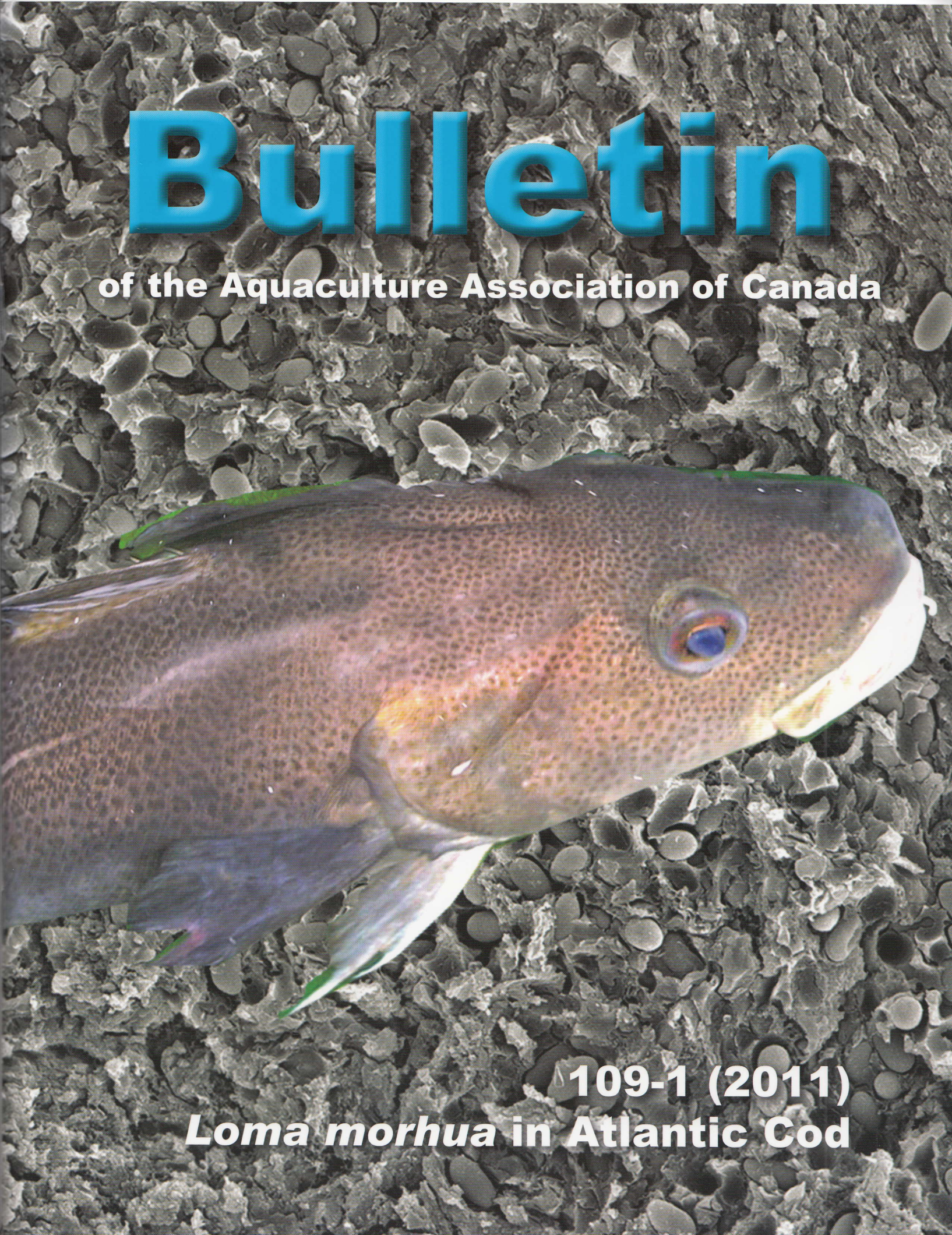
Bulletin

of the Aquaculture Association of Canada



109-1 (2011)

Loma morhua in Atlantic Cod

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de l'Association aquacole du Canada

109-1

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Cover: Created by DE Aiken from photographs of *Loma morhua* spores (courtesy of Susan Balfry of the Microscopy & Microanalysis Facility, University of New Brunswick) and of an adult cod taken by Steve Neil of the St. Andrews Biological Station, Fisheries & Oceans Canada.

Research to mitigate the negative impacts of *Loma morhua* on cod farming

The contents of this *Bulletin* represent the proceedings of a scientific workshop that was hosted jointly at the St. Andrews Biological Station of the Department of Fisheries and Oceans and on the campus of the Huntsman Marine Science Centre in St. Andrews, New Brunswick, June 24-26, 2011. The purpose of the workshop was to bring together research scientists and industrial collaborators for presentation of research findings and to entertain discussions on how best to limit the negative impact that *Loma morhua* is having on Atlantic cod aquaculture.

Atlantic cod is a relatively new species to aquaculture. As with every new farming endeavor, the culture of large numbers of organisms in close proximity to one another results in challenges due to increased potential for infection by pathogens that can cause disease. Even well established enterprises such as Atlantic salmon aquaculture face ongoing challenges with pathogens such as sea lice. It must be recognized that it takes time to learn and understand the basic biology of the pathogen in order to have any hope of success with preventing or limiting infections. In addition, it must be appreciated that no single approach (e.g., vaccination) is likely to eliminate infections by complex eukaryotic pathogens such as *Loma morhua*. Vaccines are employed commonly against viruses and bacteria but there are very few vaccines that show efficacy against more complex pathogens such as *Loma morhua*. Accordingly, we adopted a multifaceted approach towards three main goals:

1) Limit parasite transmission to cod at aquaculture sites using husbandry-based approaches;
2) Identify cod families that show genetic resistance to *Loma* for their selection and use in breeding programs;
3) Identify drugs that can block or eliminate these parasite infections in live fish.

Loma morhua was first identified as a potential problem for Atlantic cod aquaculture during a Collaborative Research and Development project that was funded by the Natural Sciences and Engineering Research Council (NSERC) of Canada from 2004 to 2007. This program was spearheaded by Mick Burt at the University of New Brunswick (Fredericton) in collaboration with numerous sponsors associated with the Atlantic cod aquaculture industry. Of 23 species of parasites identified from wild cod, *Loma morhua* was determined to hold the greatest concern for the aquaculture industry based upon >70% prevalence of infection observed in cod from New Brunswick cage sites. This parasite is particularly pathogenic in juvenile fish and can

result in near complete stock losses in a hatchery setting. Older fish seem to be more resilient but show reduced growth rates and even death when subject to additional stressors such as elevated water temperatures.

The research presented in this issue of the *Bulletin* was funded through the Strategic Project Grants program of NSERC with Kelly Cove Salmon (a division of Cooke Aquaculture Inc.) as the industrial sponsor. Our application was submitted through a special directive that sought to improve Canada's industrial base in the target area of aquaculture. The mandate of this funding program is to partner scientists with industry towards information exchange in support of research that can be used to diversify and improve the viability of the aquaculture industry.

The research on *Loma morhua* is ongoing and the team of investigators includes scientists from New Brunswick (Mike Duffy and Tillmann Benfey, University of New Brunswick; Ed Trippel, St. Andrews Biological Station, Department of Fisheries and Oceans) and Ontario (Lucy Lee, Wilfrid Laurier University; Nels Bols, Waterloo University), as well as researchers in Iceland (Matthías Eydal, University of Iceland), Denmark (Kurt Buchmann, University of Copenhagen) and Scotland (Catherine Collins, Fisheries Research Services Marine Laboratory). Numerous graduate and undergraduate students are involved in conducting this research (Aaron Frenette, Maeghan O'Neill, Hilary Byrne and Nicholas Benfey from UNB; Richelle Monaghan, Mike MacLeod and Rebecca Rumney from WLU) and we were fortunate to have the following industry representatives in attendance at our workshop: Larry Dickinson (Cooke Aquaculture Inc.), Tasha Harrold (Northern Cod Broodstock Development) and George Nardi (Great Bay Aquaculture). Previously established collaborations with the Atlantic cod industry are ongoing and we look forward to the new collaborations that were established during the workshop. This issue of the *Bulletin* represents an interim report of our ongoing research project that is funded until the end of 2013.

Sincere thanks to our 23 participants for a most productive meeting in St. Andrews!

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Tillmann Benfey, editor

Aaron Frenette

Quantitative PCR: A translational tool to help elucidate basic life history and transmission dynamics of *Loma morhua* infections towards improved feasibility of cod aquaculture

Aaron Frenette, Maeghan O'Neill, Hilary Byrne, Nicholas Benfey, Ed Trippel, Tillmann Benfey and Michael Duffy

Our research deals with a parasite that is limiting the production potential of Atlantic cod (*Gadus morhua*), a species new to aquaculture. The parasite *Loma morhua* occurs naturally in wild cod throughout their range. However, the relatively high fish stocking densities in aquaculture sites lead to heavy parasite infections that cause impaired growth and high mortality in farmed cod. We recently developed a polymerase chain reaction (PCR) assay to diagnose this parasite in cod. This test will be modified to facilitate quantification of parasites towards several translational objectives: 1) identify specific genetic lines ("families") of cod that show natural resistance to *L. morhua*, 2) determine the abundance of parasite spores released into the water column and thereby identify periods of peak parasite transmission at cage sites, and 3) determine whether blue mussels can serve as a reservoir for transmission to cod or whether these invertebrates serve to inactivate *L. morhua* spores and actually help prevent parasite transmission during aquaculture. We have adopted this multifaceted approach with the goal of limiting parasite transmission to cod during aquaculture and thereby enhance productivity of the cod aquaculture industry.

