditions found to stimulate infectivity in other microsporidian species. Tested cell lines were derived from the tissues of a haddock embryo (HEW; Bryson *et al*., 2006), the gills of

adult rainbow trout (RTgill-W1; Bols *et al*., 1994), and whole larvae of the Atlantic cod. These initial cell lines were selected for the close phylogenetic relationship between the cod and haddock (HEW), the fact that parasites of the genus *Loma* are principally found in the gills of infected fish (RTgill-W1), and for the specific host-parasite relationship between *L. morhua* and the Atlantic cod.

Evaluation of the specific modifications of culture conditions included testing the infection-stimulating effects of $MgCl_2$ supplementation, the ameliorating effects of ethelenediaminetetraacetic acid (EDTA), and the effects of pH on spore activation and germination.

Digging Deeper

There are numerous benefits to the *in vitro* production of microsporidian parasites. Perhaps the most obvious is the reduction in effort required to obtain spores in large numbers. By extension, laboratory-based culture of microsporidia facilitates the use of highly specialized techniques that would be difficult or impossible using *in vivo* infection models. The readily cultured *A. algerae* spores were used to evaluate the infection-modulating effects of the chelating agent EDTA, and for the proteomic analysis of spore wall components using two-dimensional electrophoresis (2DE). Data from infectivity trials indicated that EDTA treatment reduced spore adhesion and infection rates *in vitro*. Spore wall proteins of *A. algerae* were found to correspond closely with existing protein work conducted on the insect pathogen *Nosema bombycis* (Wu *et al*., 2008).

Such advances in the knowledge and understanding of microsporidian parasites could not be efficiently produced by work performed *in vivo*. The development of *in vitro* infection models and detailed study of infective mechanisms will aid considerably in answering questions of importance in clinical settings, apiculture, sericulture, and the aquaculture of commercially important species such as *G. morhua*.

Objectives

The research detailed in this thesis is concerned principally with three focal areas, meant to improve our understanding of the Atlantic cod and microsporidosis:

1. The establishment and characterization of a novel cod cell line
    - Characterization
    - Testing growth parameters
    - Evaluating suitability for culturing microsporidian parasites
2. Investigate infection processes using cultured spores (*L. morhua* and *A. algerae*)
    - Development of parasite protein maps using 2DE
    - Optimizing *in vitro* growth
    - Evaluating methods for inhibiting infection *in vitro*
3. The development of *in vitro* culture methods for *L. morhua*
    - Evaluation of different cell lines for supporting parasite's growth
    - Development of activation protocols
    - Enhancing infectivity through modified culture conditions

Addressing the objectives listed above required an integrative approach, with considerations across multiple levels of biological organization ranging from molecular, to cellular, to the organismal level. Additionally, many of the concepts and approaches used in this

research were informed by a variety of fields, including evolutionary biology, physiology, and cellular biology, ultimately allowing field-specific observations to be incorporated into a larger focus.

Ch. 2: Development of a Cod-Derived Cell Line: Cold Tolerance and Susceptibility to Microsporidian Infection

Abstract

Piscine cell culture has emerged as an enormously useful tool for many fields of biological study, facilitating research into fish physiology and developmental biology, aquatic toxicology, and parasitology. Despite considerable expansion, numerous commercially valuable species are poorly represented *in vitro* and many have no available cell line. The development of species-specific cell lines for popular food fish such as the Atlantic cod (*Gadus morhua*) can aid in basic research, benefitting conservation efforts and farming methods for aquacultured fish. To this end, several initial cultures were created from the tissues of larval cod. Cells from a single primary culture trial have exhibited steady proliferation, surviving more than 26 passages and two years in active culture. Seemingly representative of a stable cell line, the cultures have been characterized for physiochemical requirements, optimal growth parameters, and stem cell-like characteristics tested by the detection of alkaline phosphatase activity. DNA barcoding has positively identified the cells as being 100 per cent *G. morhua*. The cod cells require FBS supplements, exhibiting enhanced growth at concentrations of 10% (v/v) and greatest viability at the highest tested concentrations of 20% (v/v). Cells exhibit viable growth at temperatures as low as 8 degrees Celsius (°C) and do not survive incubation at 24°C. The exhibited tolerance for cooler incubation temperatures represents a trait that is somewhat unique amongst existing piscine cell lines and of potential interest for future studies.

Keywords: *Gadus morhua*, Atlantic cod, cell line, fish, *in vitro*

Introduction

Piscine cell lines are useful for a wide variety of purposes, representing a valuable tool for studies in virology (Munir and Kibenge, 2004; Xu *et al*., 2010), developmental biology (Barreda *et al*., 2004; Gabillard *et al*., 2010), toxicology (Kilemade and Mothersill, 2000; Søfteland *et al*., 2010), and physiology (reviewed in Marshall and Bellamy, 2010). They can be particularly suitable for the study of commercially important marine species too scarce, active, or large to be feasibly maintained as laboratory colonies. Despite their potential utility, few cell lines derived from cold-water marine fish are currently available (Bryson *et al*., 2006) and many species are not represented at all. As aquaculture continues to grow in importance, the need for research into matters of livestock health will certainly grow as well, necessitating the formation of cell lines derived from important food species.

Commercial aquaculture programs are faced with a myriad of challenges that can limit their success and prevent profitable production. Many of these difficulties may be ameliorated in part by studies utilizing cell culture techniques and species-specific cell lines. Juvenile production is one of the primary bottlenecks in the culture of most fish species due to the extreme difficulty in rearing larval stages (Foss *et al*., 2004; Sawada *et al*., 2005). Early life stages often experience very high mortality due to difficulty in feeding, specific environmental requirements, and high sensitivity to contaminants. *In vitro* studies utilizing embryonic or larval cell lines can be used to inform *in vivo* efforts aimed at enhancing survival rates of young fish (reviewed in Villena, 2003). This may be accomplished through a variety of means including studying the mechanisms of pathogenicity in aquatic parasites (McIntosh *et al*., 1997; González-

Contreras *et al*., 2011), the detection of pathogens (Ariel *et al*., 2009a; Dale *et al*., 2009), and preliminary evaluations of responses to, and metabolism of toxins and toxicants at the cellular level (Segner and Cravedi, 2001; Liebel *et al*., 2011). Another prominent concern in many aquaculture settings is the extremely high prevalence of disease that can result from high stocking densities, frequent handling stress, and potentially poor water quality. The use of cell culture technology is ideally suited to the study of several important fish pathogens such as the nodaviruses and microsporidia.

The Atlantic cod (*Gadus morhua*) is a prime example of an aquacultured species suffering losses due to both poorly studied pathogens (reviewed in Bricknell *et al*., 2006) and a limited understanding of care requirements at embryonic and larval life-stages (reviewed in Naylor and Burke, 2005). Research utilizing cell culture and cell lines can provide important insights into biological responses to environmental stressors such as dissolved gases (Huynh *et al*., 2011), pH (Leguen *et al*., 2001) and temperature (Hardie *et al*., 1994). It can also aid in the production and investigation of important fish pathogens without the unnecessary sacrifice of valuable livestock (Ariel *et al*., 2009b; Grayfer *et al*., 2011).

Very few reports of continuous *G. morhua*-derived cell lines exist. Though several attempts at primary culture of cod cells have been reported (Koren *et al*., 1997; Lester, 2007; Søfteland *et al*., 2010; Tianxiao; 2010), continuous cell lines are limited to a recently established embryonic line (Holen *et al*., 2010) and a gonadal culture established in the 1980s (Jensen and Christensen, 1981). The limited number of existing cod cell lines prevents their ready availability for study, leaving potential lines of research unexplored and hindering continued aquaculture

efforts. To further enhance the number of commercially-important fish species represented *in vitro* and to facilitate research aiding commercial-scale aquaculture operations, a continuous cell line was sought from the tissues of larval cod, following previous, unsuccessful attempts at the establishment of cell cultures from adult tissues lost due to contamination. The cell line was characterized for response to growth conditions, physiochemical requirements, markers indicative of stem cell-like characteristics, and susceptibility to microsporidian infection *in vitro*.

Methods

Cod Larvae

Larval cod (Figure 2.1a) were obtained from the University of Maine's Center for Cooperative Aquaculture Research (CCAR; Figure 2.1b) at 14 days post-hatch and approximately 720mm in length. Prior to use for primary cultures, larval fish were been reared in 14°C seawater and fed rotifers on an ad libitum basis (Brown, 2010, p. com.). Several larval fish were transported in 1L jugs to the nearby Mount Desert Island Biological Laboratories (MDIBL) where primary cultures were initiated in subsequent days.

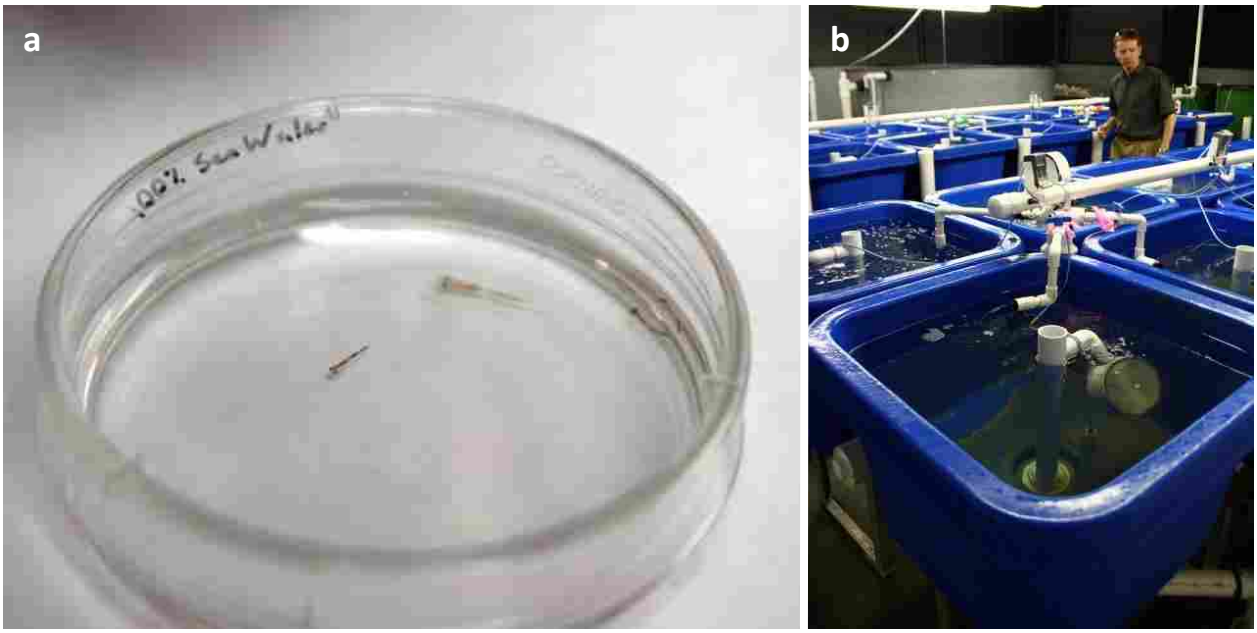

Figure 2.1: a) Cod larvae (day 14) used for primary culture obtained from the University of Maine's Centre for Cooperative Aquaculture Research (b). Petri dish measures 6cm in diameter.

Initiation of Larval Cell Culture

Primary cultures were initiated using explant outgrowth methods mirroring those used in the establishment of other cold-water marine species such as the Pacific herring (*Clupea harengus pallasi*; Ganassin *et al*., 1999) and the haddock (*Melanogrammus aeglefinius*; Bryson *et al*., 2006). Using aseptic technique in a laminar flow hood, five *G. morhua* larvae were treated for fungal and bacterial surface contamination in sterile seawater containing 2X penicillin-streptomycin-amphotericin (Caisson). The fish were then cut into pieces approximately $1mm^3$ in size using a scalpel and sharp probe sterilized in a glass bead sterilizer. Dissected sections were transferred to separate wells of 6-well Corning CELLBIND plates and 12-well BD Falcon tissue culture plates, with a small amount of growth media. Following a one-hour attachment period, the wells were gently filled with Leibovitz's L-15 growth media

(Thermo Scientific) containing various supplements of sterile seawater (SSW) and fetal bovine serum (FBS; Sigma), with 1% (v/v) penicillin-streptomycin-amphotericin. Cultures were incubated at 15°C and observed for outgrowth. Most adhered explants exhibited outgrowth of cells by 24 hours post-plating. Ten days after plating, trials exhibiting substantial outgrowth were trypsinized using TryplE (recombinant trypsin, Invitrogen) and plated onto 12.5 cm^2 tissue culture flasks (Falcon). A single trial (number five) plated with 5% SSW (v/v) and 10% FBS (v/v) survived and was successfully subcultured. Due to slow initial growth after first passage, cells were moved to a 20°C incubator and growth media was supplemented with additional serum (15%). After several months, stable repeatable growth was established, and the cells have now been subcultured for more than two years, surviving 24 population doublings. The stable, long-term growth seems to suggest the culture now represents a stable cell line that has been designated GML-5 for *Gadus morhua* larvae, trial 5.

Routine Maintenance

Cells in active culture were maintained in L-15 media supplemented with 15% FBS and 1% PS? Growth media was kept within appropriate osmolarity values used previously in cod tissue culture (Clow *et al*., 2004; refer to Figure A.1, Appendix for medium osmolarity). Every 7 to 14 days, media was removed from culture vessels, cells were dissociated with TryplE for five minutes, suspended in culture media, and centrifuged at 213 relative centrifugal force (RCF) for five minutes. Following centrifugation, the supernatant was removed and the cell pellet was split evenly between two culture flasks.

Cell pellets collected from two 75 cm^2 tissue culture flasks (Falcon) at passage 12 were suspended in growth medium containing 10% (v/v) dimethyl sulfoxide (DMSO), then frozen in liquid nitrogen.

Growth Characteristics

Growth characteristics and responses to culture conditions were evaluated using Alamar Blue (AB; Invitrogen) assays. AB measurements made using a SpectraMax Pro spectrophotometer (excitation: 530nm; emission 595) were used as an index of cell viability and cell proliferation for the majority of growth characterization experiments, following established protocols for fish cells (Dayeh *et al*., 2004). A standard curve for the correlation of cell numbers to relative fluorescence units (RFU) was performed in 96-well plates as previously used for other fish cell lines (Dayeh *et al*.). A dilution series (100%, 60%, 40%, 30%, 20%, 10%, 5%, 1%, 0.1%, and 0.01%) was prepared from GML-5 cells at passage 10 and suspended in growth media at a 100% concentration of $8.95*10^5$ cells/ml. 200µl of each dilution was plated onto a 96-well plate with eight replicates per concentration. The cells were allowed 24 hours for adherence, after which all media was removed from the wells, which were then rinsed with L-15 ex (Schirmer *et al*., 1997) and re-filled with 100µl of 10% (v/v) AB in L-15 ex. The plate was incubated for one hour at room temperature, and fluorescence was subsequently measured by spectrophotometry. Regression analysis was used to convert RFUs into approximate cell numbers for subsequent assays.

Cell response to incubation temperatures and supplemental FBS concentrations were evaluated in order to determine optimal growth conditions. Optimal FBS concentrations were

assessed by plating cells on 96-well plates (Falcon) with 200µl of GML-5 cells suspended in L-15 growth media containing 0%, 10%, 15%, and 20% FBS (v/v). Measurements were taken as per standard AB protocol for each FBS concentration at three, six, and nine days post-plating.

Response to incubation temperatures was evaluated by both AB assay measurements and direct quantitation by haemocytometer. Cells suspended in growth media were plated onto 96-well plates with 200µl, and incubated at 8°C, 18°C, 21°C, and 24°C. Measurements were taken following the same protocols used to both construct a standard curve and evaluate response to various FBS concentrations.

For physical quantitation of cell numbers, 2ml of GML-5 cells at passage 24 were suspended in growth medium and plated onto several 6-well plates (Falcon) at $1.75*10^5$ cells per ml, and incubated at 8°C, 18°C, and 21°C. Cells were trypsinized, centrifuged for five minutes at 213 RCF, resuspended in L-15 and counted at 24 hours, three, six, and nine days post-plating. Each treatment was tested in triplicate.

Response to Chemicals

To test the effects of lead citrate and aluminum hydroxide, GML-5 cells were plated onto 96-well tissue culture plates in 200µl L-15 media at initial cell densities of $2.0*10^5$ cells/well (lead citrate) and $3.4*10^5$ cells/well (aluminum hydroxide). Following a 24 hour incubation at 18°C, wells were rinsed and filled with 200µl of L-15 ex at various dilutions of lead citrate and aluminum hydroxide (100µg/ml, 80µg/ml, 40µg/ml, 20µg/ml, 10µg/ml, 5µg/ml, 1µg/ml, 0.1µg/ml, 0.01µg/ml, and 0.001µg/ml), incubated again for 24 hours and analyzed by AB assay.

Cell Identity and Attributes

DNA barcoding (reviewed in Frézal and Leblois, 2008) performed at the University of Guelph (Guelph, Ontario) was used to confirm the identity of the GML-5 source tissue. Alkaline phosphatase activity—elevated in pluripotent embryonic cells (NIH, 2009)—was detected using an alkaline phosphatase assay (Sigma) as a marker for stem cell-like characteristics (Müller *et al*., 2009; Fujikawa-Yamamoto *et al*., 2010).

Tests for alkaline phosphatase activity were performed at the University of Waterloo (Waterloo, Ontario) by Nguyen Vo. Two ml of cell suspension at a concentration of $6*10^5$ cells/ml (passage 17) were placed on a standard microscope slide and incubated at room temperature for 20 hours. The slides were then stained as per kit instructions and observed by phase-contrast microscopy.

Susceptibility to *Anncaliia algerae*

Anncaliia algerae spores obtained from the American Type Culture Collection (Manassas, Virginia; ATCC number PRA-168) were grown in gold fish skin cells (GFSk-S1; Lee *et al*., 1997) and used to evaluate GML-5 susceptibility to microsporidian infection. Spores were isolated by lysing infected cell pellets in 3ml of cell culture-grade water, which was then mixed with 3ml Percoll® and centrifuged at 1136 RCF for 20 minutes. The purified spore pellets were suspended in growth medium and inoculated onto two confluent GML-5 cultures in $25cm^2$ tissue culture flasks, plated 24 hours prior to inoculation. Cultures were maintained at room temperature and observed daily for the appearance of infected cells and developmental stages

using phase-contrast microscopy (Nikon Eclipse TE300) micrographs were taken using the a Nikon Coolpix E990.

Statistical Analysis

Cell responses to modified culture conditions were analyzed using regression analyses. All statistical analyses were performed using Microsoft Excel statistical software.

Results

Primary Culture & Morphological Traits

Primary cultures exhibited appreciable outgrowth by 10 days post initiation, with adhered cells spreading from most of the nine tissue fragments initially plated(Figure 2.2 a,b). Cells from the fourth and fifth explant trials that survived continued maintenance in following months were passaged successfully to new tissue culture vessels (Figure 2.2c,d). Cells appeared fibroblastic in form at low cell densities, becoming epithelial-like in appearance as cultures reached confluence. As a general observation, initial growth was slow, and cells from the fourth explant were lost, but cells from the fifth explant trial exhibited steady, continued proliferation, doubling in population every five to seven days.