Introduction

With the collapse of the cod fisheries off the east coast of Canada in the late 1990s, there has been recent interest in intensive culturing of Atlantic cod (*Gadus morhua*).[1] Farming of Atlantic cod is of particular significance because of the potential marketability of cod as an alternative species for commercial coldwater culture. However, as with any new net pen endeavour, infectious disease agents pose substantial risks to successful cultivation. *Loma morhua* is an obligate intracellular parasite that was first described from Atlantic cod by Morrison and Sprague.[2] It is a fungal pathogen (microsporidian) that induces a state of hypertrophy, taking over metabolic control of the host cell and rapidly reproducing asexually to establish a xenoma, a complex comprised of both host and parasite tissue.[2,3] These xenomas appear as white cysts that are found throughout

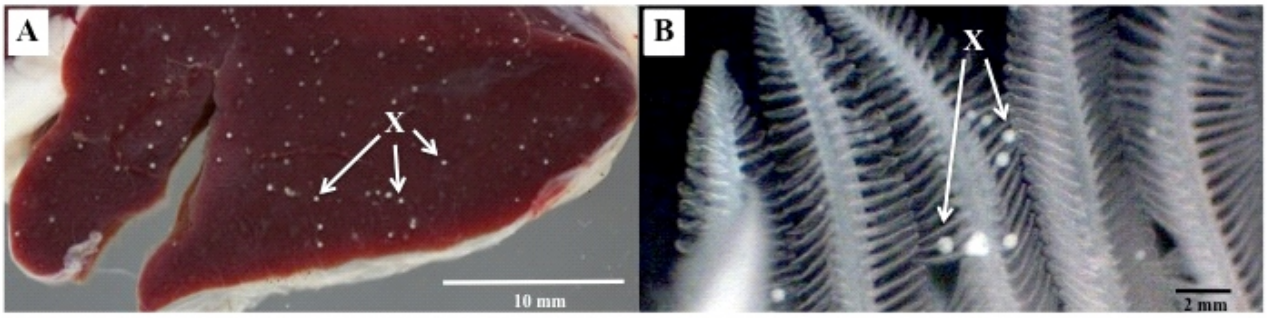


Figure 1
Gross morphology of *Loma morhua* xenomas in tissues of Atlantic cod as observed in light microscopy. Xenomas as observed *in situ* on the surface of the spleen (A), and on gill lamellae (B). Labels: X = xenoma.

vascularized tissues of cod including the viscera and gill lamellae (Figure 1). Each xenoma can contain tens to hundreds of thousands of microscopic parasite spores.

The parasite *L. morhua* is emerging as a significant pathogen to the developing cod aquaculture industry. It is not surprising that this parasite has been found in aquaculture cage sites in Atlantic Canada, given distribution of the parasite throughout the range of wild cod stocks.[4] Collections of farmed cod from New Brunswick cage sites revealed 70 to 100% prevalence of infection.[4] Infections cause impaired growth[5] and fish condition factor declines rapidly with increasing severity of infection.[4] Both juvenile[4] and adult cod[5] suffer high mortality from *L. morhua* infections when subjected to physiological stressors that occur routinely at aquaculture sites. Reduced lymphocyte counts indicate that infection with *L. morhua* serves to compromise further the health of infected cod, thereby rendering them susceptible to infection by other pathogens.[5]

There are currently no vaccines or chemotherapeutics available to treat *L. morhua* infections. The parasite life cycle has yet to be elucidated and transmission studies are important to identify clearly the route(s) of infection and factors that contribute to the epidemiology of infections during aquaculture. Given the current significance of *L. morhua* as a pathogen of both farmed and wild cod and its potential to compromise the immune status of farmed fish, we are pursuing several different approaches towards prevention of infection and disease.

No single approach (e.g., chemotherapeutics, vaccines) is likely to eliminate infections by pathogens. Accordingly, a multifaceted approach must be employed to limit parasite transmission at cage sites. Ultimately, an in-depth knowledge of the life cycle and transmission dynamics is essential for interrupting or reducing transmission. One major roadblock to effective control of a closely related parasite (*L. salmonae*) has been the inability to quantify accurately the intensity of infection in Pacific salmonids.[6] This results in an impaired ability to assess the efficacy of any control measures. Accordingly, our first major objective is to modify our PCR diagnostic assay for *L. morhua* (Frenette et al., submitted) to quantify the intensity of infection in individual cod. A quantitative PCR (qPCR) assay holds great power and is a key factor in our immediate goals of: 1) selecting established cod families that show inherent resistance to *L. morhua*, 2) identifying periods of peak transmission during aquaculture and 3) identifying potential reservoir hosts.

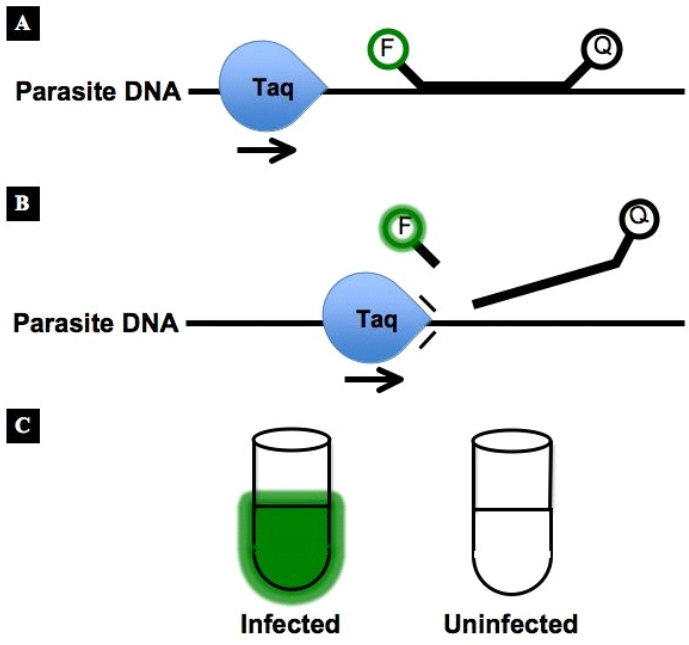
The longer-term goals of testing chemotherapeutics and assessing efficacy of vaccine preparations will also rely heavily on this qPCR assay.

PCR as a Diagnostic Tool

We have developed a PCR-based diagnostic assay that shows both high sensitivity and species-specificity in the diagnosis of *L. morhua* (Frenette et al., submitted). The specificity of our PCR diagnostic assay relies upon the fact that the parasite ribosomal DNA gene shows strong intraspecific conservation (highly similar DNA sequence within all individuals of the parasite *L. morhua*). Importantly, there are interspecific differences in the DNA sequence that also enable us to distinguish between different parasite species for specific and accurate diagnosis of *L. morhua.*

Our current PCR assay will be refined to enable determination of parasite presence and quantification of level of infection by employing quantitative PCR (qPCR). Similar assays have been developed to quantify infections by other fish microsporidians.[7] The assay relies upon use of a fluorescent DNA probe and the technology exploits two important features. Firstly, the 5 to 3 exonuclease activity of Taq polymerase results in cleavage of the fluorescent dye from an adjacent quencher molecule on the probe to yield a fluorescent signal (Figure 2). Accordingly, the amount of fluorescence in the reaction is proportional to the amount of parasite DNA in the sample and enables sensitive determination of the number of parasites for quantification of the level of parasite infection. Secondly, the assay can be used to compare infection intensity among individual fish to assess natural resistance to parasites, for determination of abundance of spores that are free-floating in the water column during parasite transmission, and in determining parasite abundance in potential reservoir hosts that could transmit the parasite to cod during aquaculture.

Figure 2
Depiction of the basic concept underlying quantitative PCR. A specific probe comprised of a fluorophore (F) and a quencher (Q) binds to parasite DNA that is present in a tissue sample (A). The enzyme Taq will cleave the fluorophore ONLY from probes that are bound to parasite DNA, thereby resulting in a fluorescent signal (B). Accordingly, fluorescence will be observed in tubes that contain infected tissues but not in tubes that contain uninfected tissues (C). The amount of fluorescence is proportional to the number of parasites in the tissue.



Below we detail the rationale for using qPCR in each of these different scenarios to improve the feasibility of cod aquaculture in the face of *L. morhua* infections.

Genetic Resistance of Cod Families to *L. morhua*

Our qPCR assay will prove invaluable in the screening of cod families for the identification of naturally resistant genetic lines and their selection for breeding and grow-out in aquaculture. We have demonstrated that *L. morhua* is found embedded within cod tissues and not simply found on the outer surface of organs. Comparable qPCR assays for other pathogens often use organ biopsy as the source of material for analysis. However, we cannot use biopsies because the parasite is not distributed evenly throughout infected organs. We invested months in identifying a methodology for complete and thorough homogenization of intact spleens. After extensive testing, we settled on a commercial device (FastPrep®-24, MP Biomedicals; Figure 3) that serves to disrupt completely an entire spleen (and any parasites) in ~40 seconds. Our preliminary qPCR assessment shows excellent promise with detection capabilities down to 10 copies of parasite DNA (= 10 *L. morhua* parasites; Figure 4). We are in an excellent position to proceed to DNA extraction from spleens from defined family lines of cod exhibiting natural *L. morhua* infections following cage culture.

Figure 3
The MP Biomedicals FastPrep® -24 homogenizer that is being employed for complete disruption and homogenization of cod tissues prior to qPCR.

Resistance to *L. morhua* infection will be compared in cod families that were established by the Atlantic Cod Genomics and Broodstock Development Program (CGP) in collaboration with Cooke Aquaculture Inc.[8] Variation in host responses to pathogens is observed among individuals of a species, and heritable resistance to fish pathogens has been documented previously.[9] We have already collected spleens from over 1100 cod following three years of aquaculture in cage sites in the Bay of Fundy. These fish represent 28 families and we have preserved materials in ethanol for DNA extraction and analysis by qPCR. Gross examination of these spleens revealed ~40% with *L. morhua* infections and the remaining 60% putatively uninfected due to an absence of xenomas (i.e., lesions) on the outer surface of these spleens. Since not all infections (e.g., parasite life stages) show visible lesions, and since not all lesions are visible on the organ surface, we will use qPCR to detect *L. morhua* and to quantify the level of infection for indication of genetic resistance. We developed a collaboration with Northern Cod Broodstock Company recently to expand our analysis to include another 44 cod families that were established by the CGP in Newfoundland. These cod were infected by *L. morhua* during their maintenance in a land-based aquaculture facility, presumably due to spores from wild fish entering the facility with ocean water. Our use of these two cod populations will help to ensure that we have defined broodstock available to produce resistant family lines that we require for our subsequent experimental work. Determining relative resistance of a large number of families to *L. morhua* becomes essential because various other traits are also desired for cod production during growout. Accordingly, resistance to infection will serve as

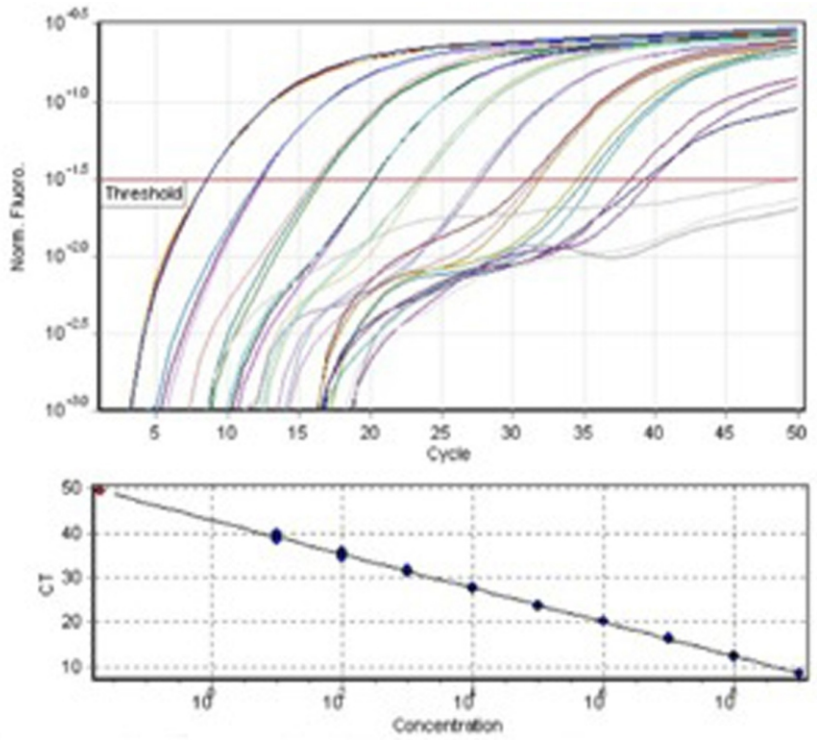


Figure 4
Standard curve to demonstrate assay sensitivity in the detection of target DNA using quantitative PCR. Copy Number of Target Sequence: 10^9 – 10^1 copies.

only one criterion for selection of broodstock. The identification of genetically-susceptible cod families will also prove essential for our longer-term goals of assessing the efficacy of therapies in the treatment/prevention of infection and disease because we must have the potential for inducing a high rate of parasite infection in susceptible fish in order to demonstrate that a therapy is effective.

Determination of Parasite Abundance in Water to Identify Periods of Peak Transmission

Infected cod develop *L. morhua* xenomas in their viscera and in gill lamellae. While ingestion of infected fish is a known route of infection, it is the rupture of these gill xenomas and the release of spores into the water column that is the principle route of parasite dissemination. Cod are known to possess gill xenomas throughout all months of the year (unpublished data) but it remains unknown whether spores are released at all times or whether there is a season of peak parasite transmission during aquaculture.

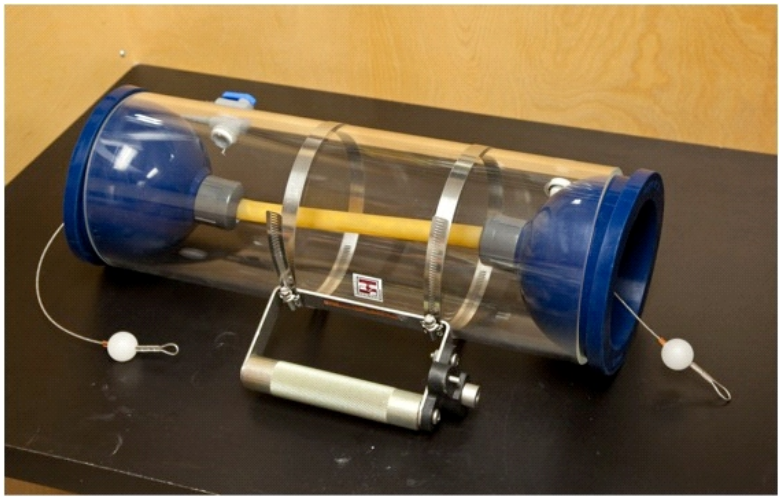


Figure 5
A Niskin device used to sample water and organisms from different depths (surface, 4 m, and 8 m) at aquaculture sites in St. George, New Brunswick. Sample fractions will be assessed using our qPCR assay for quantification of *L. morhua* spores in both water and organisms.

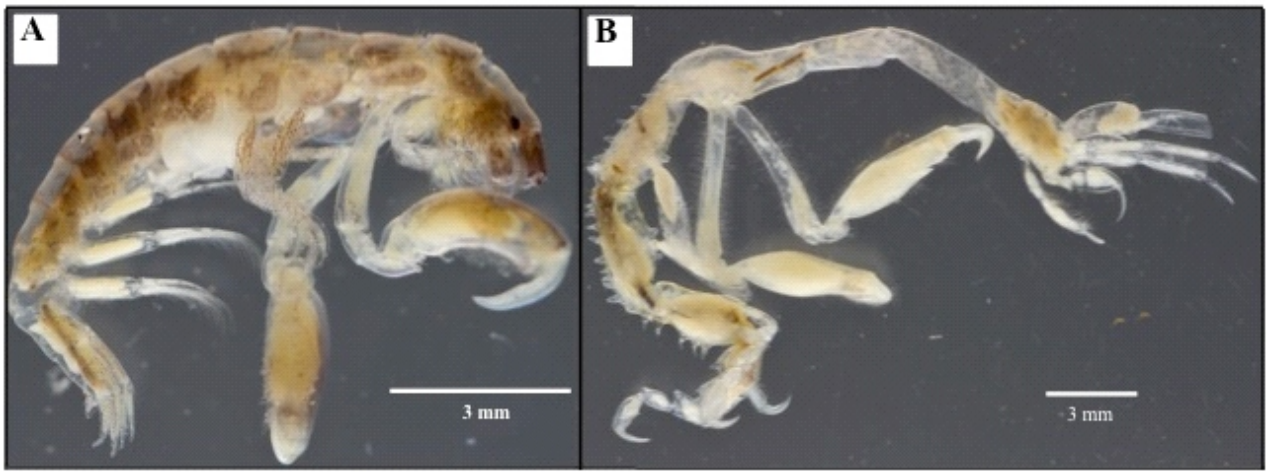


Figure 6
Amphipods from the suborders Caprelliea (A) and Gammeridea (B) that were collected from the water column at aquaculture sites in St. George, New Brunswick.

If we are able to identify discrete periods of peak transmission, modifications to husbandry practices could be employed to help prevent parasite transmission at cage sites.

Water samples were collected during monthly visits to cod aquaculture sites in St. George, New Brunswick. Water was sampled from different depths (surface, 4 m, 8 m) using a Niskin device (Figure 5) for future quantification of *L. morhua* spores in the water column using our qPCR assay. Water was clarified using serial passage through sieves (250 µm, 106 µm, 45 µm) to collect different size classes of invertebrates (Figure 6). Organisms were collected and preserved in 95% ethanol for subsequent qPCR analysis. The water was then filtered using vacuum concentration (0.2 µm filters) to collect spores (Figure 7). The 0.2 µm filters were

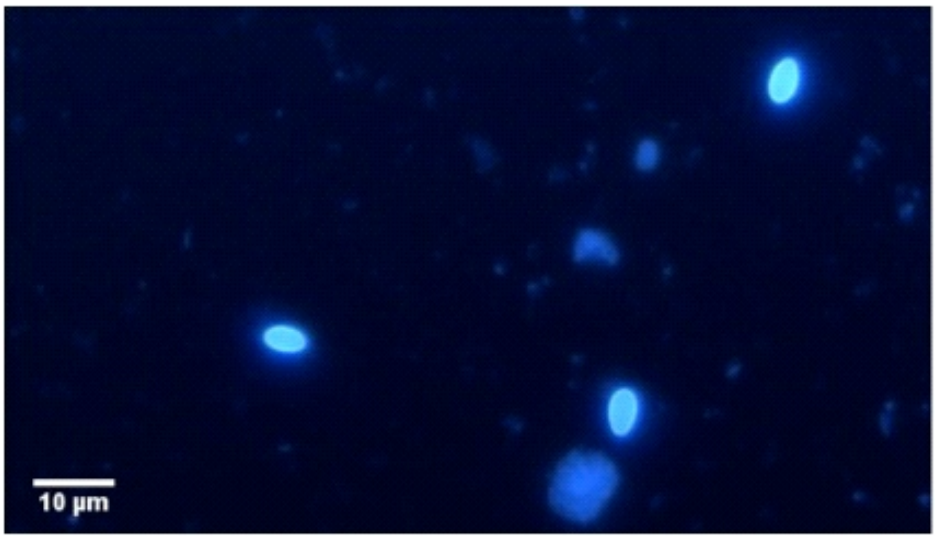


Figure 7
***Loma morhua* spores stained with Calcofluor White as observed in light fluorescence microscopy.**

stored in 95% ethanol to preserve DNA for pending qPCR analysis. This assay might show utility in predicting epidemics by assessing infection status of invertebrates and/or spore density at cage sites.

Identification of Reservoir Hosts that Transmit *L. morhua* to Cod

At a cage site setting there is typically a high fish stocking density that contributes to high potential for parasite transmission. An important consideration during aquaculture is that there are numerous other organisms that colonize cages and could act as vectors for parasite transmission. For example, blue mussels are common biofouling organisms, as are barnacles and algae.[10] Free-swimming crustaceans are found commonly at cage sites as well.[10] Blue mussels feed by filtering small particles from surrounding water with high efficiency.[11] The filter-feeding characteristic of mussels, in particular, suggests they are ideal organisms for bioaccumulation of small particles such as *L. morhua* spores. Furthermore, Atlantic cod are opportunistic feeders[12] and it is known that they feed on blue mussels during aquaculture (unpublished data).

Microsporidian parasites are known to infect oligochaetes, crustaceans, and various fish.[13] In addition, Lom and Nilson[14] suggested that the close relationship of fish microsporidians with those known from invertebrates might be indicative that fish-infecting *Loma* species could utilize paratenic or reservoir hosts. Importantly, filter-feeding organisms such as zebra mussels can bioaccumulate spores from human microsporidian parasites and thus they represent a potential reservoir for transmission of microsporidian pathogens.[15] Blue mussels have also been documented to bioaccumulate human bacterial pathogens[16] and so they represent a logical target in investigations on the epidemiology of *L. morhua* infections at aquaculture sites.