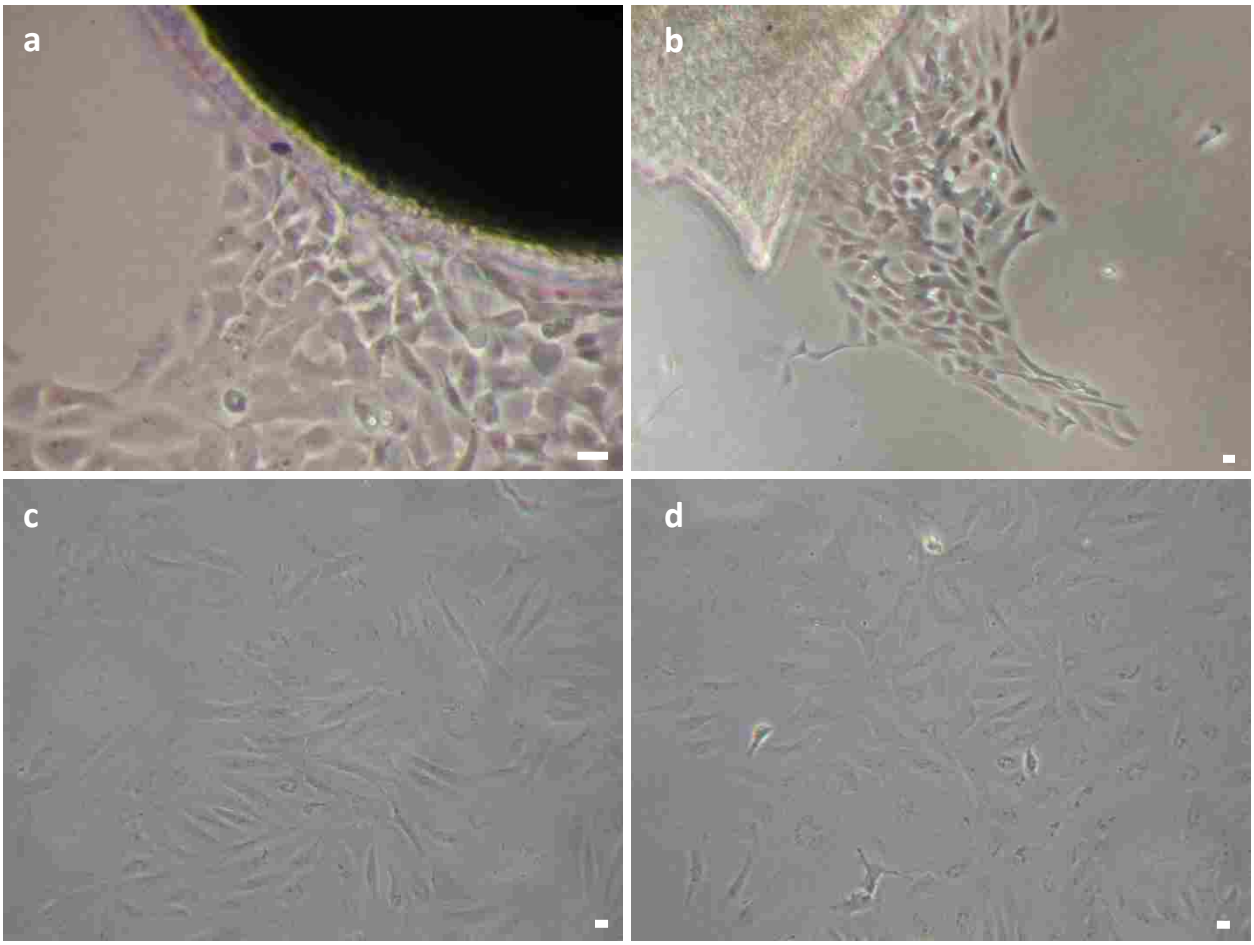

Figure 2.2: a) Micrograph of initial cell proliferation around the eye of a cod larvae adhered to a tissue vessel one week post-plating. b) Cod cell outgrowth from a tissue culture fragment one week post-plating. c,d) Cod cells adhered to tissue culture flask 24 hours after initial passage. Scale bars = 50µm

Characterization

Cells plated from a dilution series were used for the formation of a standard curve for correlating of cell numbers to Alamar Blue RFU values. Measured values exhibited a strong, direct correlation between cell numbers and RFU values (linear regression; r^2= 0.923; DF= 1. 105; p=3.993*10^{-60}; Figure 2.3). GML-5 cells exhibited low RFU values compared to haddock-derived (HEW) cells (Figure A.2, Appendix), despite similar numbers of plated cells. Alamar Blue assays indicated that GML-5 growth rates were stimulated by both elevated FBS concentrations

(Figure 2.4) and increased incubation temperatures (Figure 2.5a). The stimulatory effects of increased incubation temperatures were also observed by haemocytometer counts (Figure 2.5b). Cells incubated at temperatures at or above 24°C did not survive beyond 24 hours. Incubation in L-15 ex resulted in a steady decline in RFU values over a 10-day treatment period (linear regression; r^2= 0.928; DF= 1. 30; p= $1.18*10^{-18}$; Figure 2.6). Cells incubated in L-15 ex exhibited substantial changes in appearance over the exposure period (Figure A.3, Appendix). GML-5 cells tested positive for alkaline phosphatase activity (Figure 2.7), and their genetic origin was determined to be 100% *G. morhua* by DNA barcoding. Initial 24 hour toxicity curves for lead citrate and aluminum hydroxide suggest that cells are insensitive to the substances (Figure A.4, Appendix).

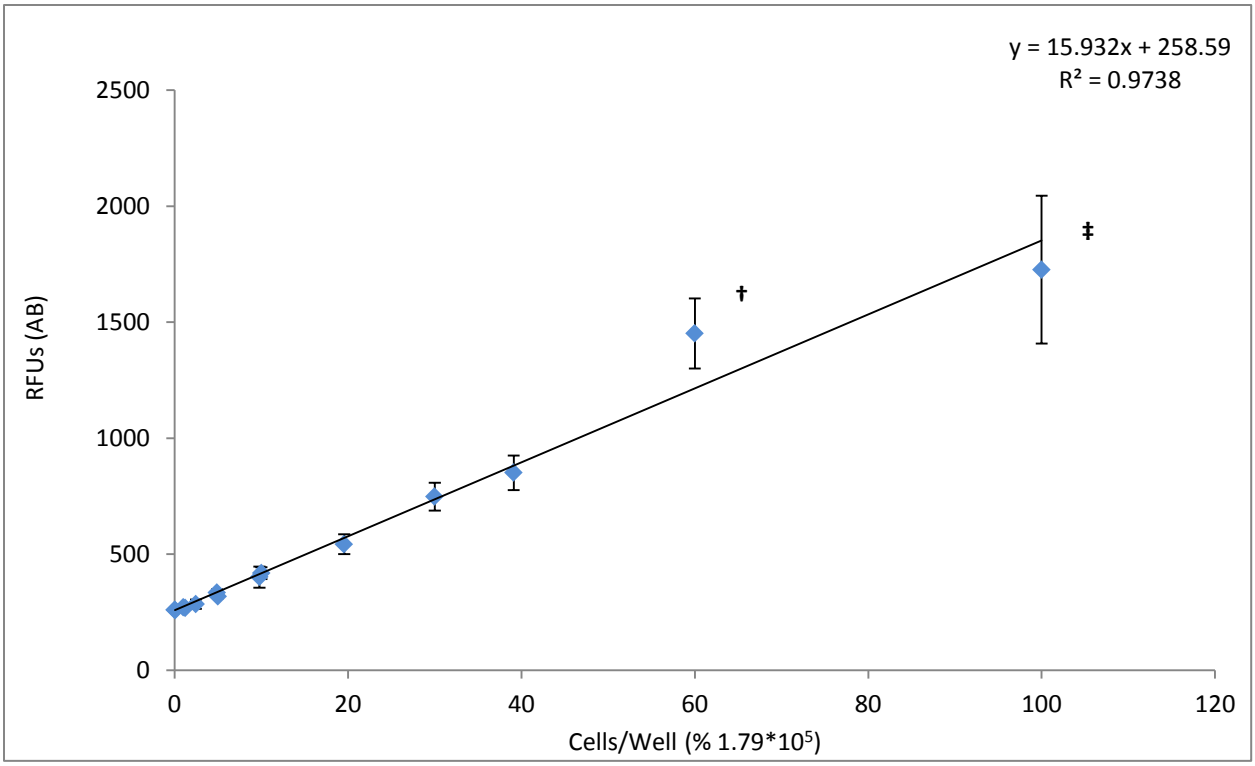

Figure 2.3: Standard curve for the correlation of Alamar Blue relative fluorescence units (RFU) to cod cell (GML-5) numbers from cell densities of ~18 cells per well to 179,000 cells per well. Error bars indicate 95% confidence intervals (n=8, $7^{\dagger}$, $5^{\ddagger}$).

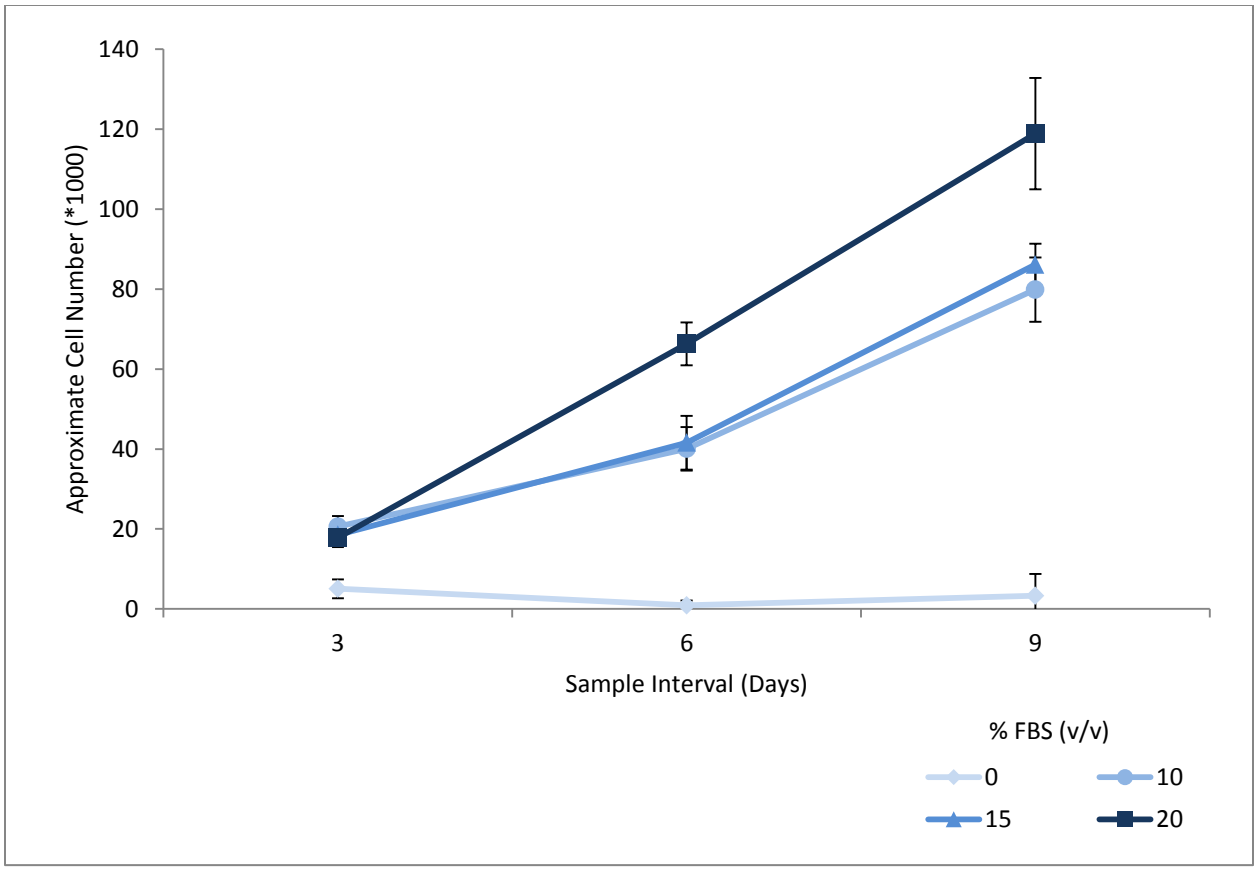

Figure 2.4: Cod cell (GML-5) response to fetal bovine serum (FBS) concentration (v/v) in growth media over a nine day observation period. Relative fluorescence units (RFU) are used as an index of viable cell numbers. Error bars indicate 95% confidence interval (n=8).

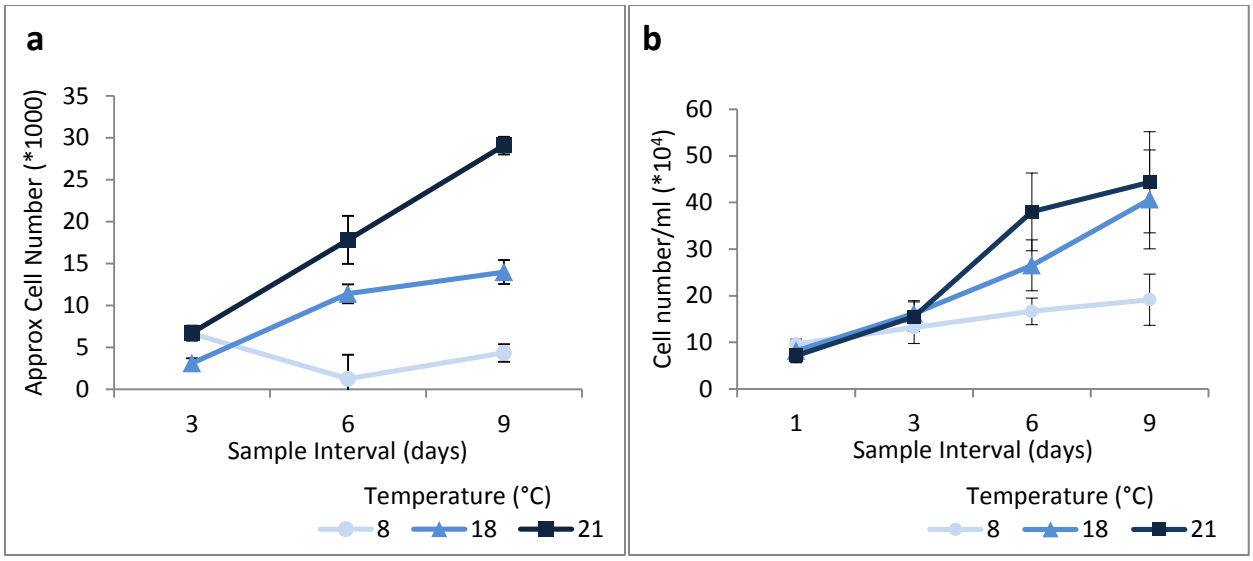

Figure 2.5: a) Cod cell (GML-5) response to incubation temperatures over a nine day observation period. Alamar Blue relative fluorescence units (RFU) are used as an index of viable cell numbers. b) haemocytometer counts of cod cells maintained at different incubation temperatures over nine days. Error bars indicate 95% confidence interval (a, n=22; b, n=3).

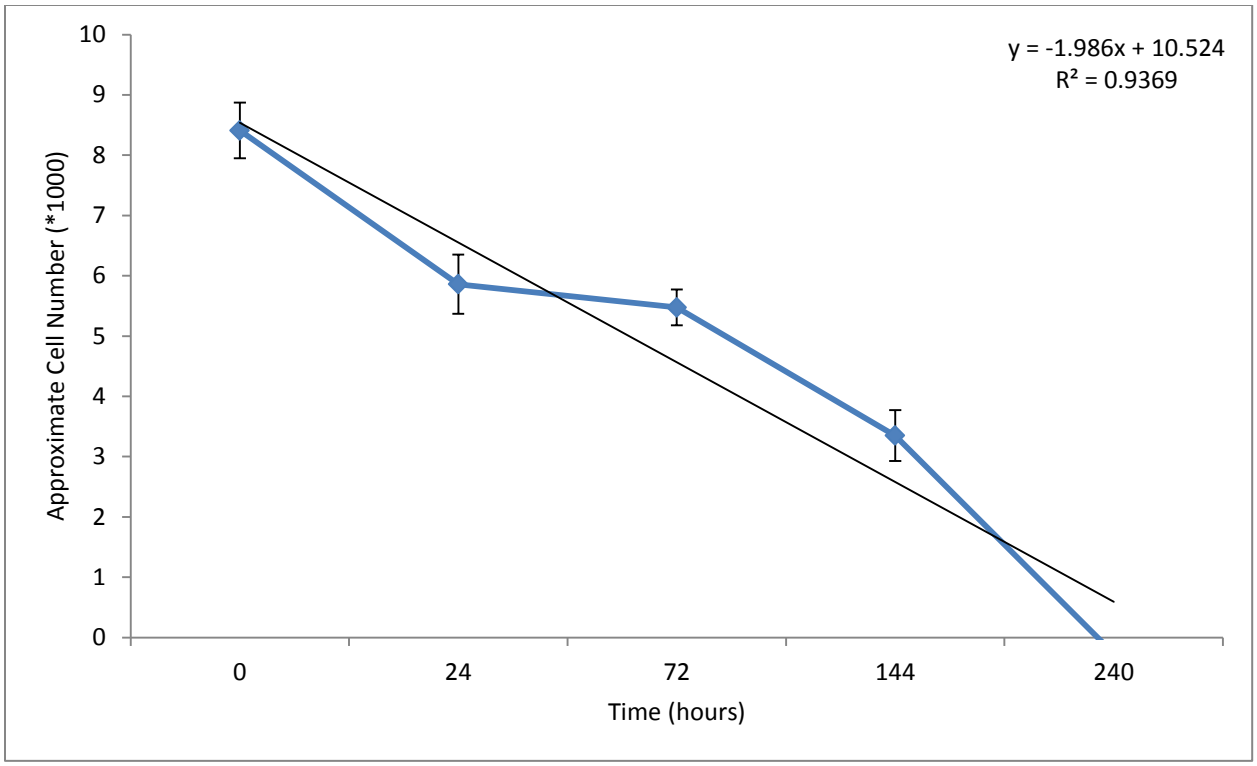

Figure 2.6: Cod cell (GML-5) response to incubation in simple exposure media (L-15 ex) over a 10 day observation period. Relative fluorescence units (RFU) are used as an index of viable cell numbers. Error bars indicate 95% confidence interval (n=8).

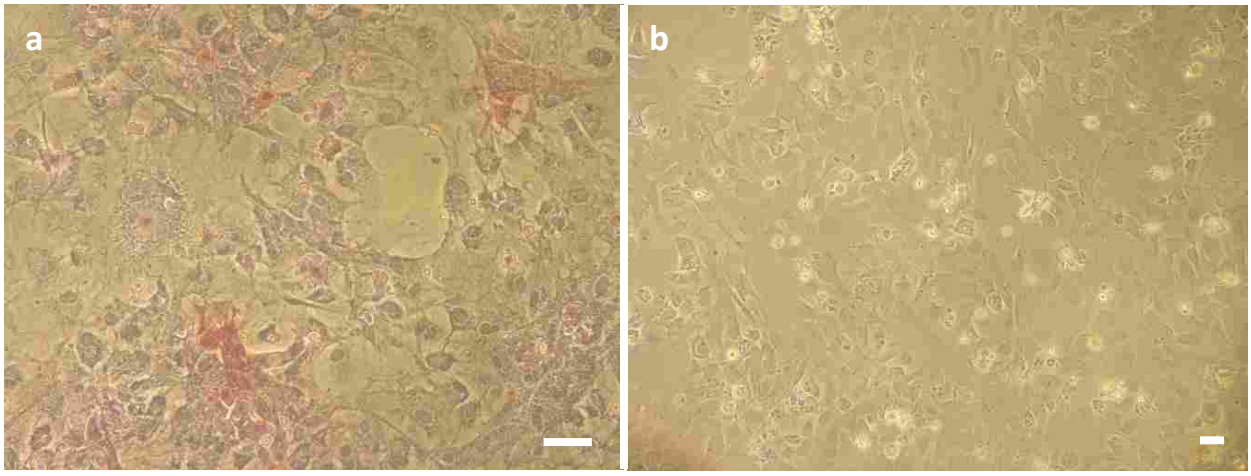

Figure 2.7: Micrographs of cod cells (GML-5) exhibiting positive alkaline phosphatase activity, apparent as bright pink granules in treated cells (a), absent in un-stained controls (b). Scale bar = 50µm (Photos by Nguyen Vo, 2012).