The obvious hypothesis is that cod eat blue mussels that have accumulated *L. morhua* during aquaculture and thereby become infected. However, an unrelated but fortuitous experiment in our lab provides an alternate hypothesis. Our preliminary work has shown that enzymatic digestion of parasites can serve to inactivate *L. morhua* spores. Given that spores are subjected to digestive enzymes within the mussel gastrointestinal tract, it remains possible that mussels might actually serve to inactivate spores before they encounter cod. Experiments are currently being conducted to make definitive conclusions about spore viability in mussels. Accordingly, the alternate hypothesis, that mussels filter the water and inactivate spores, could result in a lower prevalence and intensity of *L. morhua* infection in cod than if mussels were not present on the sea cages. Regardless of the result of the viability assays, two scenarios become relevant to the aquaculture industry: 1) proceed with use of copper wire-laced cages to prevent byssal thread attachment by mussels if they are found to serve as a reservoir of viable *L. morhua* infection or 2) proceed with use of a copper wire-laced inner cage and a conventional cage externally if the mussels are found to inactivate spores in their digestive tract. The second net scenario requires additional explanation. Mussels produce an interesting substance called pseudofeces that does not actually enter their digestive system. Not being subjected to digestive enzymes, this particulate matter might contain viable spores clumped in mucus and so mussels would need to be kept away from the cod to reduce potential transmission via pseudofeces during aquaculture. These observations have relevance for aquaculture enterprises beyond Atlantic cod based upon the current interest in integrated multi-trophic aquaculture[17] and a potential role for mussels in disease prevention. For example, blue mussels have

been documented to inactivate viral pathogens such as infectious salmon anemia virus.[18] In addition, a recent paper documents that blue mussels can inactivate larval sea lice of Atlantic salmon[19] and so the potential exists for the broader use of blue mussels to control infectious disease agents during aquaculture.

Conclusions

This study will provide important foundation information on the life history and epidemiology of *L. morhua* that is essential not only for husbandry-based initiatives that can be implemented by the industry but for longer-term therapeutic objectives of limiting the negative impact of *L. morhua* on aquaculture of Atlantic cod. Husbandry practices have long been recognized as an important approach to controlling pathogens in hatcheries and research laboratory settings.[20,21] The current research project has provided the necessary resources to identify periods of peak parasite transmission and potential vectors of importance during aquaculture. Preserved samples of invertebrates and water will be analyzed with enthusiasm once the qPCR assay has been optimized fully. We anxiously await the results of experimental exposure of mussels to parasite spores to determine whether their growth should be promoted or discouraged in support of limiting *L. morhua* transmission during Atlantic cod aquaculture.

Acknowledgements

We thank Larry Dickison and Frank Powell of Cooke Aquaculture Inc. for providing the water and blue mussel samples for use in this study. We also thank the Atlantic Cod Genomics and Broodstock Development Program, Cooke Aquaculture Inc. and Northern Cod Broodstock Company for providing cod spleens for assessment of genetic resistance to *L. morhua* to help identify parasite-resistant family lines. This research was funded by fellowships from the Natural Sciences and Engineering Research Council (NSERC) of Canada (CGS-M and CGS-D to AF) and an NSERC Strategic Grant (MD and TB). AF was supported in part by the Research Assistantships Initiative of the New Brunswick Innovation Foundation.

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Preliminary experiments to establish *Loma morhua* infections in naïve Atlantic cod (*Gadus morhua*)

Maeghan O'Neill

Maeghan O'Neill, Aaron Frenette, Robyn O'Keefe, Geoffry Harrison, Steve Neil, Ed Trippel, Tillmann Benfey and Michael Duffy

Loma morhua has substantially impeded the development of Atlantic cod aquaculture in eastern North America. Infections cause mortalities and reduced growth rates in both juvenile fish at hatcheries and adults during grow-out. Development of a reliable infection model is needed to investigate life history features of *L. morhua.* Naïve juvenile cod were exposed experimentally to spores of *L. morhua* by intraperitoneal (IP) injection and by gastric intubation but none developed visible xenomas. However, decreased body size and skin lesions support parasite development in IP-injected fish. Coincidentally, fish from the same cohort in a different facility became infected naturally with *L. morhua*. Infections in these fish suggest that low water temperatures might promote parasite development and xenoma formation. Subsequent experiments will investigate water temperature and parasite exposure levels to identify a definitive and efficient infection model for xenoma production by *L. morhua*.

Introduction

The collapse of the wild fishery in Canada and elsewhere has prompted recent interest in culturing of Atlantic cod (*Gadus morhua*) based upon successes with Atlantic salmon (*Salmo salar*) and the potential for an alternative coldwater species for commercial culture. However, as with any new farming endeavour, issues have arisen as consequences of problematic pathogens. *Loma morhua* is a microsporidian parasite that poses a substantial threat to cod aquaculture due to infections characterized by mortalities and reduced growth rates in both juvenile cod at hatcheries and adult cod during grow-out.[1,2]

While little is known about the life history of *L. morhua*, investigations of the closely related *L. salmonae* that infects salmonids have shown that ingestion of spores, either naturally through cohabitation or by feeding of infected gill tissue, leads to infection.[3-6] Intensity of parasite exposure shows a positive correlation with the intensity of xenoma establishment in fish tissues.[7] It is important to note that direct placement of spores on gill tissue does not result in infection and that ingestion of spores is required.[6]

There are two modes of parasite exposure that could occur naturally in an aquaculture setting: first, ingestion of spores present in the water column when cohabitating with infected fish; and second, ingestion coinciding with feeding on smaller infected fish, on the tissue of dead infected fish, or perhaps invertebrate reservoir hosts. Fish fed infected gill tissue were found to have higher infection

intensity and a faster rate of xenoma development than fish infected during cohabitation.[4] However, cohabitation results in longer lasting infections than occur in fish infected by ingestion of tissue,[4] presumably due to the lower number of spores leading to ongoing and chronic infections.

The current research project sought to determine the most reliable mode of infection for use in further laboratory studies.

Materials and Methods

Collection of the parasite Loma morhua

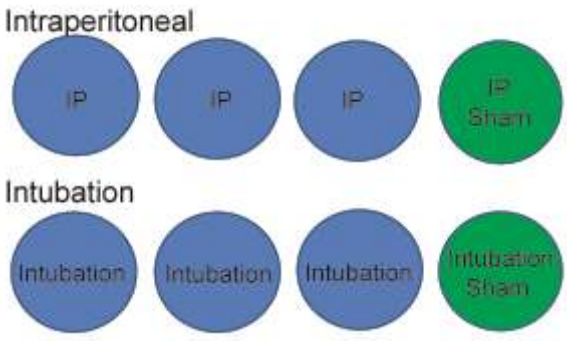
Adult Atlantic cod acquired from an aquaculture site in Back Bay, New Brunswick, were used as our source of *L. morhua* spores. Xenomas were removed from gill filaments and were ruptured with fine forceps to release spores. The mixture of spores and sterile saline represented the spore stock. Spores were quantified using a Spencer haemocytometer (VWR, Mississauga, Ontario). Spores were left to settle for five minutes and then counted in the central grid of each chamber. This process was repeated for a total of 12 chamber counts. Dilutions of the spore stock were prepared at a concentration of 2000 spores/100µL/fish. Tubes were kept on ice until experimental exposure of fish the following day.

Exposure of Atlantic cod to Loma morhua

Two hundred and forty fish of approximately 10 g were allocated randomly to eight tanks at the quarantine facility of the St. Andrews Biological Station (SABS; Department of Fisheries and Oceans, St. Andrews, New Brunswick). During allocation, fish were anaesthetized with tricane (MS 222; Sigma-Aldrich, Oakville, Ontario) at a concentration of 80 mg/L by bath immersion to allow for length and weight to be measured and recorded. Fish were left to recover and were permitted to acclimate for a period of one week prior to exposure to parasites.

Fish were not fed for two days prior to exposure to parasites. Fish from individual tanks were placed in holding containers. Ten fish were anaesthetized at a time by bath immersion in MS 222. Length and weight measurements were obtained prior to experimental manipulations. Control group manipulations were completed before those of fish being exposed to *L. morhua* spores to reduce the possibility of inadvertent parasite exposure.

Figure 1
Outline of the basic experimental design for the exposure of naïve juvenile Atlantic cod to spores of *L. morhua* in the quarantine facility at the SABS in St. Andrews, New Brunswick.



Intraperitoneal (IP) Injection: Anaesthetized fish were placed in dorsal recumbancy. A 30-gauge needle attached to a 1-mL syringe was used to inject 100 µL of solution into the peritoneal cavity just right of the ventral midline, approximately 5 mm anterior to the vent. One tank of 30 fish was injected with 100 µL 0.85% saline to serve as a negative sham control. Three tanks of 30 fish were injected with 100 µL of a solution containing 2000 *L. morhua* spores (Figure 1).

Gastric Intubation: Anaesthetized fish were placed in dorsal recumbancy and the mouth was opened to visualize the esophageal opening. A 1.25-inch 22-gauge dos-

ing needle (VWR, Mississauga, Ontario) was inserted through the esophageal opening and into the stomach where 100 µL of solution was delivered from a 1-mL syringe. One tank of 30 fish was exposed to 100 µL of 0.85% saline to serve as a negative sham control (Figure 1). Three tanks of 30 fish were exposed to 100 µL of a solution containing 2000 *L. morhua* spores (Figure 1).