GML-5 cells incubated in co-culture with *A. algerae* were found to be susceptible to microsporidian infection, with the appearance of intracellular developmental stages observed by five-days post-inoculation (PI) (Figure 2.8).

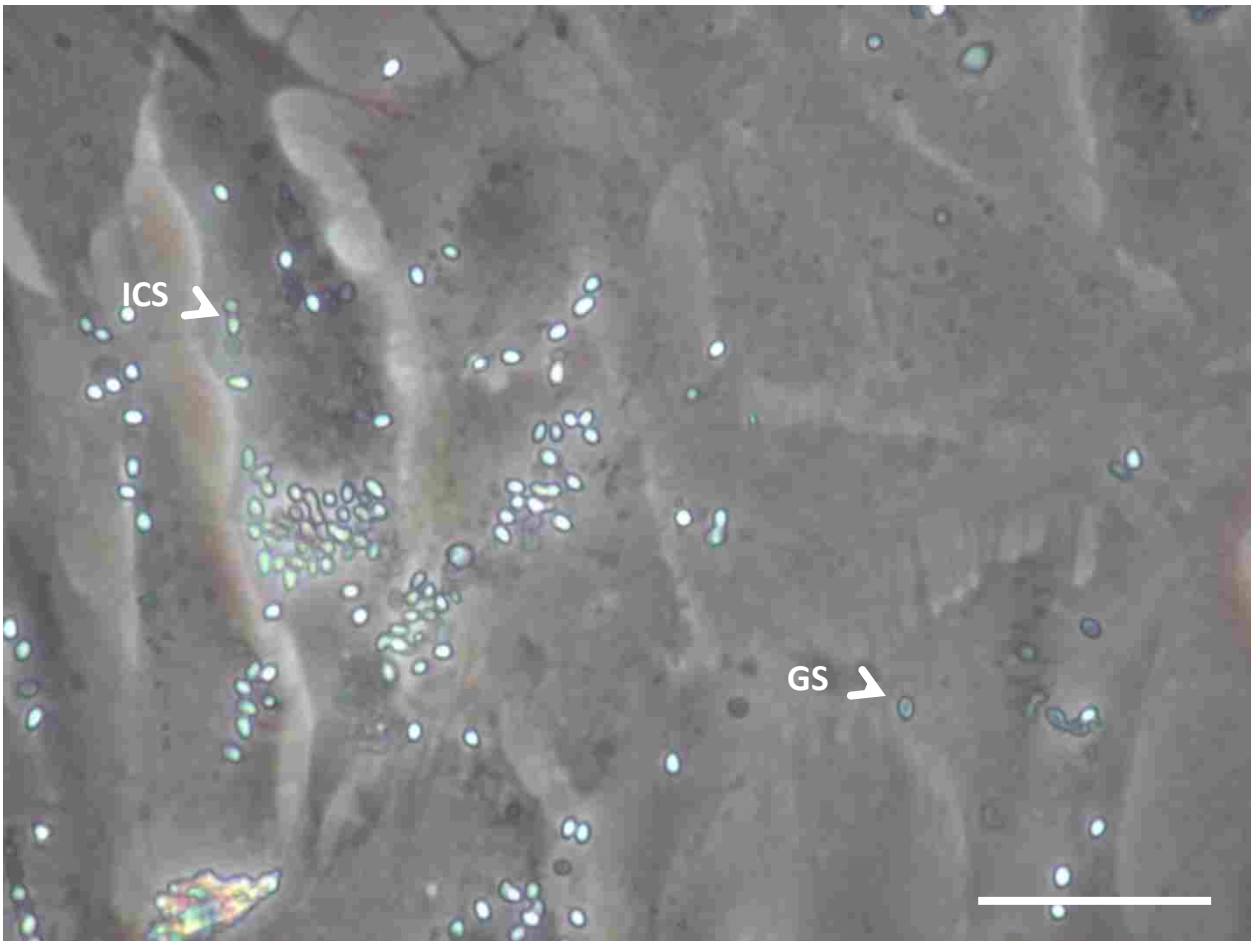

Figure 2.8: Phase-contrast micrograph of *Anncaliia algearae* spores infecting cod-derived GML-5 cells *in vitro* five days post-inoculation. Micrograph taken after rinsing and removal of media, intracellular spores are those observed within the bounds of the host cell membrane and correspond well to *A. algerae* developmental stages observed in other infected cell lines. Arrows indicate germinated spores (GS) and intracellular spores (ICS). Scale bar = 50µm.

Discussion

Growth of a cell line derived from the tissues of larval Atlantic cod was successfully established, with cultures actively maintained in standard growth media mixes of L-15 supplemented with 10-15% FBS. Under optimal conditions, cells required approximately three days to double in number, though growth appears to slow considerably as cultures reach confluence. The culture has been maintained for over two years and survived over 26 population doublings. The continued, consistent proliferation of GML-5 cells over long-term

active culture, coupled with positive detection of a marker indicative of stem cell-like characteristics, suggests that the cells represent a stable cell line.

The addition of excess FBS supplements had a stimulatory effect on cell proliferation, as has been observed with other fish-derived cell lines. Evaluations of the toxicity of aluminum hydroxide and lead citrate did not suggest that the cells are particularly sensitive to these compounds. However, this may be a result of problems with solubility and further testing will be required to better evaluate the sensitivity of GML-5 cells to environmental contaminants. The response to incubation temperatures, however, yielded more intriguing results. Increased incubation temperatures resulted in apparent increases in metabolic activity (as demonstrated by AB assay) and proliferation rates. Qualitative observations of cells incubated at 21°C yielded some signs of stress, including irregular morphology and increased vacuolation. Exposure to temperatures at or above 24°C resulted in rapid cell death, causing complete loss of cultures within 24 hours. The observed intolerance to high temperatures and continued growth under cool conditions is not particularly surprising, and is likely a reflection of the cold-water habitats in which cod thrive (Jordaan and Kling, 2003; Bosman *et al*., 2011). Few currently available cell lines exhibit such intolerance of elevated incubation temperatures or positive growth at lower incubation temperatures (Bryson *et al*., 2006), and many exhibit best growth up to 5°C higher than the thermal optimum of the living fish (Bols *et al*., 1992). GML-5 and other cell lines from cold-water marine fish may be useful for evaluating mechanisms responsible for heat-induced death at the cellular level, as well as studies on heat shock proteins previously detected in a cod-derived primary culture (Søfteland *et al*., 2010) and researched using other piscine-derived cell lines (Kondo *et al*., 2004).

Given the difficulties caused by pathogens in wild and farmed Atlantic cod, one of the principal benefits of a stable cod-derived cell line is its potential utility for *in vitro* studies into economically important diseases such as the microsporidia and piscine viruses. Initial efforts at culturing a readily available microsporidian parasite (*A. algerae*) resulted in the appearance of developmental stages by five-days post-inoculation, suggesting that the cell line is susceptible to microsporidian infection, and potentially suitable for the study of additional microsporidian parasites, such as the cod-infecting *Loma morhua*.

Ultimately, the formation of a novel cod larvae-derived cell line has the potential to aid research on the biology of fish, including their physiology (Marshall and Bellamy, 2010), growth and nutritional requirements (Kawano *et al*., 2011), and response to waterborne contaminants (Taju *et al*., 2012). GML-5 cells may also assist in the study of parasites currently confounding aquaculture efforts, serving as a complement to *in vivo* methodologies as demonstrated with other piscine cell lines (Lee *et al*., 2009). Such novel lines of research have the potential to greatly improve our understanding of the captive requirements of Atlantic cod and to inform disease management efforts in aquaculture farms, improving the reliability of production as well as the prospects for commercial-scale production of fish for human consumption.

Ch. 3: *Anncaliia algerae*: Their Capsule Proteins & Factors Affecting *in Vitro* Infection Rates

Abstract

The microsporidia are a unique group of intracellular fungal parasites that infect a host by extruding their infectious spore contents (sporoplasm) into host cells via an everted polar filament. For successful infection to occur, the spore must be in close proximity to a suitable host prior to activation of the polar filament. Consequently, spore germination processes are presumed to be mediated by physiochemical conditions specific to the site of infection and, possibly, spore adhesion prior to activation. This research was conducted to evaluate the infection modulating effects of the MgCl2 and the chelating agent ethylenediaminetetraacetic acid (EDTA). It was observed that *In vitro* infection rates of the arthropod-infecting *Anncaliia algerae* in the goldfish skin cell line, GFSk-S1 are enhanced by supplemental magnesium, and that the EDTA interferes with infection processes, significantly reducing infection rates in cultured cells. These findings support the importance of magnesium in spore adhesion and infection efficiency in a previously untested microsporidian species.

Keywords: Microsporidia, *Anncaliia algerae*, parasitology, infection, adhesion

Introduction

The microsporidia are a group of unique fungal parasites that are highly adapted for life as obligate intracellular parasites. Known mostly for their ability to cause disease in immunocompromised individuals such as AIDS patients (Conteas *et al*., 1997), the microsporidia have recently been identified in otherwise healthy non-HIV-positive individuals as well (Didier and Wiess, 2011). The parasites are able to infect virtually all animal groups, including insects (Tokarev *et al*., 2010), crustaceans (Stentiford *et al*., 2007), fish (Kvach and Winkler, 2011; Abdel-Ghaffer *et al*., 2012), reptiles (Jacobson *et al*., 1998), birds (Malcekova *et al*., 2011), and mammals (reviewed in Wasson and Peper, 2000). Given the range of potential hosts, it is not surprising that infections caused by microsporidia (microsporidosis) are a growing concern in not only clinical, but also agricultural applications. Several commercially important animal species, including honey bees (Traver and Fell, 2011), silkworms (Rao *et al*., 2004), and a variety of farmed fish (Caffara *et* al., 2010; Nylund *et al*., 2011), are host to one or more microsporidian parasites, all with the potential to hinder reliable production of, and cause excessive mortality in livestock. Due to the potential economic impact and human-health concerns associated with microsporidosis, considerable effort has been put into better understanding the parasite, its evolutionary history (reviewed in Keeling and Fast, 2002), biochemistry (Méténier and Vivarès, 2001), and susceptibility to therapeutic treatments (Beauvais *et* al., 1994; Didier, 1997; Didier *et al*., 2005). The vast majority of the work produced to date, however, has been focused on the human-infecting microsporidia of the genus *Encephalitozoon*, owing to both its clinical significance (Rossignol, 1998) and the ease with which it can be cultured *in vitro* (Visvesvara,

2002). Consequently, a growing gap exists in our knowledge of microsporidian parasites with non-mammalian hosts.

One of the most unique aspects of the microsporidia is the process by which they infect host cells: when spores are in close proximity to a suitable host, a hollow tube is everted that pierces the host cell membrane and transfers spore contents into the host. This is followed by a proliferative stage in which developing and mature spores fill the host cytoplasm, causing the cell to rupture and release new infective spores (reviewed in Bigliardi and Sacchi, 2001). The polar filament is only 50-150µm in length (Keohane and Weiss, 1998), meaning successful infection requires the spore to be in extremely close proximity to a suitable host prior to the initiation of the germination process. It is probable that this is achieved by the detection of a suitable host prior to infection, likely through a combination of chemical conditions indicative of the host species' physiology and adhesion to host membrane constituents. Southern *et al*. (2006) suggests that spore adhesion may be essential to microsporidian activation, and that infection efficiency in mammal-infecting *Encephalitozoon sp.* may be augmented by the divalent cations magnesium (Mg^{2+}) and manganese (Mn^{2+}).

Another unique feature of the microsporidians is the highly-restricted size of their genome and proteome—amongst the smallest of all eukaryotes (reviewed in Texier *et al*., 2005; Peyretaillade *et al*., 2011), which is likely a direct consequence of both their extremely small size and intracellular life cycle. This genomic compaction helps simplify the task of characterizing the parasites at the proteomic-level. Due to the evolutionary constraints placed

on microsporidians, it is likely that species will exhibit a great deal of similarity in the type, number, and quantity of proteins expressed.

This study was performed to test the effects of Mg^{2+} on infective processes in a previously un-tested arthropod-infecting microsporidian species (*Anncaliia algerae*), determine the effects of a chelating agent on infection rates *in vitro*, and construct protein reference maps using two-dimensional electrophoresis. If magnesium plays a role in the adherence of *A. algerae* spores to host cells prior to infection, supplemental Mg^{2+} is predicted to enhance infection rates, but not intracellular proliferation of the parasite. Conversely, chelation treatments should result in reduced infection rates proportional to treatment concentrations, but not alter infection intensity. Proteins isolated from *A. algerae* samples are predicted to be few in number and to conform to proteins isolated from other microsporidian species such as *Nosema bombycis* (Wu *et al*., 2008).

Methods

Routine Spore Culture & Purification

Percoll® (Sigma) centrifugation was used for the isolation of spores adhered to, or contained within living cells. Samples of *Anncaliia algerae* spores were cultured in goldfish skin cells (GFSk-S1; Lee *et al*., 1997), following methods developed by Monaghan *et al*. (2011). Infected GFSk-S1 and *A. algerae* co-cultures were dissociated using TryplE (recombinant trypsin, Invitrogen), placed in 15ml centrifuge tubes, and centrifuged for five minutes at 213 RCF in an International Equipment Co. (IEC) clinical centrifuge (Model CL). The supernatant was removed and the pellet containing both spores and cells was re-suspended in 3ml of cell-culture-grade

water to lyse the GFSk-S1 cells. Following a 24-hour lysis period, 3ml of Percoll was added and the lysis suspension was centrifuged for 20 minutes at 1136 RCF (IEC clinical centrifuge). The supernatant was again removed and the pellet—now containing only spores—was re-suspended in L-15 growth media, producing purified spore samples. The spore suspension was then used to inoculate additional flasks for continued spore production, infectivity experiments, and proteomic work.

Infectivity Experiments

To evaluate parameters affecting microsporidian growth *in vitro*, GFSk-S1 host cells were suspended in L-15 growth media containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS), plated onto multi-well tissue culture plates and given 24 hours to adhere. Following adherence, media was removed and wells were inoculated with spores suspended in treatment media mixes containing supplemental $MgCl_2$ (0, 0.01, 0.1, 1, and 10mM) and EDTA (0, 100, 200, and 400µM). Co-culture plates were incubated at 24°C and micrographs were made using phase-contrast microscopy. Quantification was made at eight days post-inoculation by means of random phase-contrast micrograph fields and haemocytometer counts. For micrograph counts of spores contained within infected cells, 20-40 fields (For $MgCl_2$ and EDTA treatments respectively) were photographed using a digital camera (Nikon Coolpix E990) attached to a Nikon Eclipse TE300 phase-contrast microscope (20X objective). The camera's digital zoom was set to allow the widest possible view while still allowing positive identification of intracellular spores. Micrographs were manually counted for intracellular spores per field, infected cells per field, and number of spores per infected cell.

Spore counts made by haemocytometer were performed on media removed and rinsed from wells as an index of free spores, and spores recovered from lysed cell samples by Percoll® purification as an index of spores associated with infected cells. These values were combined to show differences in proliferation between treatments as all cultures were inoculated with the same initial number of spores.

2D Electrophoresis

Protein samples were prepared by centrifuging spore suspensions (approximately two million spores) collected from 75cm^2 tissue culture flasks at 213 RCF for five minutes, removing the supernatant, re-suspending in 1X phosphate-buffered saline, repeated centrifugation and supernatant removal, and suspension in a lysis buffer containing urea, thiourea, dithiothreitol (DTT), and CHAPS (Table A.1, appendix) for 30 minutes. Protein quantitation was performed by Bradford Assay (Bio-Rad) and samples were loaded onto 7cm ReadyStrip™ immobilized pH gradient (IPG) strips (Bio-Rad) at 5µg per strip. Isoelectric focusing (IEF) and second dimension gels were run on 10% Mini-PROTEAN® TGX™ precast gels using a Bio-Rad PROTEAN® IEF cell and Mini-PROTEAN® Tetra Cell, respectively. The resulting two-dimensional gels were then visualized using Silver stain (Bio-Rad).

In an attempt to disrupt the spore wall and isolate additional proteins, two samples of approximately eight million spores were isolated from 175cm^2 tissue culture flasks by centrifugation at 213 RCF for five minutes, removal of supernatant, re-suspension in 1X phosphate-buffered saline, re-centrifugation, and removal of supernatant. The resulting spore pellets were suspended in lysis buffer. One sample was frozen, while another was subjected to

three freeze-thaw cycles, and sonication (Omni Ruptor 250 watt ultrasonic homoginizer; Omni International).

Statistical Analysis

Differences in infection rates and intensity were compared using t-tests and one-way ANOVAs, post hoc analysis was performed using Dunnett multiple comparison tests. Dose-dependent trends were evaluated using linear regression. Regression analyses were performed using Microsoft Excel statistical software; all other statistical analyses were performed using InStat statistical software (Graphpad).

Results

Effects of Supplemental Magnesium

Infection rates (observed as the number of intracellular spores per random micrograph field) were found to increase with increasing $MgCl_2$ supplements. Infection rates were significantly affected by $MgCl_2$ supplementation (one-way ANOVA; 0mM: 26.60 spores ± 30.79, 0.1mM: 22.15 ± 75.32, 0.1mM: 54.25 ± 46.20, 1.0mM: 52.40 ± 41.83; 10mM: 101.35 spores ± 84.17; DF= 4. 95; F = 5.65; p= $4*10^{-4}$; Figure 3.1a) infection intensity was increased over control levels at 10mM supplemental $MgCl_2$ (Dunnett multiple comparison test; 0 vs. 10: mean difference= -74.75; q= 3.99; p= $1*10^{-4}$). Average numbers of intracellular spores per infected cell were not significantly affected (one-way ANOVA; 0mM: 22.78 ± 28.37, 0.01mM: 7.00 ± 10.86, 0.1mM: 32.92 ± 32.48, 1mM: 27.87 ± 29.06; 10mM: 33.90 ± 26.84; DF= 3. 61; F= 0.4480; p= 0.7196; Figure 3.1b).