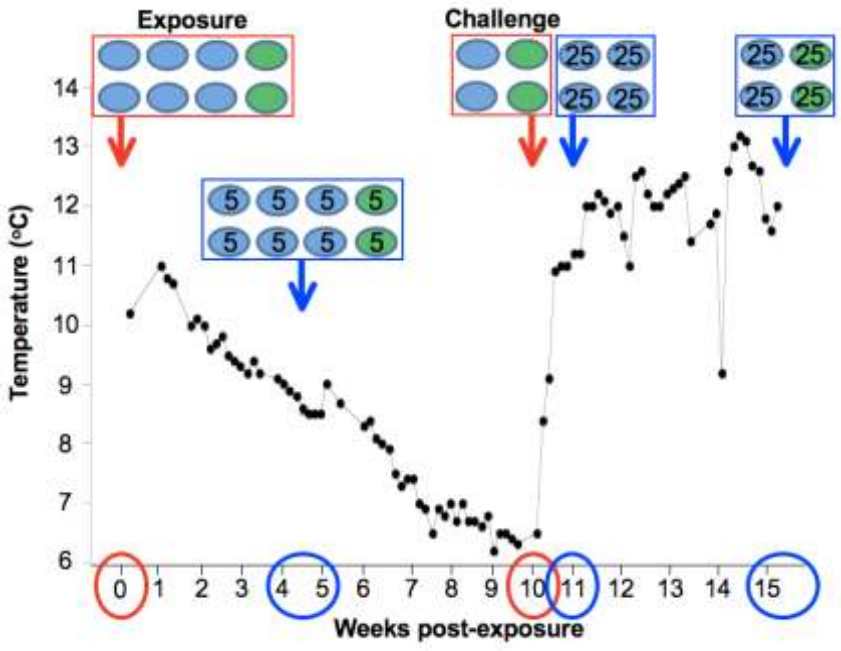
Challenge parasite exposure: At 10 weeks following the initial exposure to *L. morhua*, a challenge dose of 4000 spores was given to the 30 fish in each of four tanks: IP sham, intubation sham, IP treatment and intubation treatment (Figure 2). This challenge exposure coincided with an incremental increase in water temperature from 6.5°C to 11°C that was required for an unrelated study conducted concurrently in the quarantine facility beginning 10 weeks after the initial parasite exposure (Figure 2).

Fish harvest and necropsy

Five fish were collected from each of the eight tanks at 4 weeks post-exposure for preliminary assessment of *L. morhua* infection (Figure 2). Due to time and tank constraints imposed by unrelated experiments at the SABS quarantine facility, fish from four tanks were terminated at 11 weeks post- exposure (two IP treatment and two intubation treatment; Figure 2) with fish in the remaining four tanks terminated at 15.5 weeks post-exposure (IP sham, intubation sham, IP treatment, intubation treatment; Figure 2). All fish were subjected to an overdose of MS 222 by bath immersion. Length and weight were measured and recorded prior to blood collection via the caudal vein. Blood was collected into tubes containing either heparin or ethylenediaminetetraacetic acid (EDTA). Fish were bagged individually and placed on ice until processed at the University of New Brunswick.

Fish were examined externally to make observations of the skin of each animal. The spleen was excised and placed in a petri dish containing sterile saline (0.85% NaCl). The entire external surface of the spleen was scanned using a dissecting microscope for presence of xenomas. Scissors

Figure 2
Details of the experimental exposure outlined in Figure 1 to highlight the times of primary and challenge exposure of 240 juvenile cod to *L. morhua* (red boxes, arrows, and circles) and the times of fish harvest and necropsy (blue boxes, arrows, and circles) as they correspond to the water temperature in the quarantine facility at the SABS in St. Andrews, New Brunswick. The numbers within the blue and green ovals represent the number of fish harvested for necropsy at the corresponding times.



were used to remove the gill arches. Each arch was placed in a petri plate containing 3.4% NaCl. Filaments on each gill arch were scanned visually for presence of xenomas. Gills and spleen were placed in 1.5-mL microcentrifuge tubes following visual examination and were stored at -20°C for subsequent analysis by a quantitative PCR assay that is currently being developed.

Results

None of the 240 cod used in this trial was infected with *L. morhua* based upon visual identification of xenomas. However, cod in the intraperitoneal treatment group that received a challenge exposure to *L. morhua* were found to be of significantly lower mass ($F [3, 88] = 6.19$, $p = 0.001$) and of significantly shorter length ($F [3, 88] = 3.48$, $p = 0.019$) than the IP injected sham control cod at 15.5 weeks post-exposure. There was no significant difference in the length or mass of fish for the other treatment groups in this experiment.

Skin lesions, represented by skin sloughing, were observed in two cod in the intraperitoneal treatment group, and two cod in the gastric intubation treatment group, that received a challenge exposure. Pictures were not obtained because the lesions were visible only when the fish were live and immersed in water. Skin lesions were not observed in fish from any other treatment group.

There was no significant difference in the blood hematocrit or the buffy coat (white blood cells) proportion between tanks of fish throughout the trial.

Discussion and Conclusions

The main goal of the current study was to develop a protocol for reliable infection of cod with *L. morhua*. A reliable infection model is essential for all subsequent experimental work, especially for reliable assessment of prophylactic methods and treatments. Cod were exposed to *L. morhua* by IP injection and by gastric intubation but the results are difficult to interpret because none of the cod exhibited xenomas based upon morphological examination. The lack of xenomas could relate to spore viability but this seems unlikely. However, if the spores were not viable when delivered to cod then a productive infection would not be initiated. The spores could have been dead, empty, or insufficiently developed prior to exposure of fish, but this seems highly unlikely given that each fish received approximately 2000 spores in anticipation of not all spores being infective. The longest period of time that spores were stored was approximately one week at 4°C. Spores of *L. salmonae* were viable and able to produce visible infection in 100% of Chinook salmon after storage for up to 95 days,[8] so it is unlikely that the method or duration of storage decreased spore viability. The transfer to saline might have activated the spores to release their DNA and become empty prior to storage, but this seems unlikely because spores are naturally subjected to physiologic conditions within cod tissues. Regardless, spores will be stored in sterile seawater in the future to eliminate this possibility. Determination of the relative abundance of viable and non-viable spores will also be conducted in advance of future experiments. Staining of human microsporidian spores with the fluorescent stains Sytox Green and Calcofluor White was shown to distinguish viable from non-viable spores.[9] An aliquot of spore stock could be analyzed using this method to determine the percent of viable spores in advance of subsequent experiments. Furthermore, we now have the capacity to assess spore viability using the recently developed *in vitro* culture system for *L. morhua*.[10]

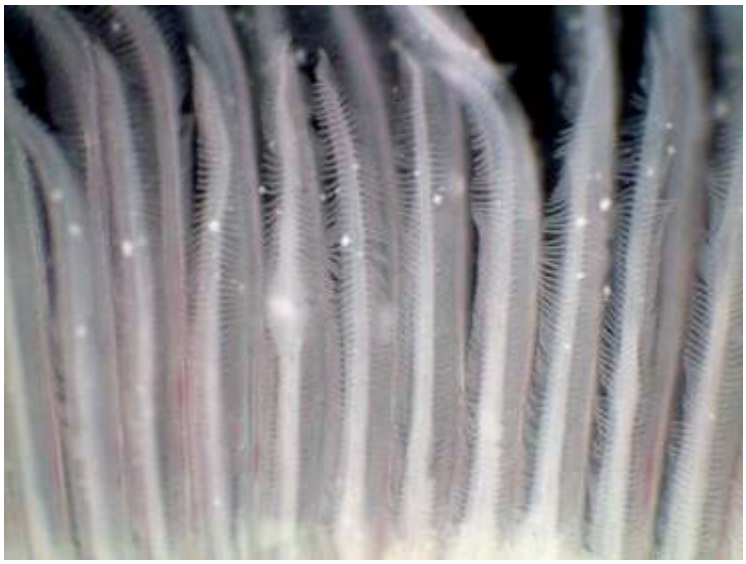
The spore concentration used in this experiment may have been lower than nec-

essary to generate a productive infection with demonstrable xenomas. Approximately 2000 spores were delivered to each fish to simulate a natural exposure dosage. Theoretically, one spore can produce one xenoma and therefore there was potential to see 2000 xenomas in each fish. It may be that a certain threshold must be reached for xenoma formation to occur; however, there is no evidence to support such a requirement. Perhaps surprisingly, studies exposing rainbow trout to *L. salmonae* used spore dosages that ranged from 100,000 to 4,800,000 spores/fish.[7,11] However, these high dosages are likely not representative of a natural exposure. For future experiments we will assess the concentration of spores that will generate a productive infection in a small-scale study prior to commencement of larger scale studies.

An increase in environmental temperature leads to increased parasite load and decreased time to disease onset for *L. salmonae*.[12] This suggests there is likely a temperature range within which xenoma formation occurs for *L. morhua.* Assuming that spores were viable in the current study, fluctuations in water temperature following exposure could have prevented parasite development and proliferation. While the initial parasite exposure of fish occurred at ~11°C, the ambient water temperature showed a steady decrease in temperature to ~6°C (Figure 2). It remains possible that these low temperatures lead to arrested development of the parasite at stages that precede formation of visible xenomas. Numerous studies have investigated the effect of water temperature on *L. salmonae* gill xenoma formation.[5-7,13,14] The permissible temperature range for *L. salmonae* to proceed to xenoma formation in rainbow trout was 9° to 20°C with maximal development at 15°C.[11] The parasite does not develop at temperatures outside this range.[11] The optimal temperature for rainbow trout growth and development (16.5° to 17.2°C)[15] is near the temperature for maximal development of *L. salmonae* (15°C).[11] Taking this into consideration, the optimum temperature for development of *L. morhua* may occur at a temperature slightly below the typical growing temperature of adult Atlantic cod (3° to 7°C).[16,17] Because the duration of exposure to that temperature range was short (Figure 2), it may not have been long enough to allow for parasite development to occur. It must be noted that a high intensity naturally-acquired infection was observed on the gills of a fish from the same cohort (Figure 3) that were reared in an adjacent facility at ambient water temperatures that declined to ~2°C over the same time period as the experimental exposures were conducted (Figure 4). This fortuitous observation indicates definitively that *L. morhua* does not require the elevated temperature conditions that are required by *L. salmonae* (15°C).[5-7,13] Furthermore, it suggests that *L. morhua* might require lower water temperatures in support of xenoma formation. Further research will be required to elucidate the effect of temperature on *L. morhua* infections.

Although a productive infection characterized by visual identification of

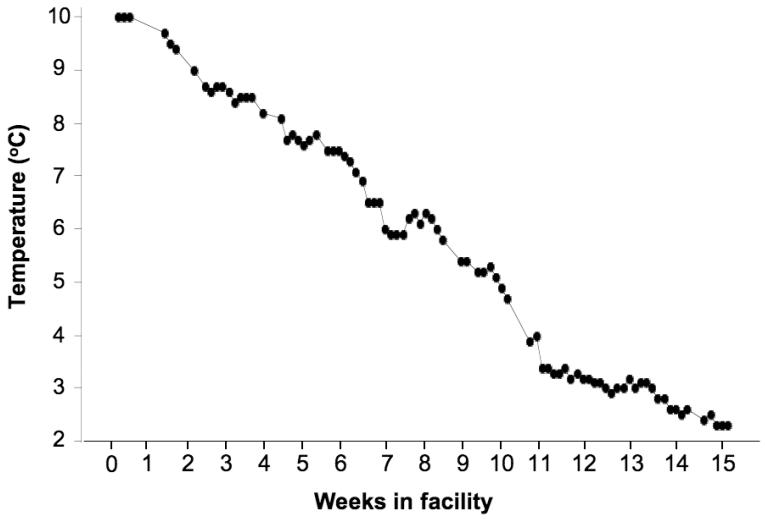
Figure 3
Observation of xenomas of *L. morhua* on the gills of juvenile Atlantic cod that became infected naturally during their maintenance in a land-based facility at the SABS in St. Andrews, New Brunswick.



xenomas was not generated, it must be noted that there was a negative effect on growth observed in the IP treatment group that received a challenge exposure of *L. morhua*. The IP treatment group was found to be of significantly lower mass and of significantly shorter length than the sham IP injected group that was treated exactly the same with exception of the original parasite exposure at time zero. A reduction in growth has been reported in rainbow trout exposed to an oral dose of *L. salmonae* spores.[18] Our observation of impaired growth suggests that the primary injection of spores resulted in infection by *L. morhua*. Fish in this group also exhibited peculiar skin lesions at the end of the experiment. While these lesions were also found in the intubation treatment group that received a challenge exposure, lesions were not found in either of the sham control groups. It remains possible that xenoma formation would have occurred had the fish been able to remain in the SABS quarantine facility longer. Regardless, definitive conclusions on the infection status of these cod will await refinement of a qPCR assay capable of detecting developmental stages that precede xenoma formation.

Loma morhua has been found to be a major problem at numerous aquaculture sites where cod are being cultured. Outbreaks of *L. morhua* have been observed in land-based Atlantic cod facilities in Newfoundland,[19] New Brunswick (as documented in the present study) and New Hampshire.[20] Chronic infections are common during grow-out of cod in sea cages along eastern North America and in Iceland.[21] Economic losses are difficult to assess during grow-out, but hatchery losses of hundreds of thousands of dollars were reported during one recent outbreak.[20] Challenges associated with pathogens differ substantially when dealing with fish in hatcheries versus grow-out at a cage site. Hatcheries and land-based culturing systems have the benefit of being able to sterilize the incoming water to prevent pathogens from entering the system. The incoming water typically enters a sand filter followed by exposure to ultraviolet (UV) light. The UV light is used to inactivate pathogens that pass through the filter; however, microsporidian parasites such as *L. morhua* produce spores that are small and highly durable, which facilitates environmental persistence. A study by Brusseau et al.[22] found that approximately 50% of spores from a related human parasite passed through a sand filter. While sand filters may trap larger pathogens it does not effectively prevent passage of microsporidian spores. Exposure to UV light inactivates spores of related human parasites,[23] and *L. salmonae* from salmon.[24] However, there is huge variability when it comes to the dosage required to inactivate common fish pathogens (2,000 to 230,000 $mW/cm^2/s$).[25] While the life history of *L. morhua* has not been

Figure 4
The temperature profile of ambient water entering the SABS facility housing fish of the same cohort that became infected naturally with *L. morhua* during their maintenance in a land-based facility at the SABS in St. Andrews, New Brunswick. The lower water temperatures from weeks 10 to 15, as compared with Figure 2, might have promoted xenoma formation by *L. morhua* in these fish.



elucidated, studies of the closely related *L. salmonae* suggest that a single infective spore gains access to a single host cell and induces a state of hypertrophy by dividing asexually to produces large numbers of spores.[12] Therefore, if a single spore were to bypass the sterilization techniques implemented in a hatchery, the infection can spread quickly throughout the entire fish population. Open-water aquaculture facilities are unable to implement water sterilization techniques; therefore, husbandry practices will likely play an important role in limiting parasite transmission.

This is the first study to employ experimental exposure of Atlantic cod to *L. morhua*. While definitive evidence of parasite establishment awaits qPCR, we have identified the duration of experiments, parasite exposure levels and water temperature as the key variables that likely influence xenoma development by *L. morhua*. Furthermore, our observation of naturally-acquired *L. morhua* infections in juvenile cod reinforces that UV sterilization systems are a necessity to help prevent disease outbreaks in land-based facilities that use a flow-through system to supply water.

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In vitro cell culture for the study of *Loma morhua*

MJ MacLeod, R Rumney, SR Monaghan, A Frenette, M Duffy and LEJ Lee

MJ MacLeod

Detailed research into microsporidian parasites using *in vivo* methodologies is challenging for a variety of reasons. The considerable time and effort needed to secure infective spores, care for livestock, and ensure controlled conditions can substantially slow the pace of research. These challenges are compounded by lengthy processing procedures and the difficulty in conclusively assigning observations of the parasite itself. While not necessarily a replacement for work with whole-organisms, *in vitro* methods are extremely useful for studies of intracellular pathogens such as *Loma morhua*, allowing observations of live parasites *in situ* under controlled conditions. Accordingly, the development of culture methods for *Loma* and other microsporidian species would facilitate novel research into the life-history, infective processes, and biochemistry of the parasites. To establish laboratory-based culture of *Loma morhua* specifically, an *in vitro* infection model has been developed using a novel cod cell line and parasite-specific culture methods. *In vitro* cultivation of parasites provides a useful model system for assessment of the efficacy of chemotherapeutics that could kill the parasites or limit infection. Infected cell cultures will be treated with a panel of fungicides to identify drugs with potential in treating live cod.

Introduction

Cultured animal cells, maintained and propagated in plastic dishes containing red liquid (Figure 1), may at first seem to bear little relationship with live animals in a complex environment. However, in certain contexts the similarity is closer than one may think. When used appropriately, cell culture can simulate specific aspects of *in vivo* systems (within an organism, such as a fish), providing meaningful results with many advantages over whole-organism studies. By using *in vitro* methods (outside of the organism such as in a test tube), fewer animals need to be sacrificed and experiments can be conducted on a smaller scale, saving time and money.

Figure 1
Atlantic cod cells being grown *in vitro* in tissue culture medium.

The use of cultured cells to study intracellular pathogens has a somewhat short history. Beginning in the early 1900s, the technology developed principally during the 1940s under the impetus of virology research. Despite its young age, cell culture practices have produced many noteworthy products benefitting clinical and agricultural research, including vaccines, monoclonal antibodies and other bio-products that would not be possible using traditional microbiology systems. Since the development of the polio vaccine, *in vitro* research has branched out beyond virology into the study of various intracellular parasites such as *Chlamydia*,[1] *Plasmodium*[2] and the microsporidians.[3,4] *Loma morhua* is a microsporidian parasite that is limiting cod aquaculture and thus our interest in capitalizing on these previous achievements and successes using *in vitro* research.

The microsporidia are a unique group of obligate intracellular fungal parasites that have been found to infect a wide variety of commercially valuable host species, both vertebrate (e.g., fishes) and invertebrate (e.g., honey bees). As intracellular parasites, the microsporidia are prime candidates for research using cell culture techniques. Consequently, a growing body of work has developed around the *in vitro* study of microsporidian parasites in recent years. While existing work has been focused principally on human and arthropod infecting species, potential applications stretch beyond clinical and silvicultural uses. When one considers the number of commercially important fish species susceptible to microsporidian infection, it becomes clear that *in vitro* research into these parasites could benefit aquaculture programs within the food and pet industry. For the sake of cod aquaculture programs specifically, laboratory-based research into *L. morhua* is a prudent step for improving our understanding of the parasite and enhancing health management practices with farmed fish.

As *in vitro* work with fish-infecting microsporidians is in its infancy, the development of culture methods for *L. morhua* required a ground-up approach. An infection model requires a representative host, as well as the ability to successfully induce infection. For the representative host, a cod cell line was developed. Infection was induced using *in vitro* culture methods for *L. morhua* which were developed using manipulated chemical conditions meant to represent the host environment at the infection site.

Cod Cells

While scattered reports of attempted cod cell culture exist, including the establishment of a gonad-derived cell line, [5] none were available when this study was initiated. As a result, a new cell line was developed using tissues from larval Atlantic cod (*Gadus morhua*) using well-established methods for culturing cells of cold-water fish, including closely related species such as the haddock, *Melanogrammus aeglefinus*.[6]

Several 14-day-old larvae, obtained from the University of Maine Centre for Cooperative Aquaculture Research, were disinfected and dissected into 1 mm^3 tissue fragments using sterile tools. The samples were plated into six-well tissue culture plates (Corning) containing a small quantity of Leibovitz's L-15 medium. The explants were observed over the following week for outgrowth and successful trials were subcultured by means of dissociation using TryplE (recombinant trypsin from Invitrogen), centrifugation and plating onto new flasks with fresh medium. Subcultures were periodically harvested for characterization and infection experiments.

Characterization of the cod cells was performed to evaluate response to various growth conditions and to determine the tolerable concentrations of potential treat-

ments and additives. This was accomplished using Alamar Blue assays, a fluorescent measure of metabolic activity in living cells that provides the means to evaluate cell viability following exposure to experimental conditions. The cells responded favourably to elevated FBS concentrations and grew most quickly at 21°C (Figure 2). The identity of the cells was confirmed as *G. morhua* by DNA barcoding performed at the University of Guelph. The cells have now been maintained for over one year, surviving more than 16 passages and expanding into a collection of nearly 60 flasks. Two flasks have been cryogenically preserved and the culture appears to represent a stable cell line.

Loma culture

As the *L. morhua* parasite had not been previously cultured *in vitro*, spore samples were obtained from the dissected tissues of farmed fish by the Duffy lab at the University of New Brunswick. White, spore-filled xenomas were removed from gill and spleen tissues and suspended in saline solution. Spores were isolated from these samples by means of Percoll® gradient centrifugation —a method for enhancing sample separation by density gradient—and subsequently used for experimental infection trials.