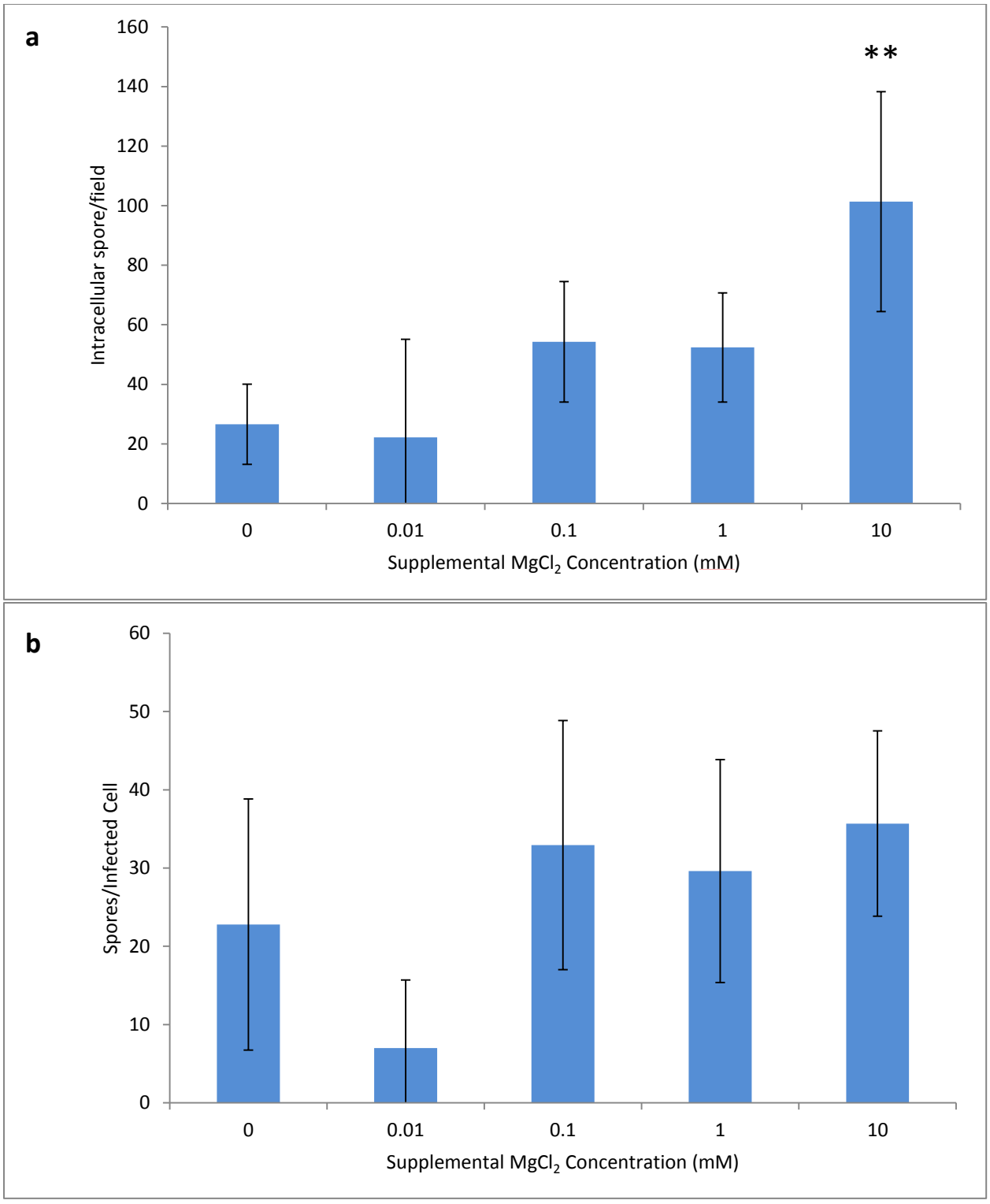

Figure 3.1: a) Magnesium-mediated enhancement of infection by *Anncaliia algerae* in goldfish skin (GFSk-S1) cells. Infection values represent counts of intracellular spores from phase-contrast micrographs (n=5 micrographs/well for 4 replicate wells/treatment). Highly significant increases in infection were observed at 10mM supplemental $MgCl_2$ concentrations ($p=1*10^{-4}$). b) Average spore numbers per infected cell were unaffected by treatment concentrations. Error bars indicate 95% CI.

Impact of EDTA

The initial infectivity experiment comparing infection rates in EDTA-treated and control GFSk-S1/*A. algerae* co-cultures resulted in reduced numbers of intracellular spores in EDTA treated flasks eight days post-inoculation (PI) (t-test; 0µM: 434.20 spores ± 157.18, 100µM: 222.98 spores ± 98.96; DF= 78; t= 7.193; p= $3.382*10^{-10}$; Figure 3.2a). The number of intracellular spores per infected cell was not significantly affected (t-test; 0µM: 63.15 spores ± 14.96, 100µM: 67.87 spores ± 25.91; DF= 78; t= 0.941; p= 0.35; Figure 3.2b).

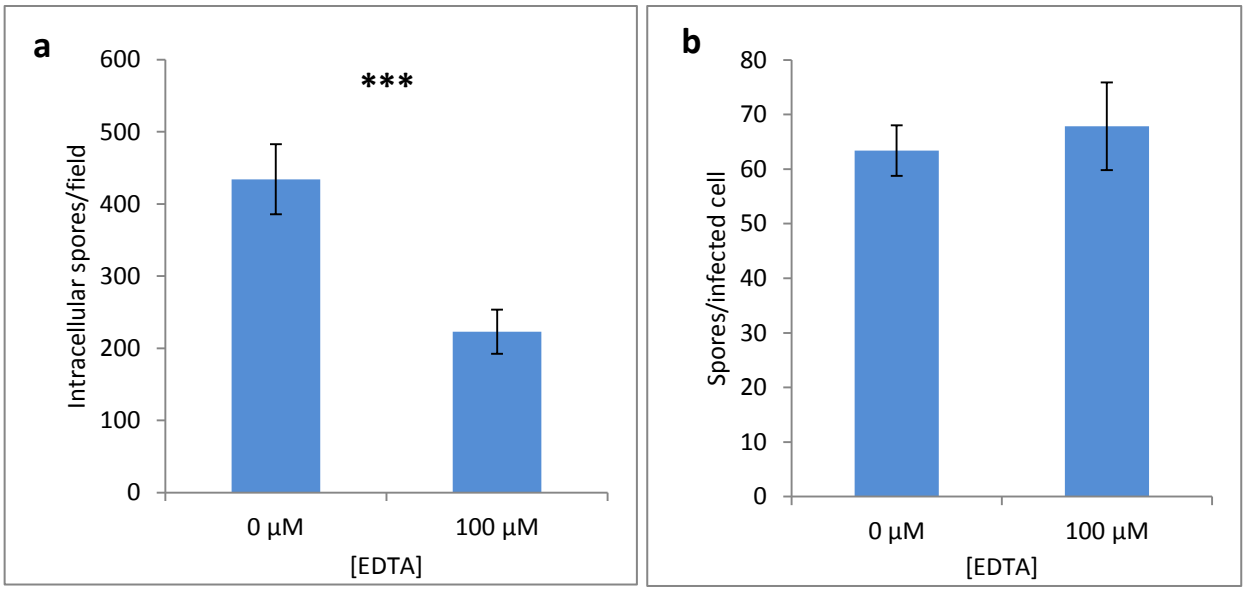

Figure 3.2: EDTA-mediated infection response of *Anncaliia algerae* in goldfish skin cells (GFSk-S1) eight days post-inoculation. Intracellular spores per field (a) were significantly reduced by EDTA treatment ($p=3.38*10^{-10}$). b) The average number of spores per infected cell was not significantly affected (p=0.35). Counts determined by random micrographs (n=5 micrographs/well for 8 replicate wells/treatment) pooled from eight replicate treatments. Error bars indicate 95% CI.

A subsequent experiment conducted using a range of supplemental EDTA concentrations showed significant differences in spore numbers between EDTA treatments for the number of spores recovered from media (one-way ANOVA; 0µM: 10.30 ± 2.05, 100µM: 12.90 ± 2.43, 200µM: 15.80 ± 1.75, 400µM: 34.90 ± 3.07; DF= 3. 16; F-ratio= 110.45; $p=6.626*10^{-11}$; Figure 3.3a), spores recovered by Percoll® centrifugation (one-way ANOVA; 0µM: 724.875 ± 78.551, 100µM: 359.6 ± 75.886, 200µM: 213 ± 28.065, 400µM: 8 ± 1.541; DF= 3. 15; F-ratio= 134.598; $p= 4.566*10^{-11}$; Figure 3.3b), and total spore numbers (one-way ANOVA; 0µM: 735 ± 79/794, 100µM: 372.5 ± 74.186, 200µM: 228.8 ± 28.702, 400µM: 42.9 ± 2.022; DF= 3. 15; F-ratio= 126.795; $p= 7.022*10^{-11}$; Figure 3.3c). Significant differences in spores recovered from media at were found at 200µM (Dunnett multiple comparison test; 0 vs. 200: mean difference= -5.50; q= 3.66; p= <0.01) and 400µM (Dunnett multiple comparison test; 0 vs. 400: mean difference= -24.60; q= 16.36; p= <0.01). Spores recovered by Percoll® preparation were different from controls at 100µM (Dunnett multiple comparison test; 0 vs. 100: mean difference= 365.28; q= 9.97; p= <0.01), 200µM (Dunnett multiple comparison test; 0 vs. 200: mean difference= 511.88; q= 13.98; p= <0.01), and 400µM (Dunnett multiple comparison test; 0 vs. 400: mean difference= 716.88; q= 19.58; p= <0.01). Total spore counts differed from controls at 100µM (Dunnett multiple comparison test; 0 vs. 100: mean difference= 362.5; q= 9.24; p= <0.01), 200µM (Dunnett multiple comparison test; 0 vs. 200: mean difference= 506.20; q= 13.86; p= <0.01), and 400µM (Dunnett multiple comparison test; 0 vs. 400: mean difference= 692.10; q= 18.95; p= <0.01). Linear regressions indicate that increasing EDTA concentrations are a significantly correlated with increasing spore counts from media (r^2= 0.873; DF= 1. 18; $p= 1.741*10^{-9}$), decreasing counts from Percoll® preparations (r^2= 0.861; DF=

1. 17; p= 1.035*10^{-8}), and reduced total spore counts (r^2= 0.846; DF= 1. 17; p= 2.516*10^{-8}).

Host-cell cultures treated with EDTA at 400µM did not survive the exposure period.

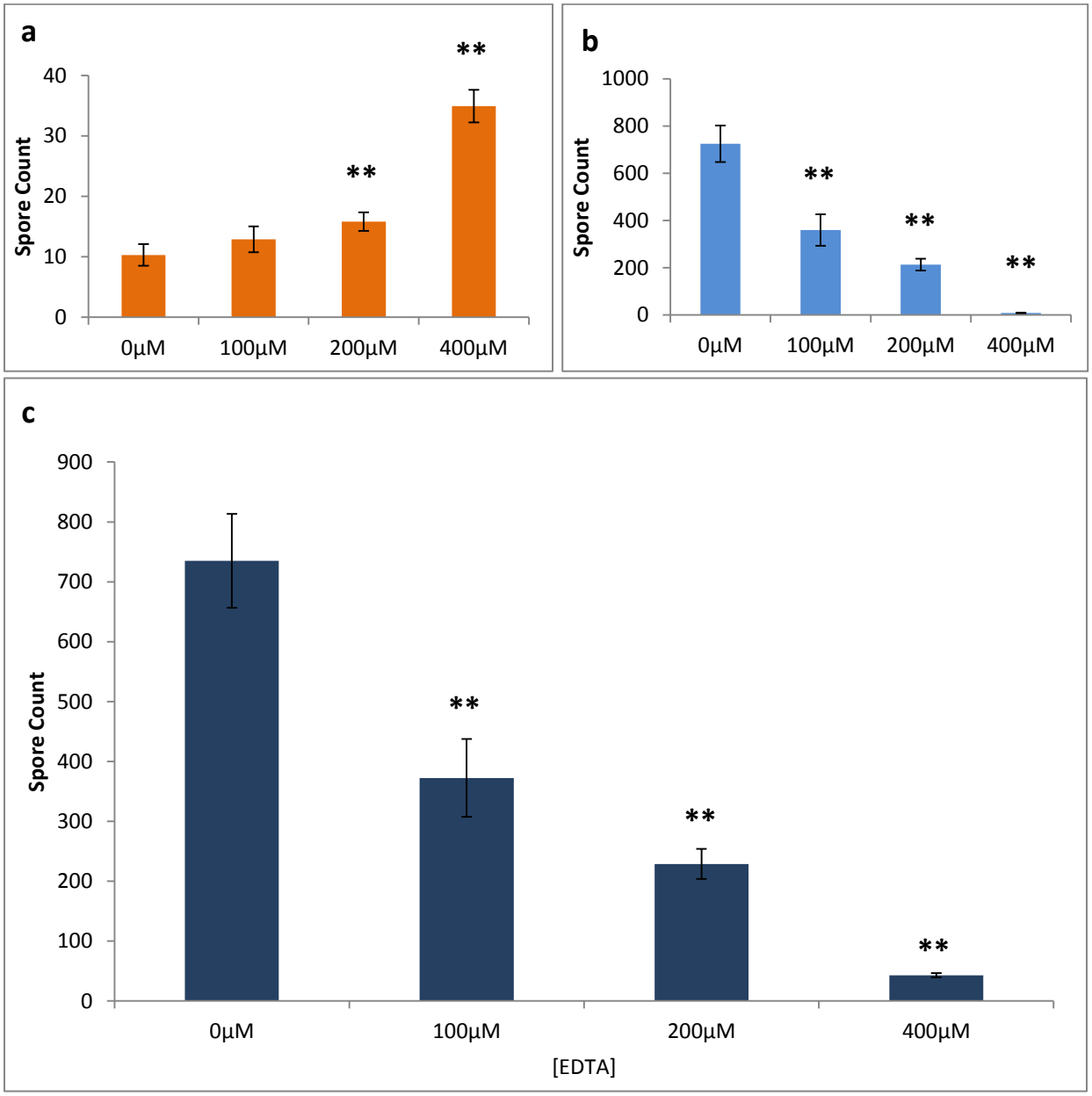

Figure 3.3: Haemocytometer counts of spores recovered from infected flasks eight days post-inoculation (n=5 replicates/treatment). a) Increased EDTA concentrations resulted in larger numbers of free-floating spores recovered from media (p=1.741*10^{-9}), (b) reduced numbers of infecting spores recovered by Percoll® centrifugation (p=1.035*10^{-8}), and (c) reduced total spore numbers (p=2.516*10^{-8}; chart c). Error bars indicate 95% CI.

Proteomics

Two-dimensional gels of spores washed with lysis buffer and loaded onto IPG strips at 5µg yielded three proteins at around 37 kDa and pH 5.5 (Figure 3.4). Gels run from samples prepared using greater initial spore numbers and treated with repeated freeze-thaw cycles and sonication produced approximately 12 protein spots, clustered around 37 kDA, 75 kDa, and 150 kDa. The majority of protein spots had an isoelectric point of approximately pH 5.5.

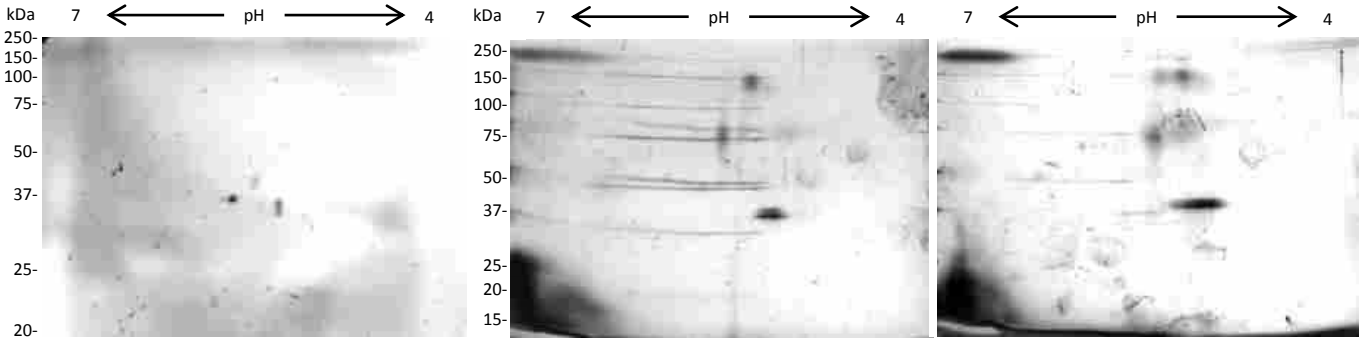

Figure 3.4: Two-dimensional gels obtained from *Anncaliia algerae* by exposing approximately two million spores to lysis buffer for 30 minutes (left), approximately eight million spores to lysis buffer followed by freezing (middle) and lysing eight million spores followed by repeated freeze-thaw and sonication (left). Spots clustered at around 37 kilodaltons (kDa) in all samples, gels run with higher protein load (right) exhibit additional spots around 75 and 150 kDA.

Discussion

The observation of enhanced infection rates at 10mM supplemental $MgCl_2$ in *Anncaliia algerae* supports previous research on the stimulatory effects of divalent cations, such as magnesium on spore adhesion and infection as first observed in human-infecting *Encepalitozoon sp.* by Southern *et al*. (2006). The apparent lack of stimulatory effects produced

by supplements below 10mM is likely a consequence of $MgCl_2$ already contained within L-15 media at a concentration of 0.95mM.

In light of the distant relationship between hosts for *Encephalitozoon* and *A.algerae*, the observation of magnesium-stimulated enhancement of infection in *A. algerae* also suggests that the response may be conserved amongst distantly-related microsporidian species. The putative role of magnesium in microsporidian infection is the activation of a spore wall protein involved in spore adhesion to host cells prior to initiation of spore germination processes (Southern *et al*., 2006). The adaptive benefit of such a system is clear—rising ionic concentrations, suggestive of a host environment, trigger adhesion to host cell glycoproteins such as mucin (Pleshinger and Weidner, 1985), which in turn stimulate the activation of the polar filament and sporoplasm discharge, ensuring that germination only occurs when spores are within sufficient proximity to a suitable host. Recent proteomic work has supported the role of spore wall components in infective processes through the identification of spore wall proteins that interact with the polar filament (Li *et al*., 2012) and protect spores from phagocytosis (Cai *et al*., 2011). In addition to this, experimental removal of spore wall proteins has been demonstrated to reduce *in vivo* infectivity in *Nosema bombycis* (Tu *et al*., 2011), further demonstrating the role of spore wall components in the mediation of successful infection in the microsporidia.

Given the apparent role of magnesium and manganese ions in the activation of infective processes in microsporidian parasites, the inhibitory effects of the chelating agent EDTA is hardly surprising. Indeed, similar work conducted by Southern *et al*. (2006) produced control-

level infections in cation treatments combined with 500µM EDTA. The chelating action of EDTA is likely inhibiting infection by interfering with additional infective processes as well, such as the role of Ca^{2+} influx in polar filament eversion. In the chelation treatment experiments the EDTA-mediated inhibition of infection was demonstrated in a previously un-tested microsporidian species. Furthermore, the inhibitory effects of EDTA appear to be related to dose concentrations, with observable effects, over the eight-day exposure period, at treatment concentrations not lethal to host cells. The observation of a positive correlation between EDTA concentrations and spores recovered from media is suggestive of inhibited spore adhesion to host cells, while the negative relationship between EDTA concentration and spore counts from Percoll® samples suggests a consequent inhibition of infection. Lastly, the downward trend in total spore count confirms that proliferation rates were lower at higher treatment doses.

Interestingly, while the number of infected cells and intracellular spores was reduced by EDTA treatment, it had no apparent effect on the number of spores per infected cell. This observation strongly suggests that while EDTA treatments decrease *A. algerae* infection rates *in vitro*, the parasite's intracellular proliferation and development is able to continue unabated following successful entry into a host cell.