Attempts at culturing the *L. morhua* parasite *in vitro* were initiated prior to the establishment of the cod cell line. As such, initial efforts were made utilizing surrogate cell lines derived from species or tissues suitable for infection by *L. morhua*. The first attempt was made using a cell line derived from haddock embryos. These were selected due to the close phylogenetic relationship between haddock and cod and the species' susceptibility to infection by related *Loma* species. Unfortunately, this initial attempt yielded no evidence of

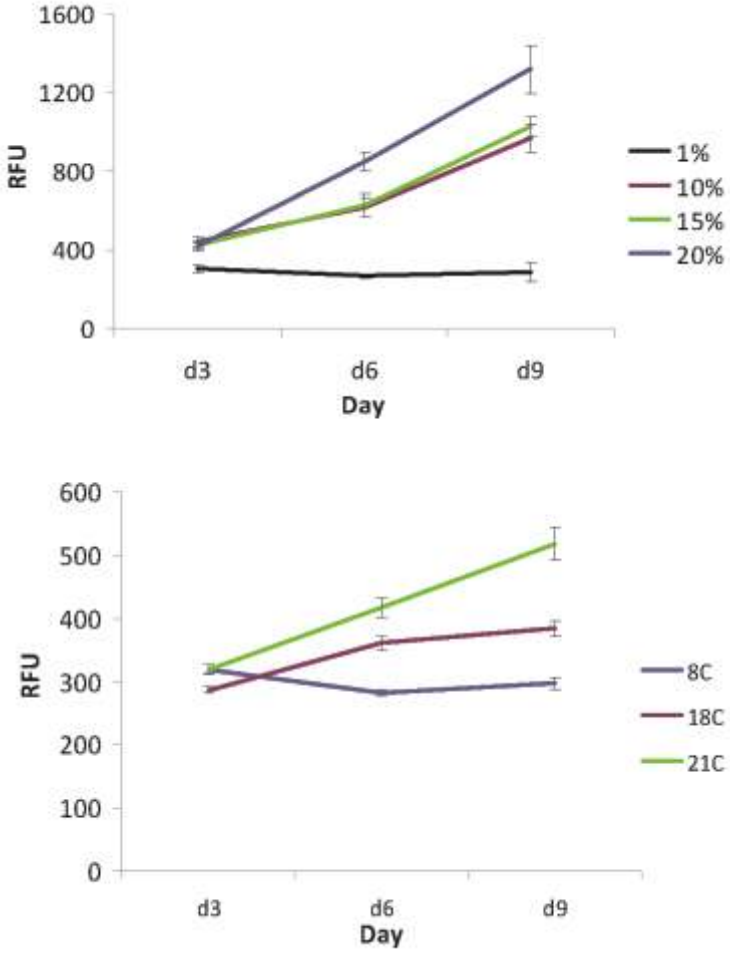

Figure 2

Cod cell growth response curves. Upper panel: response to [FBS] (v/v), showing higher growth rates to elevated serum supplements. Lower panel: response to temperature, showing higher growth rates at elevated temperatures although accompanied by poor cell appearance when maintained at 21°C. All cells died when incubated at 24°C. Error bars indicate 98% confidence intervals.

successful infection and later trials with this haddock cell line and a readily cultured microsporidian (*Anncaliia algerae*) suggested a limited capacity for supporting spore growth. A second attempt was made using a cell line derived from the gill tissues of rainbow trout, again yielding no success.

Infection trials with cod cells were initiated once cells became available in sufficient numbers. The first attempt was made by inoculating a cod flask with *Loma* spores and incubating at 8°C without any modification to culture conditions. Very little evidence of infection was observed initially, but an isolated case of intracellular spores appeared 20 days post-inoculation (Figure 3).

Due to the limited availability of *Loma* spores, simultaneous work with *A. algerae* was conducted to direct culture efforts, in the hope that conditions favouring infective processes in one microsporidian could be applied to enhance the growth of another. Following work with two human-infecting *Encephalitozoon* species,[7] *A. algerae* infectivity in fish-derived cells was found to be enhanced with supplemental magnesium (Figure 4). A *Loma* trial was conducted using cod cells and supplemental magnesium at 8° and 18°C, but no noticeable stimulatory effect was produced.

The inability to produce appreciable infection rates *in vitro* indicated that a different approach was necessary. A third trial using cod cells was attempted using modified pH conditions, which were intended to replicate the environment of the host intestine (inspired by the work of Pleshinger and Weidner). [8] This meant increasing the pH of the growth medium containing *L. morhua* in the presence of cod cells, accomplished by suspending a spore pellet in Minimum Essential Medium (MEM) that was then inoculated into a flask. The MEM-spore suspension was allowed to remain in the flask for one hour, over which time the pH rose to approximately 8. Following this, the pH was brought back down and stabilized by the addition of fresh L-15 medium. By 14 days post-inoculation, many instances of *Loma* infection were evident, developing into heavily spore-laden cells similar to those observed in *A. algerae* infection by 31 days (Figure 5).

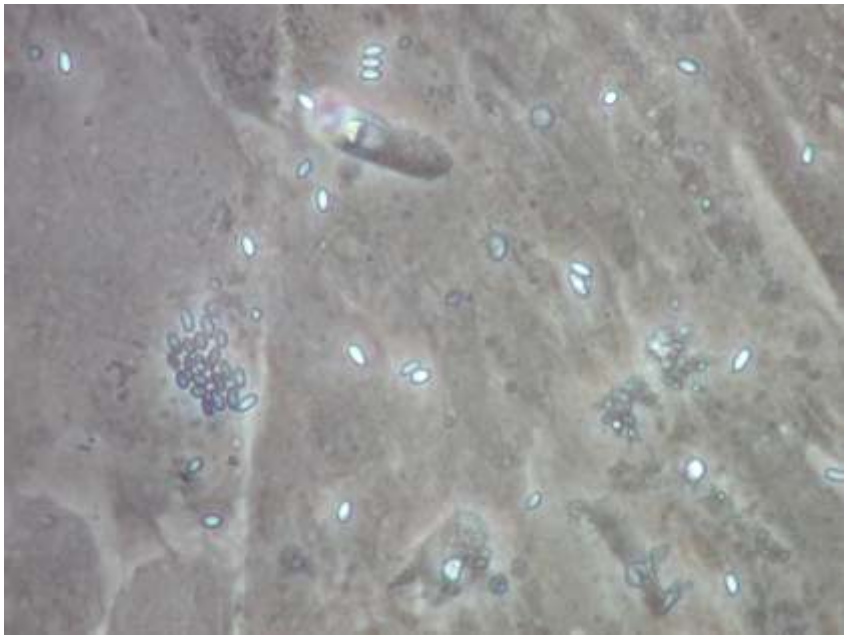

Figure 3
***Loma morhua* growing in cod cells 20 days post-inoculation. Co-culture was maintained in L-15 medium containing 10% (v/v) fetal bovine serum, 10 mg gentamicin and 0.125 mg amphotericin B, and incubated at 8°C.**

While these results represent a preliminary trial demanding further investigation into the importance of pH adjustments for *in vitro* infection, they confirm the feasibility of culturing *L. morhua* within fish cell lines. Subsequent experiments are needed to determine whether or not similar infection rates can be achieved without pH modification, the pH range over which successful infection can be induced, and the optimal temperature ranges for inducing high infection rates. For the time being, however, the combination of a stable cod cell line and methods successfully inducing *L. morhua* infection in a laboratory setting represents the solid foundation for an *in vitro* infection model.

Proteomic Characterization

By optimizing culture conditions, *L. morhua* spores can be produced in large quantities. This will facilitate additional *in vitro* studies into the parasite's life-cycle and biochemistry including the proteomic characterization of host-parasite interactions over the course of infection. This will be attempted using specialized centrifugation techniques, which have been successfully employed to produce purified samples of intracellular microsporidian developmental stages.[9] By using 2-dimensional electrophoresis to analyze parasite samples over the course of infection, up- and down-regulation of proteins may be observed, uncovering potential targets for therapeutic treatment.

As few microsporidians, *Loma* species included, have been studied at the molecular level, a steady supply of viable spores in the laboratory represents an important tool for continuing research into the parasite, its life-cycle, biochemistry and potential treatments.

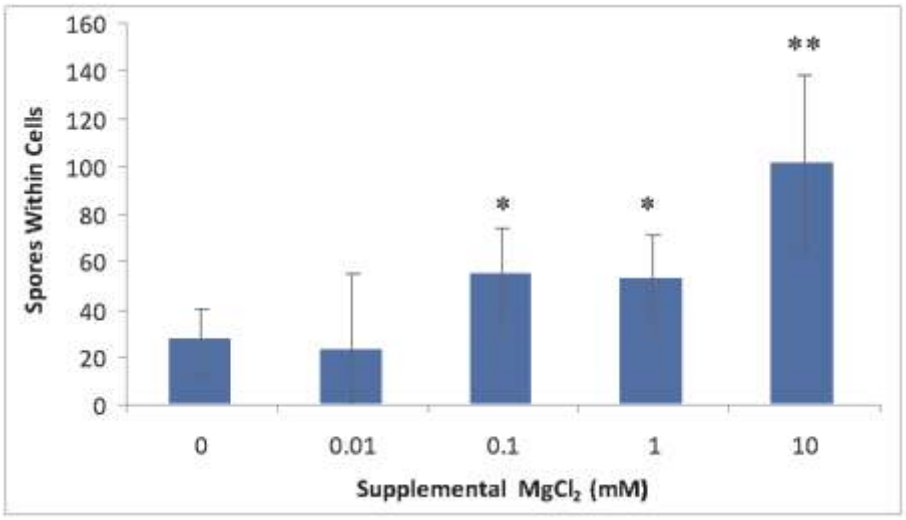

Figure 4

Enhanced infectivity of *Anncaliia algerae* spores in GFSK cells with supplemental magnesium. X-axis values indicate molar concentration of $MgCl_2$ added to stock Leibovitz's L-15 medium. Spore count reflects number of spores counted within cells from 20 random micrograph images. A significant response was observed in both 0.1 and 1 mM supplemental treatments ($p<0.05$) as well as 10 mM ($p<0.01$).

Conclusions

The establishment of a continuous cod cell line and development of *in vitro* culture methods for *L. morhua* represents the basis for a comprehensive infection model. By producing the parasite and infecting cells *in vitro*, real-time observations may be made of spores within living cells, permitting observations and insights that would be difficult or impossible with experiments using living fish. Additionally, *in vitro* studies can be used in lieu of *in vivo* studies, for preliminary work at least, thereby reducing cost, man-hours and the number of live fish sacrificed for the sake of studying parasites. We intend to use the *in vitro* model system for the assessment of a panel of potential fungicides to identify drugs with potential in treating live cod. However, continuing efforts will be necessary to ensure repeated, reliable infection of cod cells and to determine the optimal conditions for producing spores in sufficient numbers for additional research.