The appearance of three major protein spots on initial two-dimensional gels at around 37 kDa corresponds well to previous findings of three major spore-wall proteins in proteomic analyses of *Nosema bombycis* (Wu *et al*., 2008), suggesting similar extra-sporular proteomic compositions between species. Wu *et al*. also reported the detection of 11 additional proteins. It is possible that the failure to visualize additional spots on initial 2-D gels run with *Anncaliia*

algerae was the result of extremely low —approximately 5µg— protein loads, which is at the lesser end of recommended loading levels. Subsequent gels yielded additional protein spots, suggesting that the combination of freeze-thaw cycles and sonication may have disrupted the spore wall. Conversely, it is possible that the additional isolated proteins are the product of higher protein loading, and therefore represent more minor spore wall components. All gels prepared from *A. algerae* samples exhibited reduced numbers of isolated proteins when compared to samples obtained from fish cell lines (Figure A.5, Appendix), consistent with the prediction of a limited proteome in microsporidian parasites.

The observation of similar responses to culture conditions exhibited by disparate microsporidian species is strongly suggestive of highly-conserved infection mechanisms amongst species with very dissimilar hosts and life histories. Continued research is needed to evaluate the similarity of infective processes over a wider range of microsporidian parasites, and to determine if these mechanisms can be co-opted to trigger the germination and proliferation of previously uncultured microsporidia species *in vitro.* Additionally, due to the shared response to EDTA treatments between such distinct microsporidian species, it would be worthwhile to evaluate the susceptibility of *A. algerae* to treatments successfully used to modulate infections caused by other microsporidians. One such example is the use of synthetic polyamines to reduce infections *in vitro* and *in vivo* (Bacchi *et al*., 2002). Infectivity trials conducted on *A. algerae* in fish cells can be conducted using spermine at non-lethal doses (Figure A.6, Appendix) to determine the efficacy of polyamine treatments in managing arthropod-infecting microsporidian infections.

Ch. 4: Use of a Cod-Derived Cell Line and Novel Culture Methods for the *In Vitro* Culture of *Loma morhua*

Abstract

Commercial-scale aquaculture of the Atlantic cod (*Gadus morhua*) has expanded considerably since its beginnings in the late 1980s, achieving global production in excess of 20,000 tonnes in 2008. Despite this success, many challenges need to be overcome to ensure the viability of cod farming operations. High disease prevalence in cod farms is one of the greatest problems confounding efforts today, with numerous viral, bacterial, and eukaryotic pathogens causing decreased growth rates, increased incidence of deformities, and excessive mortality in livestock. Microsporidosis caused by the intracellular microsporidian pathogen *Loma morhua* is an emerging concern in North American and Icelandic fish farms, causing chronic losses that cannot be managed by conventional treatment methods. Despite the prevalence of the disease and its potential economic impact, very little is known about the biology of *L. morhua* and microsporidosis in general. *In vitro* infection models are useful for the study of intracellular pathogens, permitting observations that would be impossible *in vivo*, improving the ease with which live specimens of the parasite may be obtained, and facilitating the use of specialized laboratory-based techniques. This report outlines the formation of basic *in vitro* culture methods for the *Loma* parasite, using a novel cod larvae-derived cell line and pH shifts in exposure media to stimulate spore germination. *L. morhua* infection was successfully stimulated in a representative host cell line by pH shift treatment, representing the first reported *in vitro* culture of a *Loma* species.

Keywords: Microsporidia, parasitology, Atlantic cod, infection model, *in vitro*

Introduction

Global capture rates of Atlantic cod (*Gadus morhua*) have fallen sharply in recent decades (FAO, 2011). This decline has been particularly severe in North American waters, where historically rich fishing grounds have been pushed beyond the brink of collapse resulting in cod-harvest moratoriums and substantial economic losses (DFO, 2001). Aquaculture practices have emerged as a means of meeting continually-growing demands for fish meat, generating economic growth, and easing the burden currently placed on wild fish stocks around the globe. Commercial-scale farming of *G. morhua* was first attempted in the late 1980s, in conjunction with the decline in productivity of wild fisheries (FAO, 2010b). Initial efforts were largely unsuccessful due to poor juvenile production and profitability remained low, limiting the drive for continued efforts. Measurable success was first achieved in the early 2000s and production levels rose sharply in the following years, generating considerable interest in cod farming. The economic crisis of 2008 reduced both the profitability and productivity of existing cod farms (FAO, 2010b). However, the foundations of rearing cod in aquaculture programs have been laid, and the prospects for continued growth are good.

One of the greatest problems currently facing cod aquaculture are the heavy losses imposed by piscine diseases. As a somewhat novel species for captive rearing, *G. morhua* is host to a variety of poorly understood diseases, many of which have no known cure or treatment. The challenges presented by diseases in livestock are further exacerbated by the increased susceptibility and transmission rates created by the crowding and handling stress commonly encountered in fish colonies. If cod aquaculture is to achieve production levels comparable to

wild catches, increased research into fish pathogens will be required. Of cod pathogens, those caused by bacteria are currently the best understood; research has been conducted into vaccines for both bacterial listonellosis (Caipang *et al*., 2008) and atypical furunculosis (Lund *et al*., 2008). Other important pathogens remain poorly studied, including bacterial agents such as *Francisella noatunensis*, and intracellular pathogens such as the microsporidian parasite *Loma morhua*.

A fungal parasite, *L. morhua* infects host cells by extruding genetic material into their cytoplasm through a polar filament. Once inside the host cell, the parasite proliferates, eventually filling the host cell cytoplasm, leading to cell death and the release of new infective spores into the environment. Similar to other fish-infecting microsporidian parasites, *Loma* infections are typically associated with the formation of enormous, spore-filled cells known as xenomas (Lom and Dykova, 2005), which are often observed on the gills and gut epithelium of infected fish (Morrison, 1983), and inflict mortality rates as high as 63 per cent (Khan, 2005).

As intracellular pathogens, microsporidians like *L. morhua* are particularly good candidates for *in vitro* studies. Indeed, our current understanding of microsporidian parasites is based largely on research conducted with species that are readily cultured *in vitro* such as *Encephalitozoon* sp. (Katinka *et al*., 2001; Hayman *et al*., 2005; Brosson *et al*., 2006) and *Nosema* sp. (Gisder *et al*., 2011; Higes *et al*., 2011). Many novel and important insights can be produced by developing laboratory-based culture methods and infection models for the microsporidia, and the formation of such systems could aid greatly in the continued advancement of cod aquaculture practices. To this end, the development of reliable *in vitro*

culture methods for *L. morhua* was attempted using several fish-derived cell lines—including a recently developed cod cell line (GML-5)—and novel culture methods meant to simulate physiochemical conditions local to the primary sites of infection such as the intestinal epithelium (Sanchez *et al*., 2001).

Methods

Spore Samples

Mature *Loma* spores were obtained from xenomas provided by Dr. Michael Duffy at the University of New Brunswick (Saint John, New Brunswick). Mature Atlantic cod collected from aquaculture facilities were dissected and observed for signs of *Loma* infection in the gills and spleen. Infected fish were further dissected for the removal of xenomas, which were then suspended in 1ml sterile saline in bullet tubes and sent by mail to Wilfrid Laurier University (Waterloo, Ontario). Suspended xenomas were broken apart by physical agitation using sterile sharp probes, re-suspended in 3ml cell culture grade H_2O, mixed with 3ml Percoll® (Sigma), and centrifuged at 1136 RCF for 20 minutes. The supernatant was then removed and the pellet re-suspended in L-15 growth media containing 2X Gentamicin-Amphotericin B to combat any residual contamination. Purified spore samples were stored in tissue culture flasks prior to inoculation on various cell lines.

Preliminary Infection Trials

As a representative cod-derived line was not yet available, initial attempts at culturing *L. morhua* spores were made using surrogate cell lines derived from the tissues of haddock embryos (HEW), and rainbow trout gills (RTgill-W1). These cell lines were selected for the close

phylogenetic relationship between *G. morhua* and the haddock (in the case of HEW cells), and for the fact that *Loma* infections are typically observed on the gills of afflicted fish with RTgill-W1. These first trials were performed by inoculating 5ml of purified *Loma morhua* spore suspensions onto confluent cultures of surrogate cells in 25cm^2 tissue culture flasks. Co-cultures were incubated at 8°C and observed using phase-contrast microscopy for signs of spore germination, infected cells, and the appearance of developmental stages comparable to those observed in the readily-cultured *Anncaliia algerae* (Figure 4.1).

Cod cell-*Loma* co-culture

Subsequent to the establishment of a stable cod larvae-derived cell line (GML-5), an infection trial was performed using $MgCl_2$ supplements following the methods outlined in Chapter Three (page 48). Spores were suspended in media containing 0mM, 1mM, and 10mM supplemental magnesium and inoculated onto bare GML-5 cultures.

To simulate pH shifts from neutral to alkaline—previously found to stimulate germination in another marine fish-infecting microsporidian species (Pleshinger and Weidner, 1985)—spores were suspended in Minimal Essential Medium containing 10% FBS and 1% PS, and inoculated onto 12.5cm^2 GML-5 cultures in Falcon tissue culture flasks with 0.5ml of spore suspension. These were then incubated at 8°C and observed using phase-contrast microscopy. The pH of MEM rises to 8.2 in the presence of GML-5 cells (Figure A.7, Appendix), allowing pH shifts to occur while spores and cells are in close proximity. To determine if developing spores were contained within the host membrane, cells were passaged by trypsinization, centrifugation, re-suspension, and plating 31-days post-inoculation (PI).

A second MEM trial was performed following the methods outlined above with several minor alterations. Several spore samples were exposed to acidic conditions prior to suspension in MEM, by suspension in L-15 brought to a pH of 5.0 with 1.0M HCl. The spores were then re-centrifuged, and suspended in MEM media supplemented with 10mM $MgCl_2$ and 0.065% mucin (m/v)—following Pleshinger and Weidner (1985). 0.5ml of the spore suspension was then inoculated onto confluent GML-5 cultures in 12.5cm^2 tissue culture flasks. Plates were incubated at both 8°C and 18°C. All pH measurements were made using a Fisher Scientific accumet BASIC AB15 pH meter.

Due to the severe limitations in the availability of *L. morhua* samples and correspondingly limited spore numbers (three 1ml bullet tubes), all experiments were conducted on a preliminary basis. This precluded any evaluations of infection intensity and concrete comparisons with other previously-cultured microsporidian species. Statistical analyses were performed on infection trials.

Results

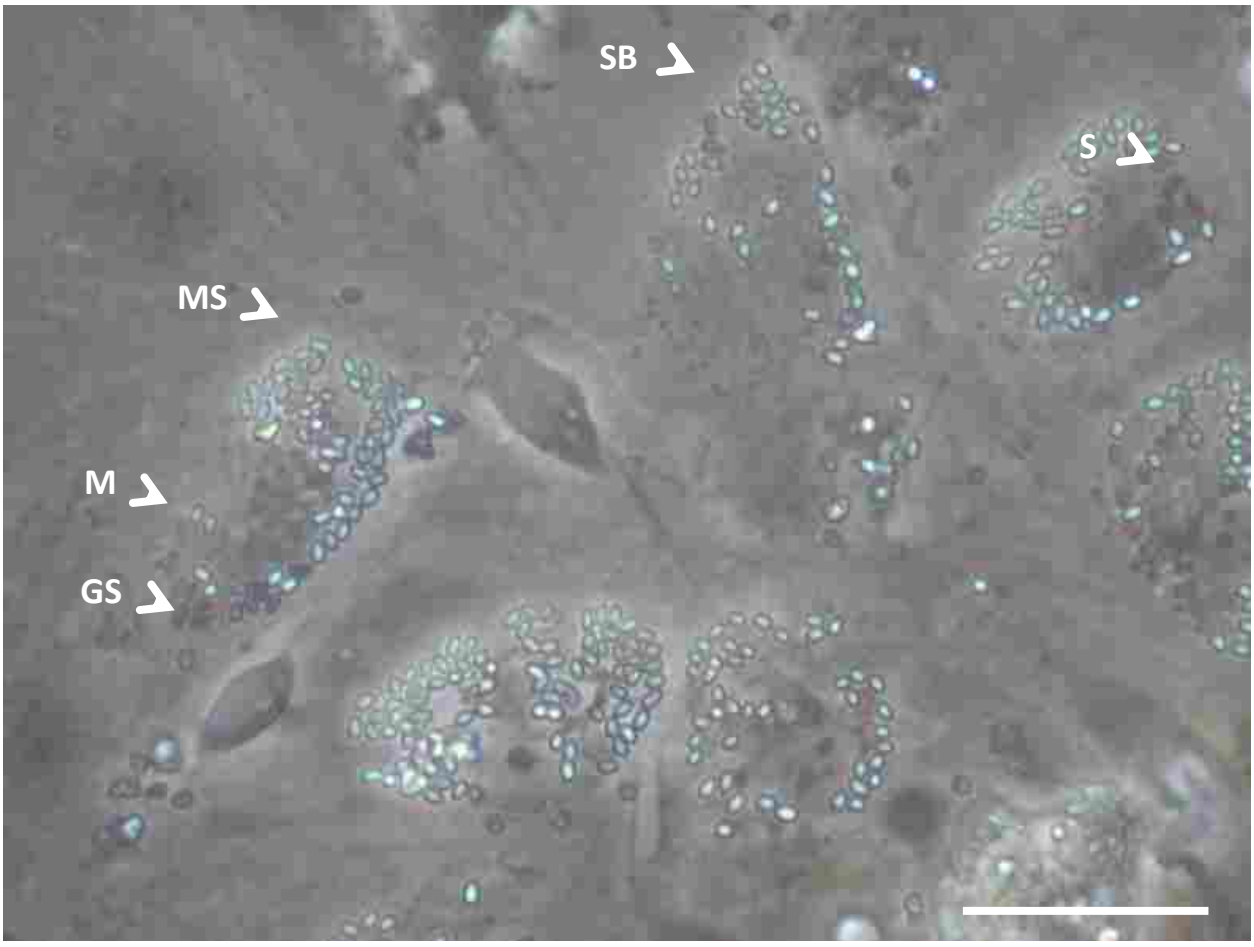

Figure 4.1: Micrograph of goldfish skin (GFSk-S1) cells infected with *Anncaliia algerae* eight days post inoculation. Spore clusters represent infected cells. Arrows indicate stages of the parasite's life cycle: germinated spore (GS), meront (M), sporont (S), sporoblast (SB), and mature spore (MS). Scale bar = 50µm.

Surrogate Cells

Co-cultures of *L. morhua* spores with stand-in HEW and RTgill-W1 cell lines did not exhibit any signs of infection over the 30-day observation period. All visible spores in inoculated flasks remained phase-bright, were freely dispersed in the media, and no developmental stages were observed (Figure 4.2).

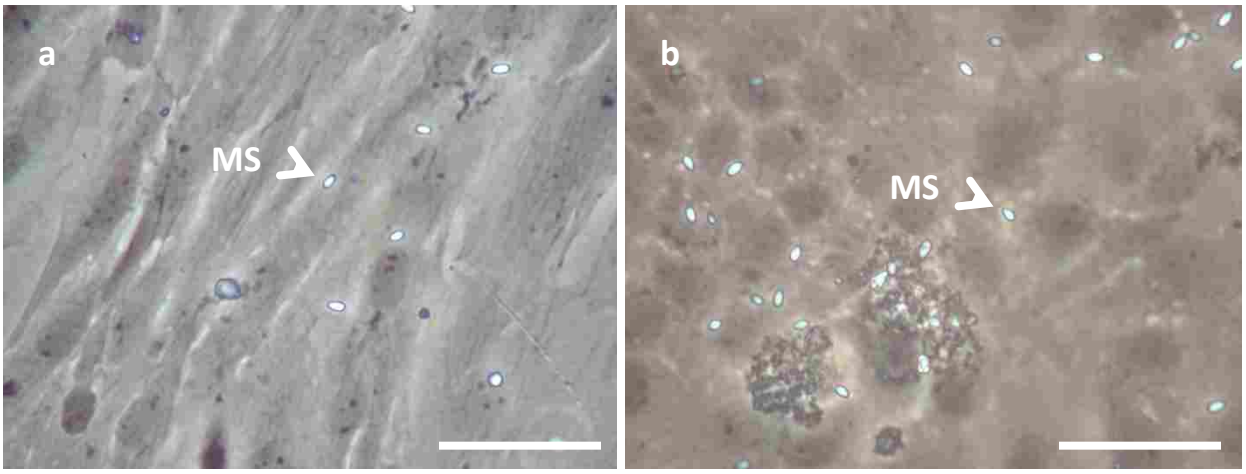

Figure 4.2: *Loma morhua* spores (MS) in co-culture with (a) haddock-derived (HEW) cells and (b) rainbow trout-derived (RTgill-W1) cells four days post-inoculation. Phase-bright appearance indicates that spores have not germinated. Scale bar = 50µm.

Cod $MgCl_2$ Trial

A single observation of an infected cell was recorded 20-days post-inoculation (PI), in GML-5 flasks inoculated with *Loma morhua* suspended in magnesium-supplemented growth media (Figure 4.3). The infected cell was observed in a control flask that was not supplemented with additional $MgCl_2$.

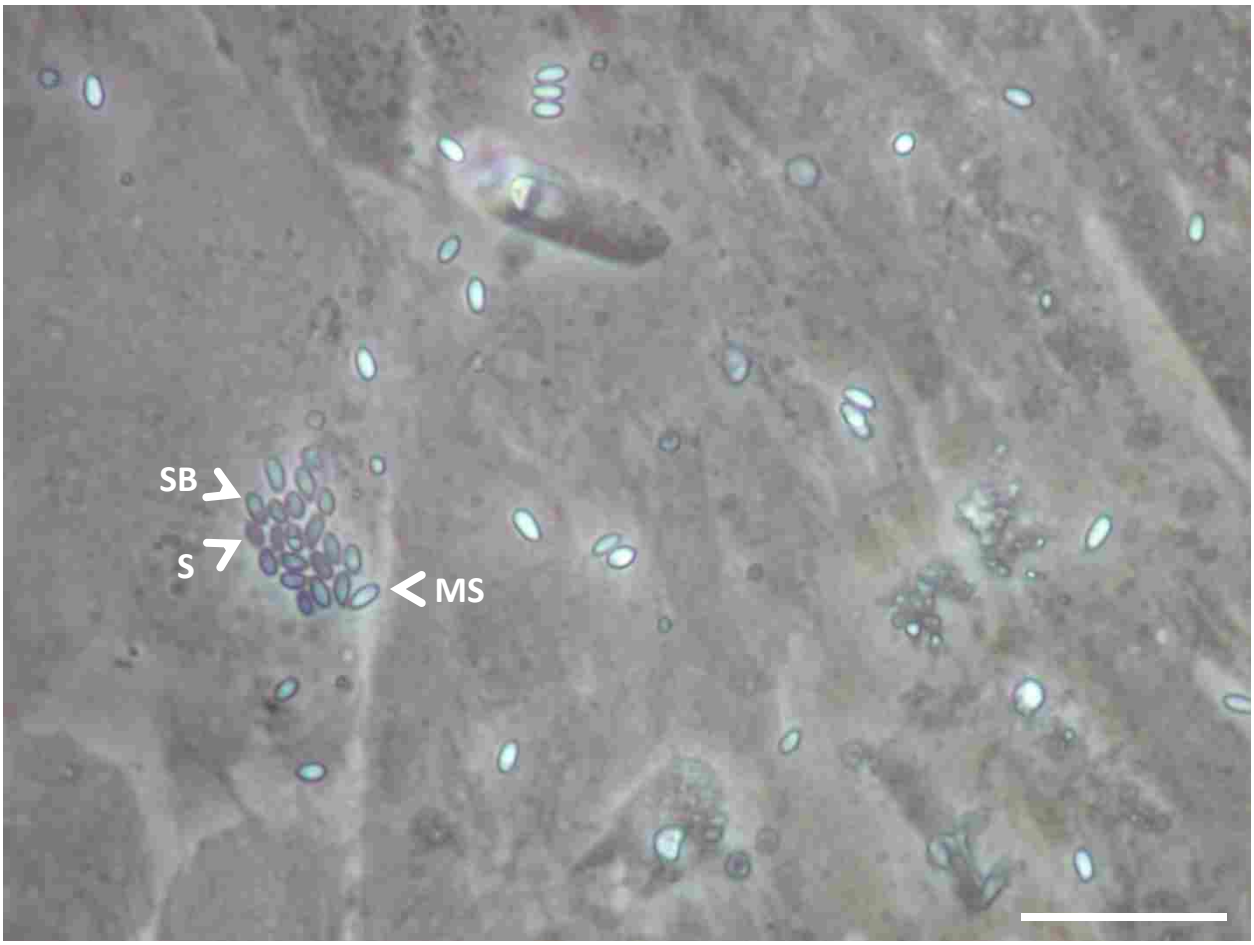

Figure 4.3: Micrograph showing intracellular development of *Loma morhua* co-cultured with cod-derived GML-5 cells 20 days post-inoculation in a control flask from magnesium supplement experiment. Arrows indicate developmental life stages of the parasite: sporont (S), sporoblast (SB), and mature spore (MS). Scale bar = 50µm.

Cod MEM Trials

Multiple instances of intracellular developmental stages were observed by 15 days PI and pH shift treatment (Figure 4.4a). The number of intracellular spores in infected cells increased considerably by 31-days PI (Figure 4.4b, c, and d). Spore-filled cells were visible in fresh flasks shortly after passaging 31 days PI (Figure 4.5). Passaged co-cultures did not produce additional observations of infected cells.

The second MEM trial resulted in numerous infected cells similar to that observed in the first trial. Multiple observations were made of free-floating spores with very dark appearance, randomly interspersed with normal phase-bright spores. Infected cells were first observed by six days PI, expanding to multiple infections and increased numbers of intracellular spores by 21 days PI. Low pH (5.0) treatment prior to suspension in MEM did not appear to have any substantial effect on infection rates, with infected cells appearing in both treatments by day 6 and exhibiting similar infection intensity by 21 days PI. No infected cells were observed in flasks incubated at 18°C. All four flasks maintained at 8°C contained infected cells.

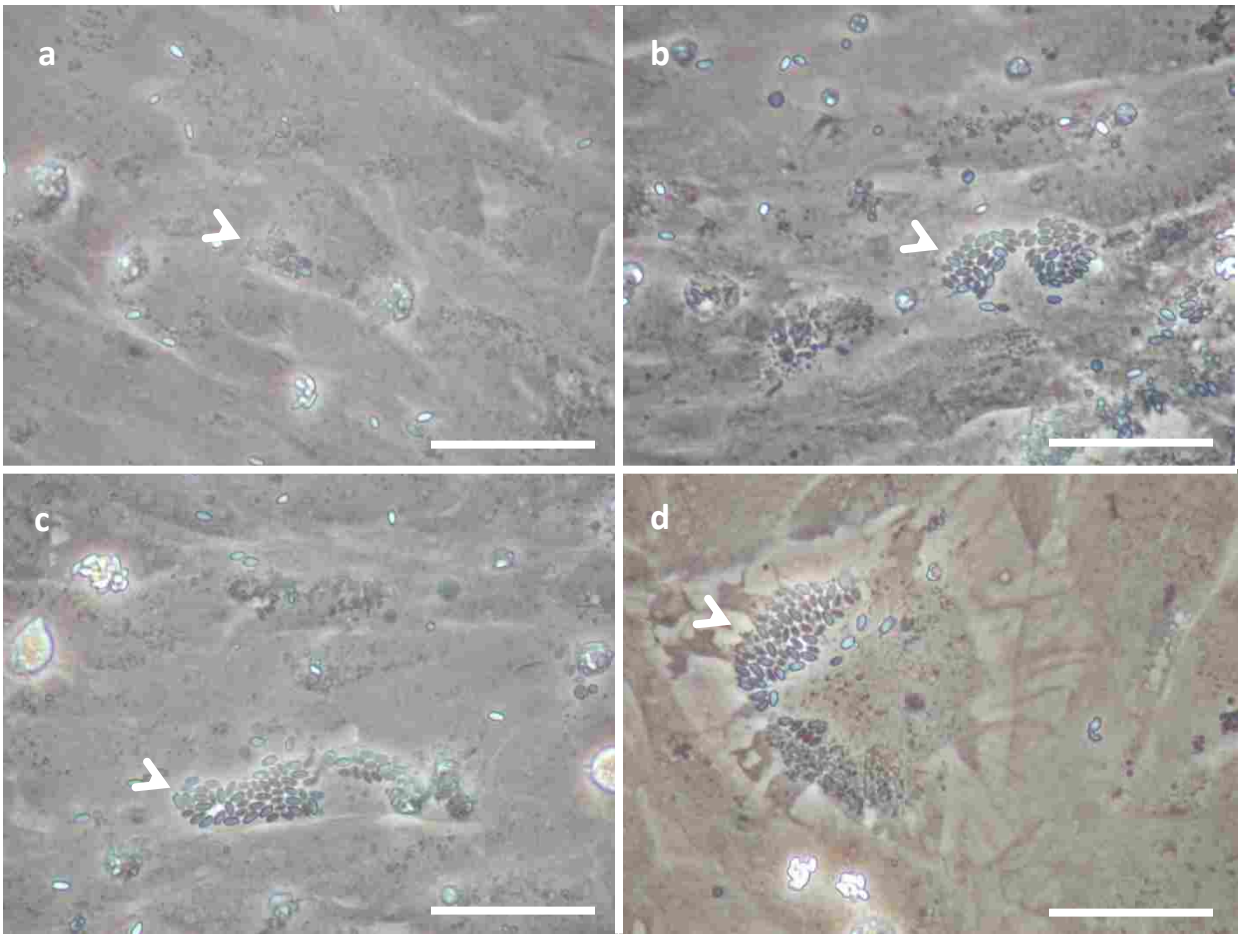

Figure 4.4: Micrographs of *Loma morhua* spores growing in cod (GML-5) cells following suspension and inoculation in minimum essential medium (MEM) 15 days post-inoculation (a) and several infected cells 35 days post-MEM treatment (b,c,d). Arrows indicate infected cells. Scale bars = 50µm.

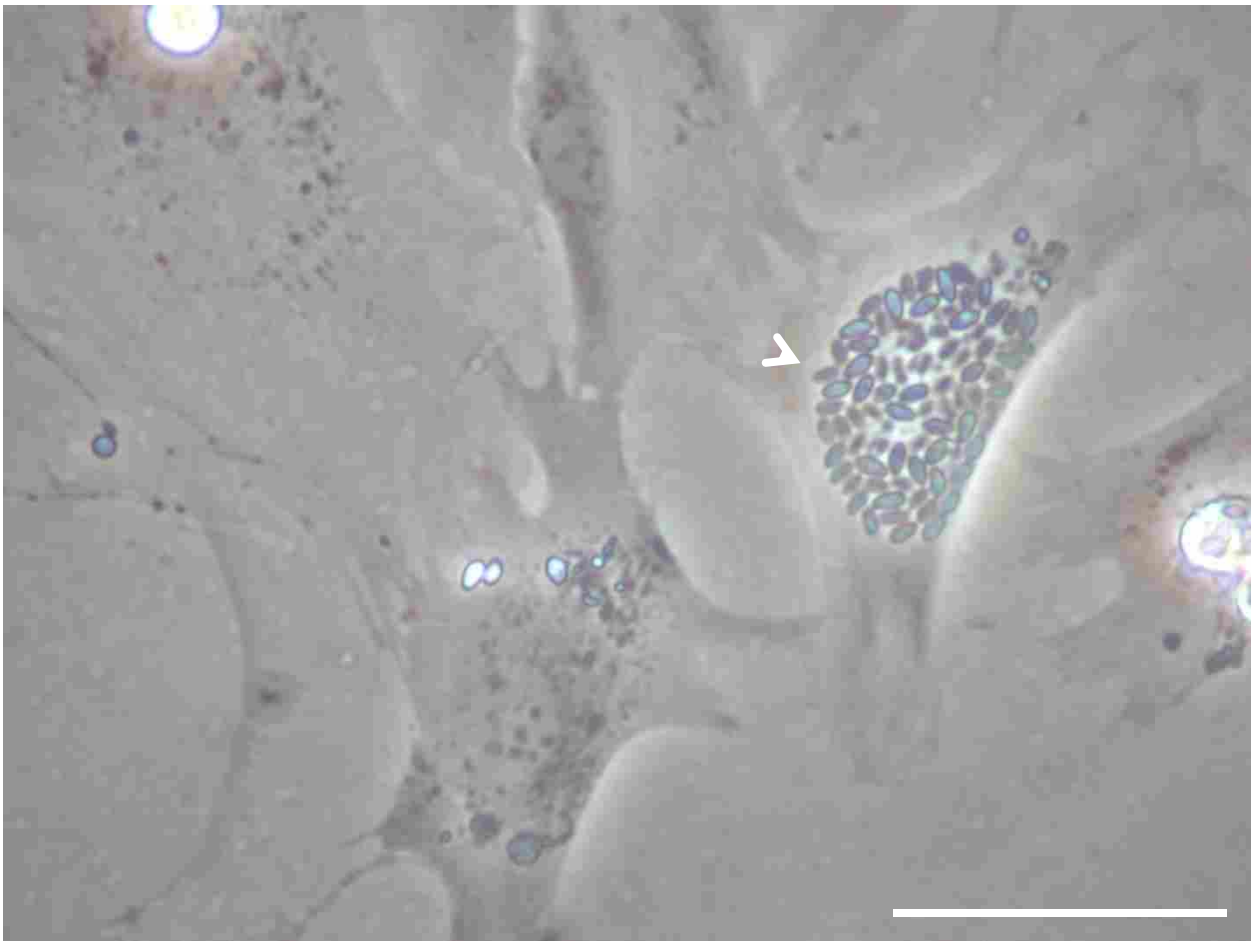

Figure 4.5: Micrograph depicting intracellular spores transferred within a cod (GML-5) cell to a fresh culture flask one-hour post-passage, 36-days post-inoculation. Arrow indicates infected cell. Scale bar = 50µm.

Discussion

The results of this study represent the first successful infection by, and production of, *Loma morhua* spores *in vitro*. Initial culture attempts using *Loma*-inoculated HEW, RTgill-W1, and GML-5 cultures failed to produce appreciable infection rates, with visible developmental stages appearing only in the cod-derived cells. Given the apparent lack of host-specificity exhibited by several microsporidian parasites (Lores *et al*., 2003; Monaghan *et al*., 2011), it is interesting to note that infected cells were only observed in a cell line representative of the

parasite's host species. This suggests the possibility of more specific host requirements than previously-cultured species. However, given the limited number of infections achieved by simple inoculation protocols, additional experimentation will be required to determine the suitability of non-host species cell lines. Based on *in vitro* research performed on other microsporidian species, the spore germination and polar filament discharge processes are associated with a sudden change in appearance from phase-bright to phase-dark (Bhat and Nataraju, 2007). The observation of only free-floating, phase-bright spores in initial inoculation attempts strongly suggests that spore germination was not occurring in culture, precluding any subsequent infections and parasite proliferation. The observation of phase-dark spores following exposure to alkaline pH conditions (by suspension and inoculation in MEM) indicate that triggering infective processes in *L. morhua* requires a shift from neutral to basic pH. This result is consistent with observations of spore activation and discharge in *Spraguea lophii*, another marine-fish-infecting microsporidian parasite (Pleshinger and Weidner, 1985). It is probable that the observed response to specific pH changes represents a means of detecting passage from the gut to the intestinal tract via pH shifts from acidic to alkaline by pancreatic secretions. Pleshinger and Weidner also noted that polar filament discharge was only observed with concomitant exposure to polyanionic compounds such as mucin, likely representing a response to cell surface proteoglycans. The conditions observed to trigger spore germination compare favourably with existing literature reporting the intestinal epithelium as the primary site of infection in other *Loma* species (Sanchez *et al*., 2001).

Modifications to MEM spore-delivery protocols yielded several interesting observations. First, exposing spores to acidic conditions (pH 5.0) prior to the shift to alkaline pH did not

appreciably change infection rates. This suggests that a pH shift from neutral to basic is sufficient for the activation of polar filament discharge in *L. morhua*, consistent with previous findings in *S. lophii* (Pleshinger and Weidner, 1985). Second, $MgCl_2$ supplements produced the earliest observations of infected cells and greater apparent rates of infection, suggesting that Mg^{2+} plays a role in the infective processes of *L. morhua* similar to that observed in *Encephalitozoon* sp. (Southern *et al*., 2006) and *Anncaliia algerae*. Third, despite the importance of glycosylated proteins in spore germination, mucin supplements did not increase infection rates beyond those observed in the first trial. If glycosylated cell surface proteins serve as a spore adhesion site on host cells, then the presence of exogenous substrates would be predicted to interfere with the infection process, rather than enhance it. Lastly, the apparent lack of infections occurring at incubations of 18°C and consistent appearance of intracellular developmental stages in co-culture held at 8°C are strongly suggestive of temperature dependence in successful cell invasion and proliferation in *L. morhua* at temperatures representative of *in vivo* conditions (Bosman *et al*., 2011), and within suitable ranges for larviculture (Jordaan and Kling, 2003).

Ultimately, the successful stimulation of *L. morhua* spore germination and subsequent appearance of developmental stages within the Atlantic cod-derived GML-5 cell line represents the foundation of a novel, laboratory-based infection model for the *Loma* parasite. Continuing efforts will be required for investigations into optimal growth conditions for the developing parasite, as well as the suitability of other cell lines for supporting *L. morhua* culture *in vitro*. As with other microsporidian species, laboratory culture of *Loma* spores can facilitate *in vitro* research into many unexplored areas of the parasite's life cycle (Schottelius *et al*., 2001; Gisder

et al., 2011), growth requirements (Southern *et al*., 2006), physiology (Weidner and Byrd, 1982; Hayman *et al*., 2005), genetics (Katinka *et al*., 2001), and proteomics (Brosson *et al*., 2006). By better understanding the parasite, valuable insights into treatment measures and improved microsporidosis management strategies for cod farming may be uncovered, enhancing livestock health and assisting ongoing aquaculture efforts.

Ch. 5: General Discussion, Implications for Microsporidian Culture, & Future Directions

Significance & Possible Uses of Cod Cells

The successful establishment of a cod-derived cell line presents a valuable tool for research into the biology of the Atlantic cod at reduced cost and without the need for large numbers of living fish. The tolerance of cool conditions and inability to withstand high temperatures exhibited by the cod-derived GML-5 cells is fairly similar to existing cell lines derived from cold-water fish (Ganassin *et al*., 1999; Bryson *et al*., 2006), suggesting possible uses in physiological research into cold-water fish species. Research conducted using GML-5 cells may also be utilized to address many of the important problems currently confounding cod aquaculture efforts. By answering questions of cod physiology, toxicology, and parasitology, conditions for livestock health can be improved, growth rates increased, and mortalities reduced. The bulk of this research has focused on the use of GML-5 cells for establishing an *in vitro* infection model for the emerging cod pathogen *Loma morhua*, but the cell line lends itself to other areas of research as well.

Insights into Microsporidian Infection

Observations of magnesium-mediated infection stimulation and inhibition by EDTA in *A. algerae* conform closely to results from similar experimentation using *Encephalitozoon* sp.—microsporidian pathogens of widely different host species (Southern *et al*., 2006). The findings not only support the role of magnesium in microsporidian infection processes, but that several

mechanisms governing the infection process may be shared amongst many microsporidian parasites.

The stimulation of microsporidian infection by supplemental magnesium suggests that the process is dependent on the presence of cations, similar to infection processes in non-microsporidian parasites (Hamer *et al*., 1994; Petrópolis *et al*., 2008). The role of magnesium in facilitating spore adhesion to host cells suggested by Southern *et al*. (2006) is supported by the observation of equal average spore numbers in infected cells from both control and EDTA-treated cultures. As intracellular spore proliferation was unaffected by chelation treatments, it would seem that the role of magnesium is served prior to the penetration of host defenses. The combination of increased free-floating spores in EDTA-treated flasks (following eight days of co-culture with GFSk-S1 cells) further supports the proposed role of magnesium in stimulating host-binding prior to infection. The chelating action of EDTA is likely inhibiting infection by a variety of means. The first is the removal of Mg^{2+} and Mn^{2+}, inhibiting adhesion to host cells. Second, the removal of Ca^{2+}—critical for polar filament eversion—likely prevents successful entry into the host's cytoplasm. Lastly, it is possible that EDTA presents an exogenous negative binding site for spore adhesion, preventing the parasite from binding to correct sites, even in the presence of sufficient magnesium concentrations.

Interestingly, the work by Southern *et al*. (2006) found that Ca^{2+} did not enhance host cell attachment, but did increase infection rates. While this may seem counterintuitive, it does not contradict previous observations of calcium's role in spore germination (Weidner and Byrd, 1982; Leitch *et al*., 1993). Instead, the observation provides evidence that spore adhesion and

activation are separate processes, mediated by distinct factors. By coupling these observations with other factors found to govern microsporidian infection, it is possible to break the infection process down into several steps: (1) the triggering of spore-wall host-adhesion by the presence of sufficient Mg^{2+} or Mn^{2+} concentrations, activating spore wall components (Southern *et al*., 2006) involved in (2) adherence to glycosylated proteins such as those found on the surface of host cells (Pleshinger and Weidner, 1985; Hayman *et al*., 2005). Adherence is followed by (3) activation of germination processes by a combination of increasing internal hydrostatic pressure by the rapid influx of calcium ions (Weidner and Byrd, 1982; Hayman *et al*., 2005) and the possible cleavage of intrasporular trehalose stores to glucose (reviewed in Méténier and Vivarès, 2001), both potentially mediated by interactions between a recently identified spore-wall protein and the polar filament (Li *et* al., 2012). These processes are followed by (4) the discharge of the polar filament, subsequent extrusion of sporoplasm into the host cell, and initiation of the intracellular proliferation phase of the parasite's life cycle (Figure 5.1). Several studies have provided evidence for additional factors that serve a probable role in mediating infective processes such as the presence of digestive enzymes such as pepsin (Lores *et al*., 2003) and changes in pH (Pleshinger and Weidner, 1985; Lores *et al*., 2003; Kucerova *et al*., 2004).

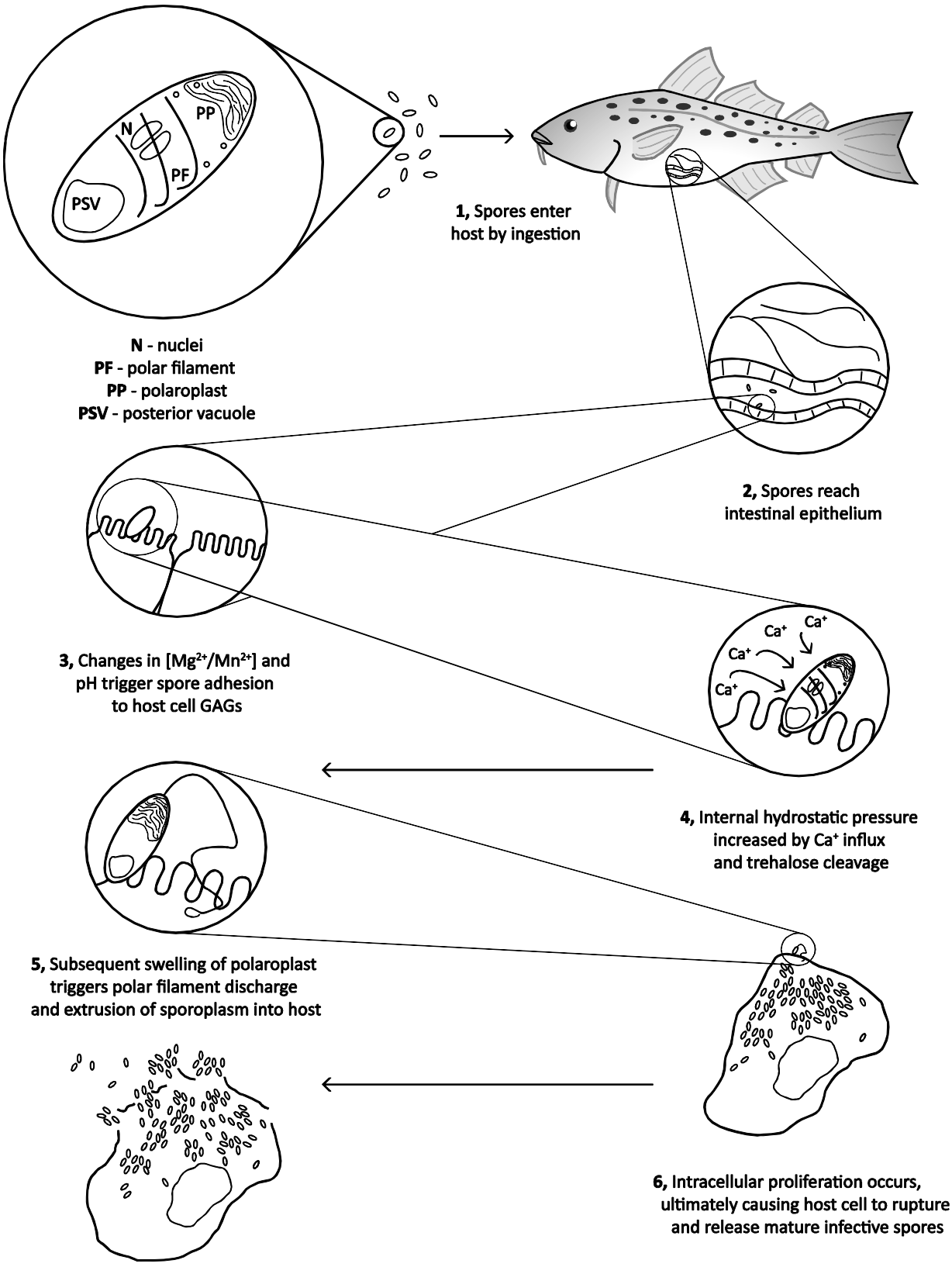

Figure 5.1: Pathway and mechanisms of microsporidian infection in fish. Image © Mike MacLeod, 2012.

The infectivity response to EDTA treatment (observed by haemocytometer counts) provides evidence of a dose-dependent inhibition of spore adhesion at concentration levels that do not impair host-cell viability *in vitro*. The work performed by Southern *et al*. (2006) demonstrated a reversal of infection augmentation using short-term EDTA treatments of 500µM, beyond the 400µM treatments that proved fatal to host cells over an 8-day period in this study. Treatments of 100µM and 200µM produced proportional reductions in host-cell infection rates without destroying the host.

The *Loma* Parasite

Using growing knowledge of parameters affecting microsporidian infection, it was possible to successfully induce *Loma morhua* infection in representative host cells (GML-5), effectively creating an *in vitro* infection model for the *Loma* parasite and the Atlantic cod. The use of MEM media as a spore delivery vehicle allowed for the exposure of spores to high pH conditions while in intimate proximity to hosts, producing appreciably higher infection rates than by simple inoculation processes. It is probable that the upward shift in pH experienced by the *Loma* spores was the critical factor in stimulating their germination and subsequent infection in cell culture, supporting observations of pH as an important factor in triggering spore germination in other species of microsporidia (Pleshinger and Weidner, 1985; Kucerova *et* al., 2004).

Though pH likely plays a key role in inducing germination, the infection-enhancing effects of Mg^{2+}, Mn^{2+}, and Ca^{2+} observed in this and other studies may likely be employed to

further improve infection rates *in vitro*. Additionally, though the critical importance of glycosoaminoglycans in spore adherence observed in other microsporidian species (Pleshinger and Weidner, 1985; Hayman *et al*., 2005) may be satisfied by existing cell-surface proteins expressed by GML-5 cells, infection rates may be further enhanced by utilizing cell lines with higher levels of mucous production. By improving infection rates *in vitro* it will be possible to enhance spore production levels, facilitating further research into an important cod pathogen and potentially uncovering methods for treatment of microsporidosis in fish.

Continuing Research: Extensions to Other Microsporidians

The successful use of GML-5 cells to support *in vitro* infection and growth of *L. morhua* demonstrates the potential efficacy of simple alterations to standard spore culture protocols used for *A. algerae*. However, although *Loma* infection was successfully achieved, infection rates remained low, and development occurred slowly when compared to other previously-cultured microsporidian species. Several steps will be necessary to evaluate the feasibility of improving *Loma* infection in cultured cells, such as employing altered culture methods found to enhance infection rates in other species and determining optimal incubation temperatures for parasite growth and development. Given the limited host-specificity exhibited by other microsporidian parasites *in vitro* (Monaghan *et al*., 2011), it may also be worthwhile to evaluate the suitability of additional cell lines for better supporting *Loma morhua* infections.

Triggers for producing appreciable *in vitro* infection rates appear to be highly conserved among distinct microsporidian groups with widely different host species. Mg^{2+} has been found to enhance infection in arthropod-infecting (this study) and mammal-infecting (Southern *et al*.,

2006) species. Glycosylated cell surface components have been found to play an essential role in successful infection by both fish-infecting (Pleshinger and Weidner, 1985) and mammal-infecting (Hayman *et al*., 2005) microsporidians. As well, pH has been observed to mediate germination in species with arthropod (Kucerova *et al*, 2004) and piscine (Pleshinger and Weidner, 1985; Lores *et al*., 2003) hosts. Additional supplements such as calcium may also be useful for stimulating greater infection rates *in vitro.* Given the apparent similarity in infection processes exhibited by different species of microsporidia, it is likely that advances in the culture methods of one species may also be applied to others.

Despite many similarities, it is apparent that various microsporidian species differ in the specificity of their growth requirements and their sensitivities to infection-inducing conditions. For example, *A. algerae* infectivity appears to be enhanced by incubation temperatures of 24°C and above (Figure A.8, Appendix), well beyond levels suitable for the growth of *L. morhua*. Spore sensitivity to pH conditions appears variable as well—Kuverova *et al*. observed that a pH of 9.5 was optimal for inducing the germination of *Anncaliia* (*Brachioloa*) *algerae*, similar to results observed in *Spraguea lophii* by Pleshinger and Weidner (1985) and *Loma morhua* in this study. *A. algerae* infection in cell culture, however, does not seem to require any modulation of pH conditions. These differences likely reflect specific aspects of host physiology and differences in the proportion of the parasite's life cycle spent in intimate affiliation with a suitable host. Arthropod-infecting microsporidian species have been found to be transmitted vertically (Goertz *et al*., 2007; van Frankenhuyzen *et al*., 2007), potentially to the exclusion of horizontal transmission in some cases (Ironside *et al*., 2003). It would be adaptive for microsporidian parasites with freely dispersed life stages to require conditions more specific to

suitable hosts for the initiation of germination. Any attempts at culturing additional microsporidian species will therefore require consideration of both parasite and host biology.

The methods used to stimulate infection by *Loma morhua* may be useful for the *in vitro* culture of additional piscine-infecting microsporidians of commercial and scientific importance. Such species include the salmonid-infecting *L. salmonae*, the zebrafish pathogen *Pseudoloma neurophilia*, and the ornamental fish-infecting *Plesitophora hyphessobryconis*. A better understanding of the parasites and greater control over the damage they cause can be attained through continuing efforts in the development of novel *in vitro* infection models, improved spore production using cell culture systems, and research using laboratory-produced spores. The insights yielded by *in vitro* work may then be used for *in vivo* applications, enhancing aquaculture methods, piscine-veterinary technology, and the efficiency with which farmed fish can be produced. Such advancements would not only serve the scientific community, but enhance aquacultured food production in a time when wild fish populations face an uncertain future.

Summary

The cod fisheries of the Canadian Atlantic have been pushed beyond the point of collapse and the formerly productive fishery now yields only meagre harvests. As a result of poorly regulated fishing practices, cod harvests suffered severe declines through the early 1990s, and it became clear that wild populations could not sustain unrestricted fishing indefinitely. Poor capture rates resulted in the imposition of a Canadian cod-fishing moratorium

in 1992. Despite this, cod populations have exhibited few signs of recovery and fishing harvests remain well below former levels.

As demand for fish meat increases with growing human populations, aquaculture technology has emerged as a potential means of meeting food-production requirements while concurrently reducing demands on wild fish. Early commercial-scale cod production systems were initiated in the late 1980s, but appreciable success was not achieved until the early 2000s. Production levels have exhibited promising growth, but many difficulties have yet to be overcome.

Perhaps the greatest challenge currently posed to cod farming operations is the prevalence of disease and the undeveloped state of piscine veterinary medicine. Wild cod are host to numerous viral, bacterial, and eukaryotic pathogens, many of which occur with higher prevalence in aquaculture systems due to the high stocking densities required by commercial farms and the difficulty in maintaining high water quality. Of diseases affecting the Atlantic cod, microsporidosis (caused by the parasite *Loma morha*) has emerged as a particularly troubling problem. Despite a growing body of research, the parasite remains poorly understood.

This research was conducted with an integrative focus, calling on multiple sub-disciplines within biology, including evolution, physiology, and cell biology to inform and direct efforts meant to facilitate a better understanding of the *Loma* parasite and aid in ongoing aquaculture efforts through three parallel lines of study:

1. The development of a cod cell line
2. The use of culture spores to study factors affecting microsporidian infection
3. The development of *in vitro* culture methods for *Loma morhua*

Each of these general objectives was met. First, the development of a cod cell line was successfully achieved using the tissues of larval cod, producing stable continuous proliferation in culture and growth characteristics similar to those of other cell lines derived from the tissues of cold-water marine fish. Second, cultured spores of a readily cultured microsporidian, *Anncaliia algerae*, were successfully used to test the infection-modulating effects of $MgCl_2$ and EDTA *in vitro* and for the construction of protein reference maps, demonstrating shared infectivity features and proteomic similarities between microsporidian parasites with distantly-related host species. Lastly, the cod-derived (GML-5) cell line was used in combination with novel culture methods to stimulate the *in vitro* infection and development of *L. morhua* spores.

Further research should be conducted to evaluate methods for improving *Loma* infection and proliferation rates *in vitro* by using additional cell lines and culture method modifications. The *in vitro* culture of additional fish-infecting microsporidians may also be attempted using MEM treatments and appropriate host cell lines. Lastly, proteins isolated from laboratory-cultured spores by two-dimensional electrophoresis should be positively identified to allow more detailed study into the mechanisms that underlie infection processes in piscine microsporidia.

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Appendix:

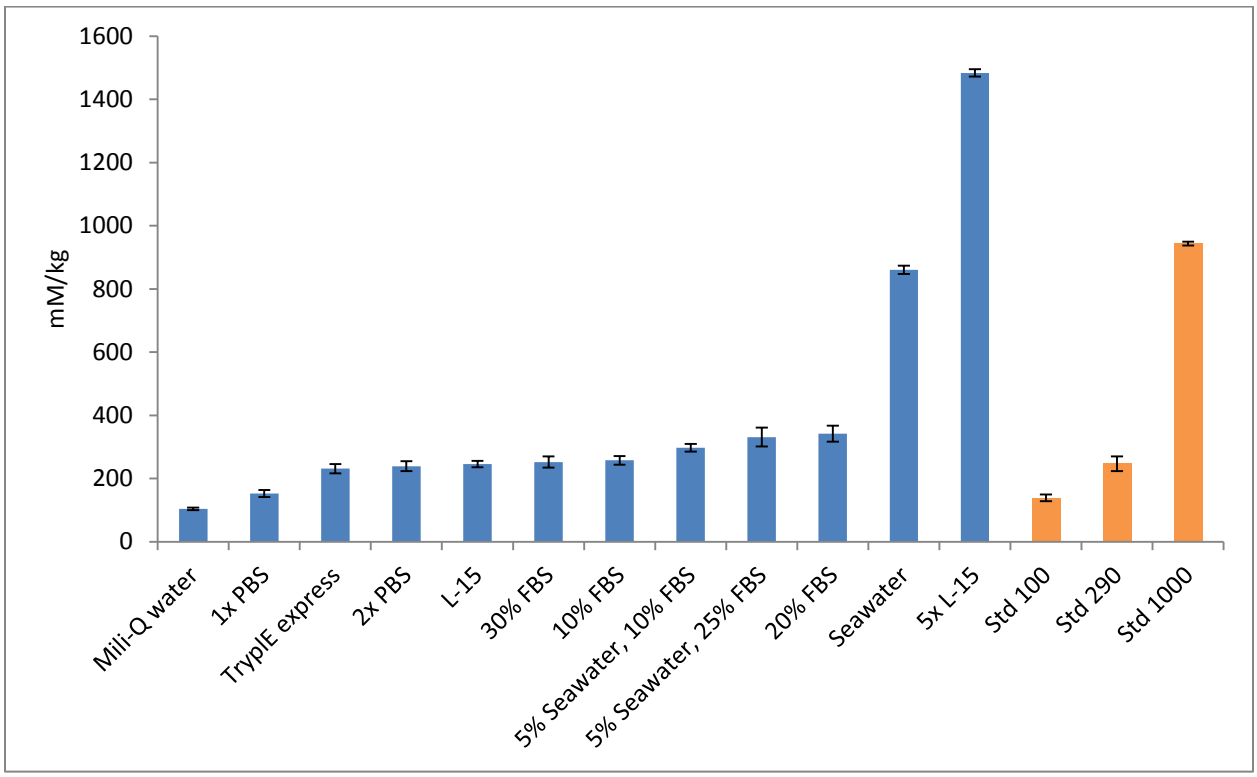

Figure A.1: Osmolarity values (in mM/kg) cell culture fluids and media mixes (blue) and standards (orange). Error bars indicate 95% confidence interval (n=3).

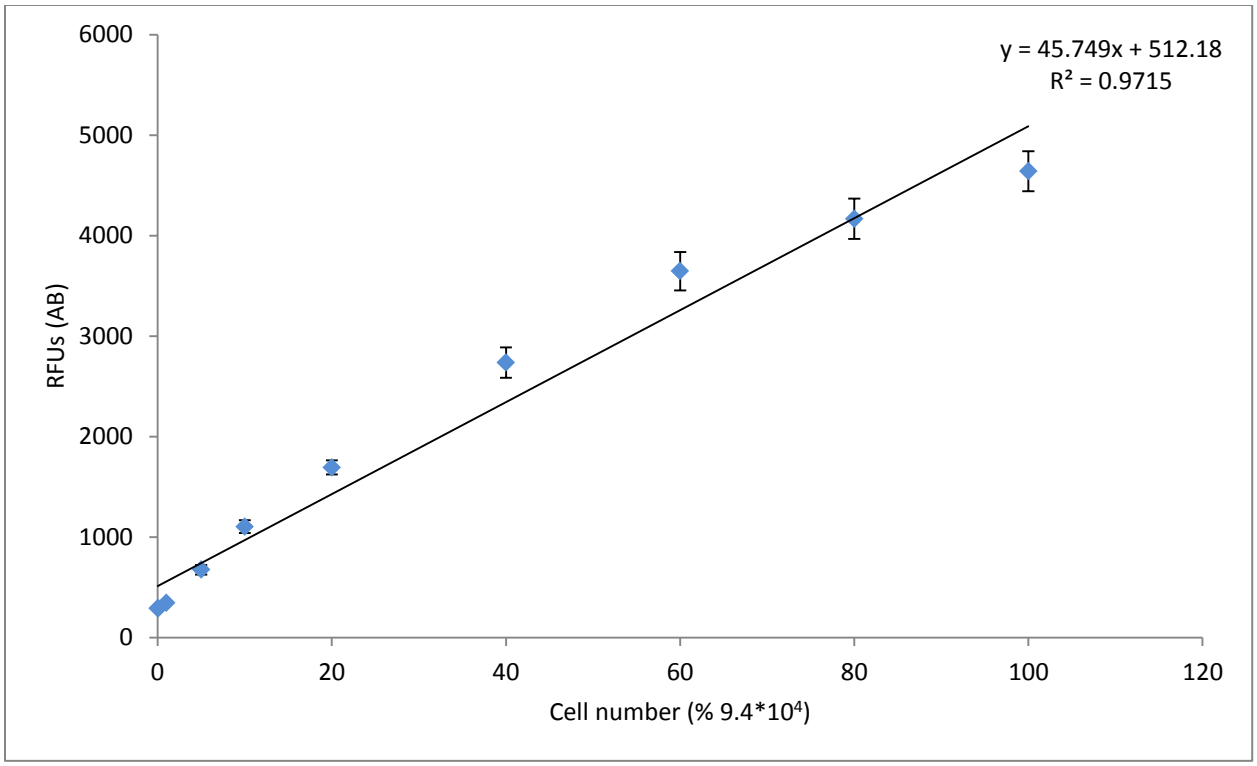

Figure A.2: Standard curve for the correlation of Alamar Blue fluorescence values to cell numbers of haddock-derived (HEW) cells. Error bars indicate 95% CI (n=8).

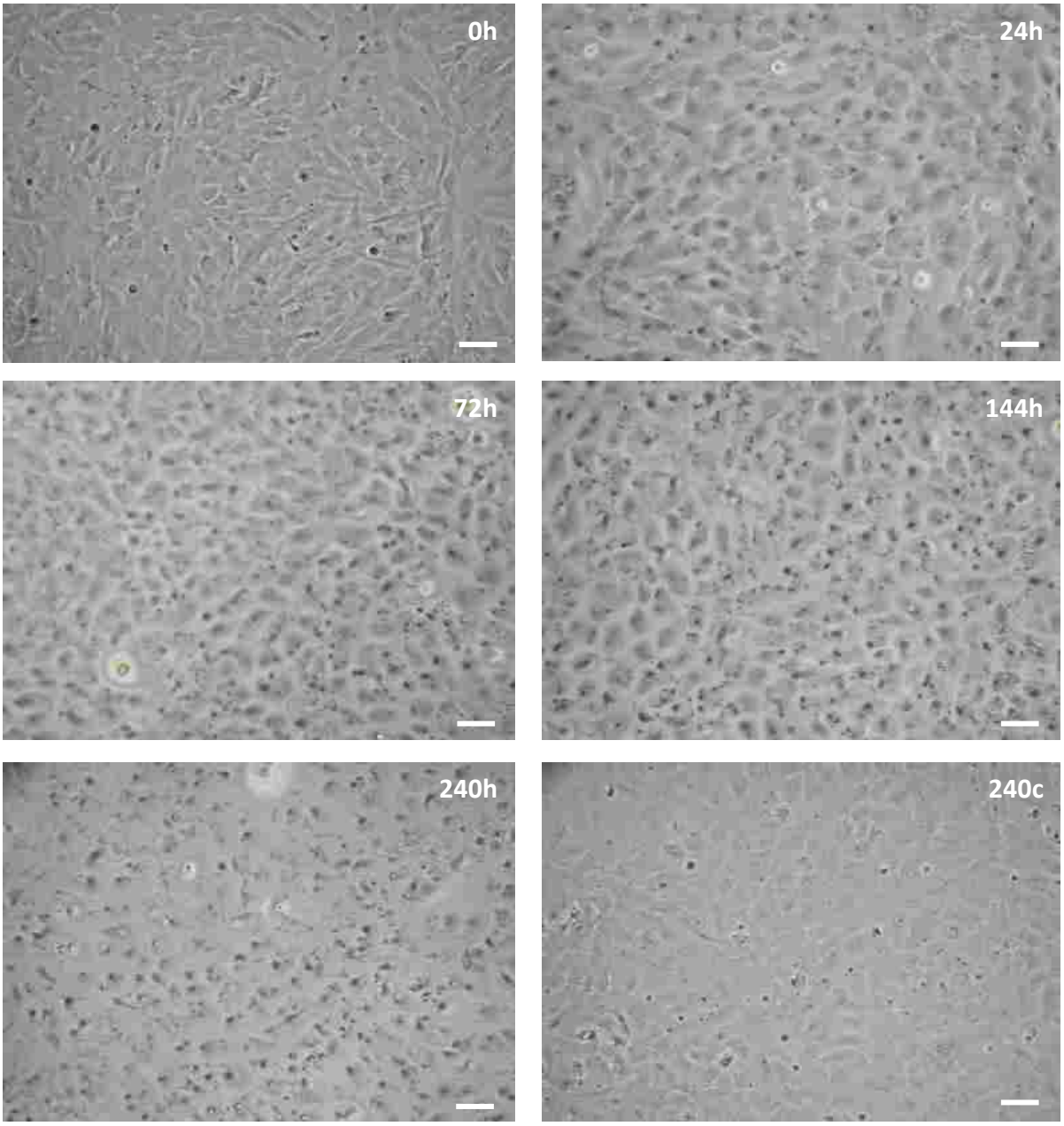

Figure A.3: Micrographs of cod cells following exposure to L-15 ex at 18°C. From top, left to right: immediately following exposure, 24 hours, 72 hours, 144 hours, 240 hours, and control cells at 240 hours. Scale bars = 50µm.

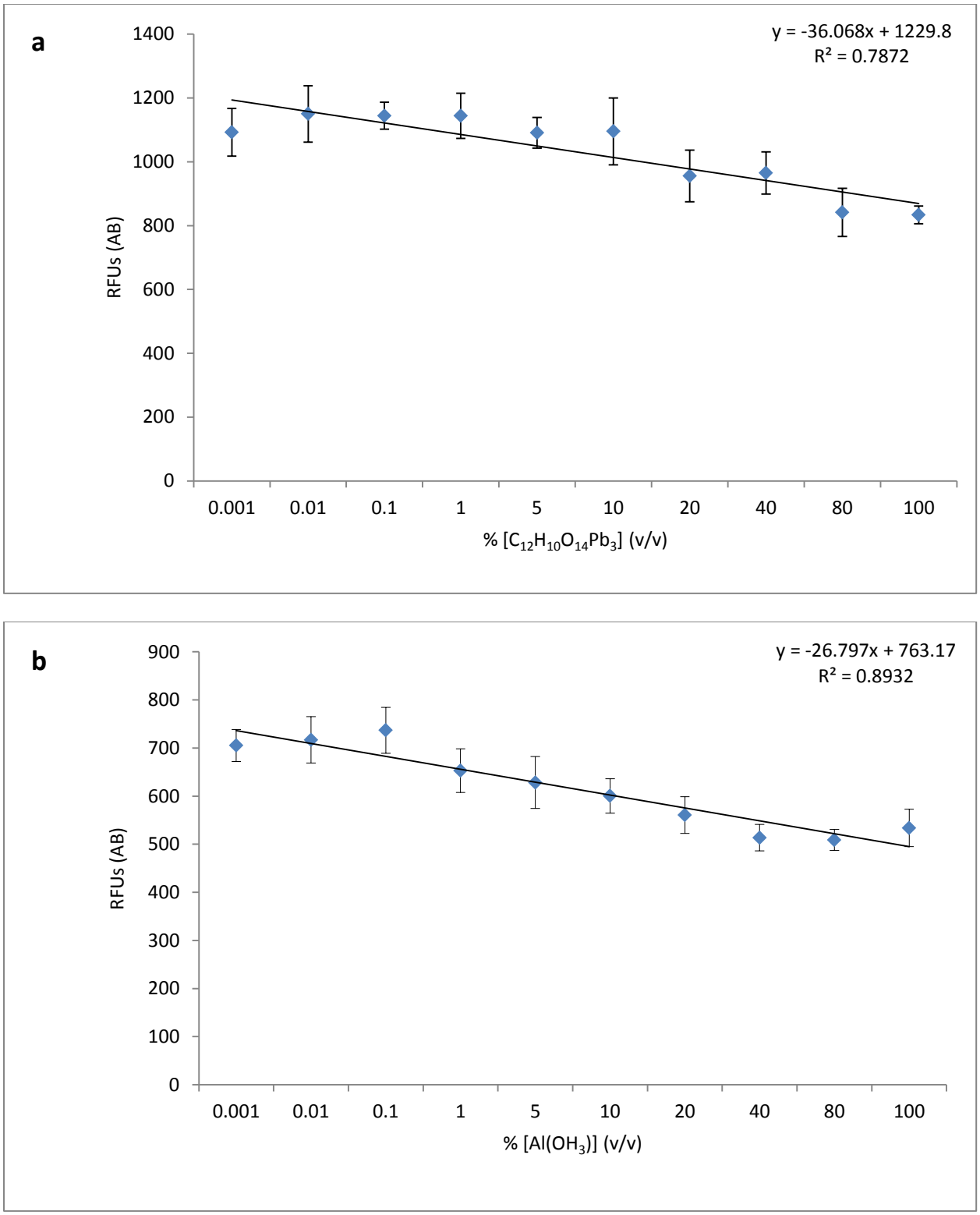

Figure A.4: Alamar Blue assays of 24 hour toxicity of: a) Lead citrate($C_{12}H_{10}O_{14}Pb_3$) and b) aluminum hydroxide($Al(OH_3)$) to cod (GML-5) cells. Cells exhibit slight downward trend in viability with increasing concentrations. Error bars indicate 95% CI (n=8).

Table A.1: Recipe for two-dimensional electrophoresis lysis buffer for a final volume of 50ml

Step	Reagent	Quantity	Molarity
1) Stock solutions	Tris HCL (ph 7.4) DTT EDTA	5ml 5ml 0.25ml	0.5M 0.5M 0.2M
2) Combined with following reagents to make 50ml	Glycerol Ampholytes Nono-pure water	5ml 7.5ml 22.75ml	
3) Add final reagents	Urea Thiourea CHAPS	24g 3.8g 1g	

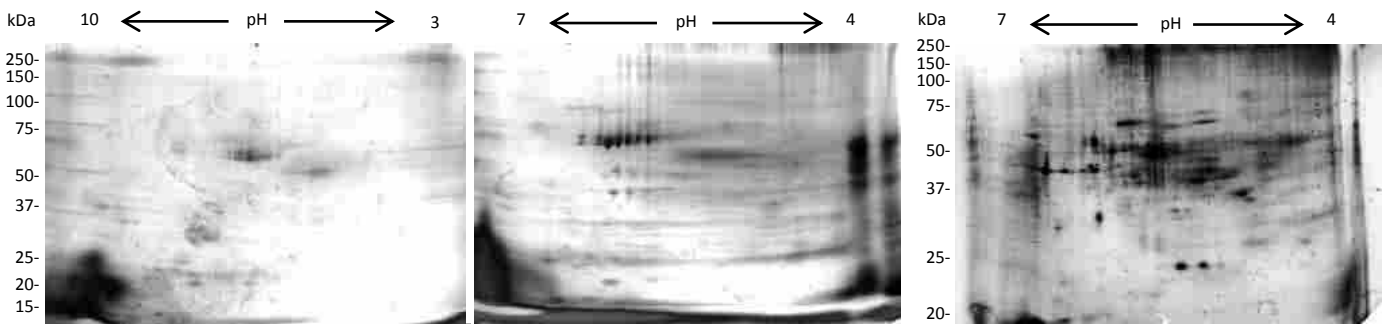

Figure A.5: Two-dimensional electrophoresis gels obtained from cod-derived GML-5 (far left and middle) and haddock-derived HEW cell lines by exposure to lysis buffer and immediate isoelectric focusing.

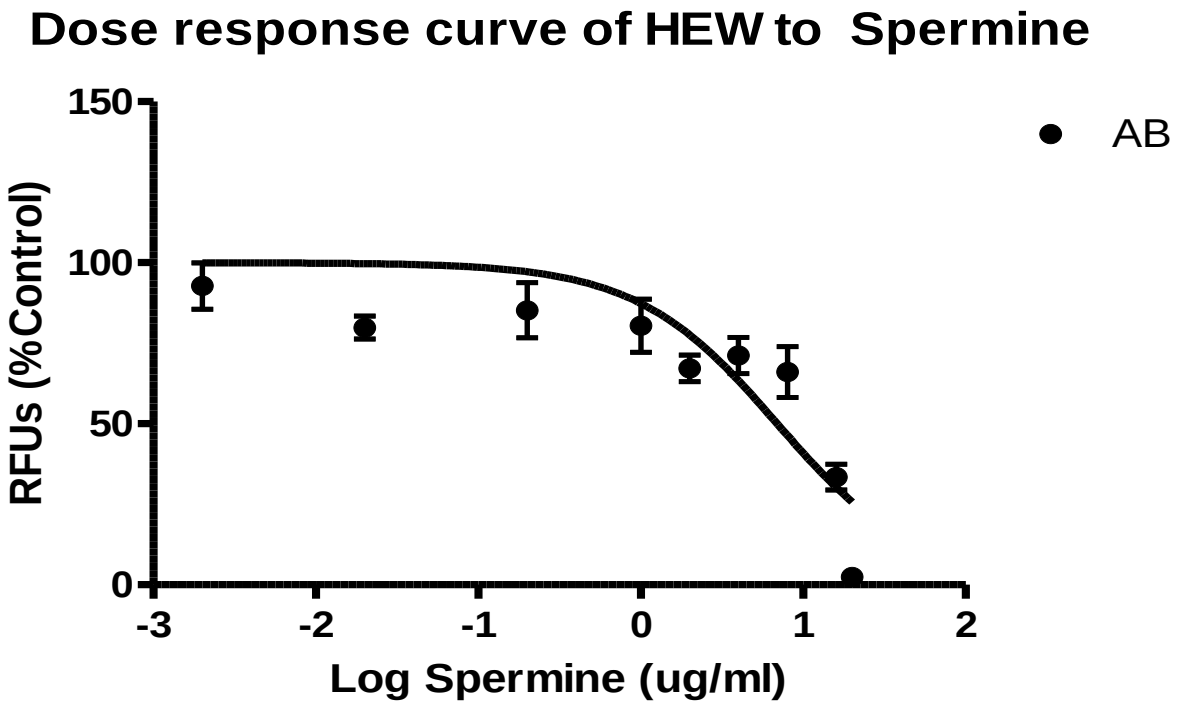

Figure A.6: 24-hour toxicity curve for haddock-derived (HEW) cells to spermine (0.01%, 0.1%, 1%, 5%, 10%, 20%, 40%, 80%, and 100% (v/v)).

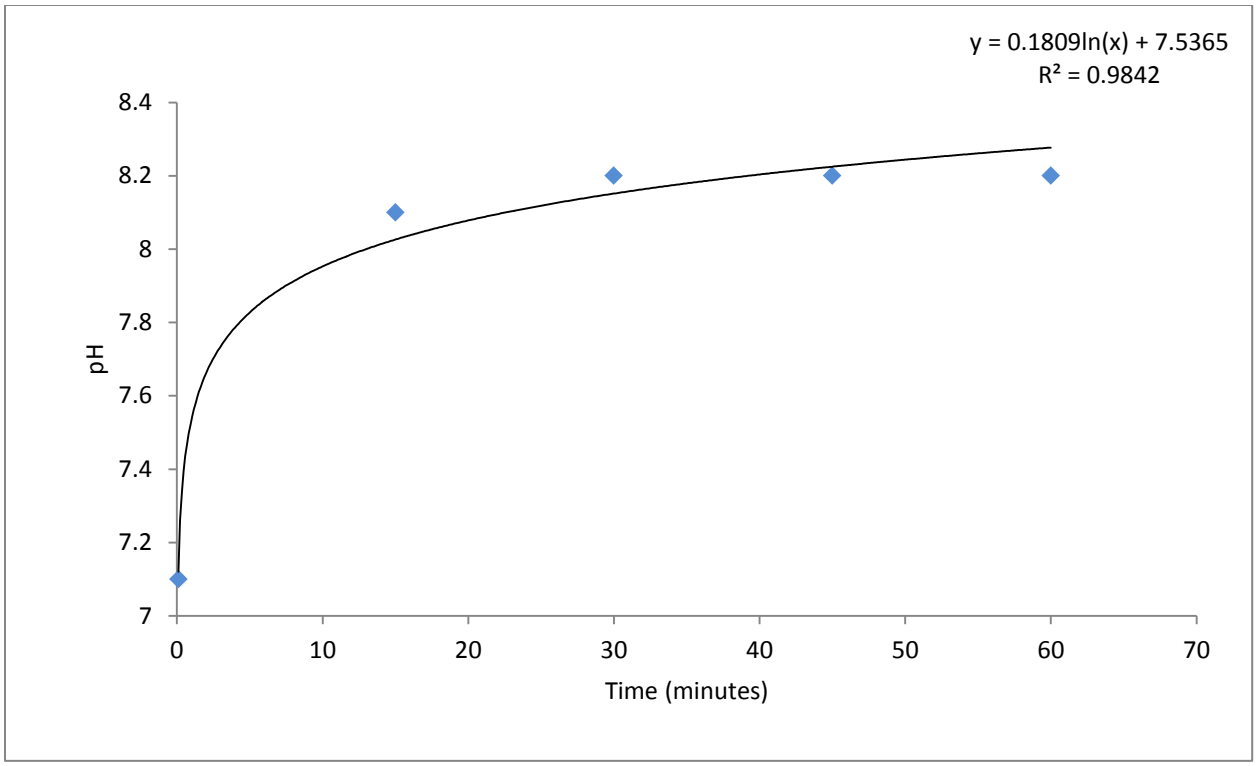

Figure A.7: Change in pH of minimum essential medium (MEM) exposed to cod (GML-5) cells over a one-hour period. pH values obtained from 1ml MEM plated onto confluent GML-5 cultures in 25cm^2 TC flasks.

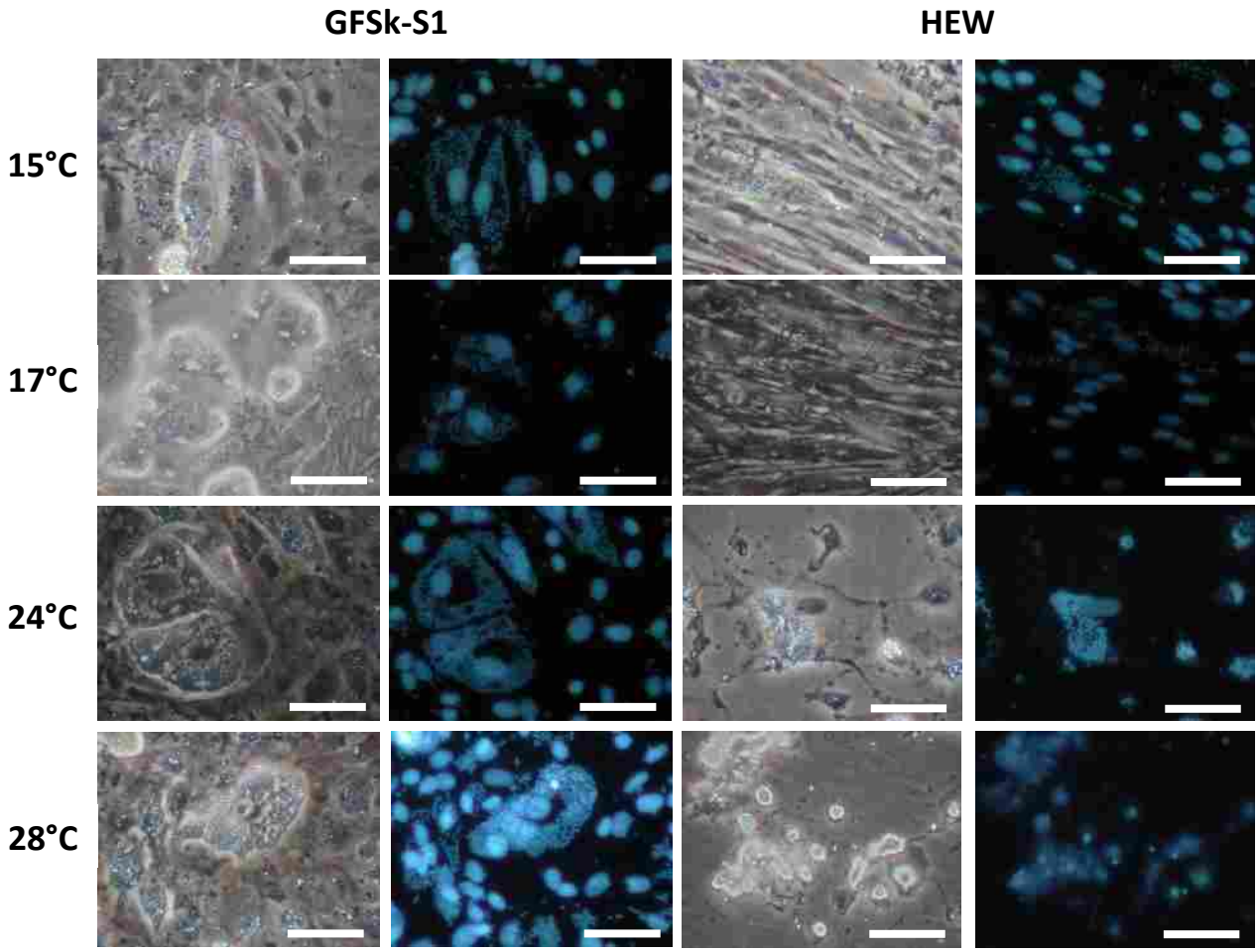

Figure A.8: Phase-contrast and fluorescence (DAPI) micrographs of *Anncaliia algerae* infecting goldfish skin (GFSk-S1) and haddock (HEW) cells (left and right respectively) 18 days post inoculation (PI). Higher incubation temperatures increase apparent infection rates. HEW cells exhibit less susceptibility to infection than GFSk-S1. Scale bar = 50µm.