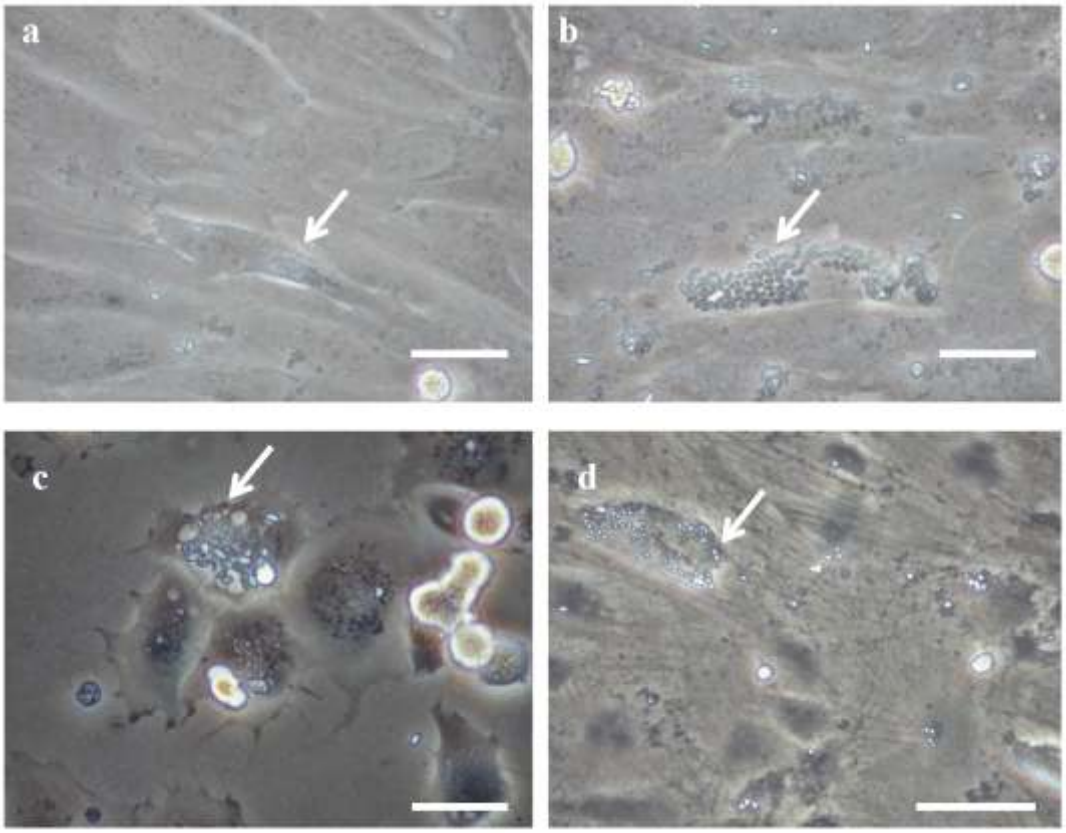

Figure 5

Micrographs illustrating *Loma morhua*-filled cod cells following MEM treatment, with *Anncaliia algerae* infected GFSK micrograph for comparison. Arrows indicate infected cells. Scale bar = 25 μm.

a: Intracellular *L. morhua* spores, 14 days post-inoculation.

b: Heavily infected cell, 31 days post-inoculation.

c: Post-passage micrograph showing *Loma* spores carried over to fresh flask within cell.

d: *Anncallia*-infected cell containing mature spores and developmental stages.

By using laboratory-produced spores to investigate the mechanisms of microsporidian infection at the molecular level, potential targets for therapeutic treatment may become evident. The unique advantages of this type of research may be employed to supplement or direct *in vivo* studies, and *L. morhua* may be better understood than otherwise possible with only whole-organism studies. Ultimately, the insights provided by both *in vitro* and *in vivo* research will aid in the development of therapeutic treatment for *Loma* infection. This will, in turn, play a part in maintaining the health of cultured Atlantic cod, improving fish farming practices and ensuring the availability of cod for human consumption.

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TJ Benfey

The production of single-sex and sterile populations of Atlantic cod *(Gadus morhua)* for aquaculture: fish health considerations with a focus on *Loma morhua*

TJ Benfey, NJ Feindel, S Lin, JA Whitehead, DJ Martin-Robichaud, EA Trippel and M Duffy

The high rate of pre-harvest sexual maturation of farmed Atlantic cod (*Gadus morhua*) is a major constraint to the commercialization of cod aquaculture. Sexual maturation has been eliminated in other species of fish by using all-female triploid stocks. This paper describes the methods used to develop this technology successfully for Atlantic cod. However, little data are available on the culture characteristics of triploid cod. They are predicted to be more susceptible to mortality from *Loma morhua* infection because of the well documented effects of triploidy on blood cell size. An experimental infection trial is currently underway to test this prediction.

The commercialization of Atlantic cod *(Gadus morhua)* farming in Atlantic Canada is constrained by sexual maturation of production fish for several reasons: they divert energy from somatic growth to gamete production (Figure 1) and spawning, there can be high mortality of unspawned "egg-bound" females, and there is the potential for release of eggs and larvae from within-cage spawning into the surrounding environment.[1,2] All of these constraints can be addressed by using female triploids, which invest very little energy into gonadal growth (Figure 2) and are sterile.[3]

Figure 1
Diploid female Atlantic cod showing ovary size at maturity. [Photo: Nathaniel Feindel]

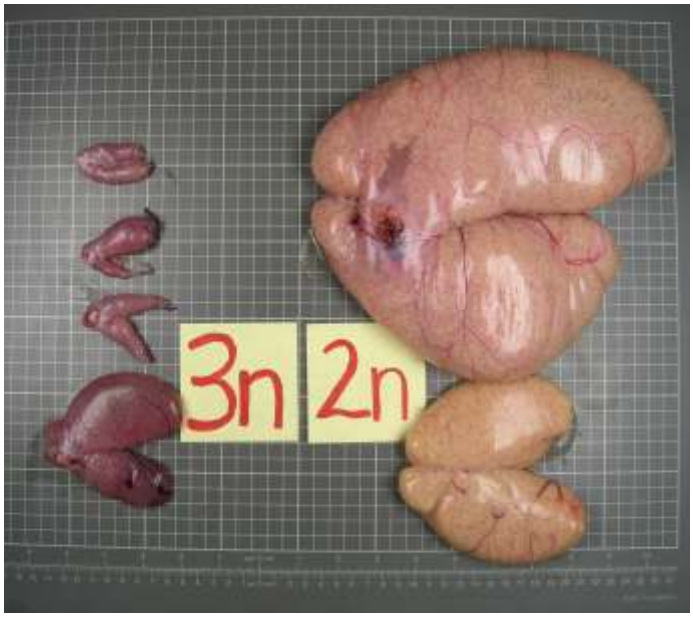

Figure 2
Dissected ovaries from equal-sized sibling triploid (3n) and diploid (2n) Atlantic cod.
[Photo: Nathaniel Feindel]

A collaborative research program between the University of New Brunswick (Fredericton, NB) and Fisheries and Oceans Canada (St. Andrews, NB), in partnership with Cooke Aquaculture Inc. (Blacks Harbour, NB), has been focussing on developing protocols for the production and evaluation of female triploid Atlantic cod populations for aquaculture. This paper summarizes progress made in this program and outlines the rationale for including triploids in the current *Loma morhua* research project.

Production of Female Triploid Cod Populations

Mixed-sex triploid populations of Atlantic cod have been produced in both Canada and Norway using simple thermal and hydrostatic pressure treatments similar to those developed for other teleost species,[4,5] and commercial-scale pressure systems designed for this purpose are produced in New Brunswick (Figure 3). As is typical for fishes, gonadal development in triploid Atlantic cod is suppressed to a much greater extent in females than in males,[6] and triploid males are capable of producing functional sperm that can activate development in eggs, although progeny from crosses between triploid males and diploid females are aneuploid and die early in development.[7,8] For maximum advantage in aquaculture, the production of triploid Atlantic cod should therefore be combined with the production of all-female populations.[6]

In species in which male parents determine the sex of their offspring (i.e., species having female homogamety and male heterogamety, equivalent to the mammalian XX-female/XY-male sex determin-

Figure 3
TRC-APV-M^{TM} pressure system used to produce triploid Atlantic cod (TRC Hydraulics Inc., Dieppe, NB) [Photo: Roger Smith]

ing system), all-female populations can be produced by crossing functionally masculinized females ("neomales") with normal females.[9] Although androgens (i.e., masculinising sex steroids) are used to produce neomales, it is their untreated offspring that are then used as production fish (Figure 4). All-female populations of rainbow trout (*Oncorhynchus mykiss*) and Atlantic halibut (*Hippoglossus hippoglossus*) produced in this way are already farmed in North America and Europe. However, not all fish species have the male-heterogametic sex determining system.[10] Gynogenesis, whereby the paternal genome is excluded and the maternal genome duplicated, can be used to determine the genetic basis of sex in fish, and this approach has been used to confirm female homogamety in Atlantic cod by demonstrating that gynogens are invariably female.[11,12] Female homogamety has also been demonstrated in this species by the observation that all-female populations result from crosses using sperm from hermaphrodites to fertilize eggs from normal females, with hermaphrodites in this case having been produced from partially effective androgen treatments designed to produce neomales.[13]

The effective integration of this technology into commercial breeding programs requires the reliable production of "strippable" neomales that do not need to be killed to obtain their milt. Achieving this goal requires optimizing sex reversal protocols, by determining both the best steroid dose and the best time to begin and end steroid treatment. Based on detailed histological observation, the optimum treatment for masculinisation of Atlantic cod has been shown to encompass the size interval from 8 to 46 mm standard length; when androgen is fed to the fish over this time interval, there is a dose-dependent shift from a mixed-sex to an all-male population.[14,15] Given that untreated populations have a sex ratio not different from 1:1, approximately half of the males in such an all-male population should be neomales. These fish are currently being reared at the St. Andrews Biological Station for future use in Atlantic cod breeding programs. At this time, the only reliable way to distinguish normal male Atlantic cod from neomales is by determining the sex ratio of each individual male's progeny; this approach was used successfully to identify and retain neomales for breeding purposes in Atlantic halibut.[16]

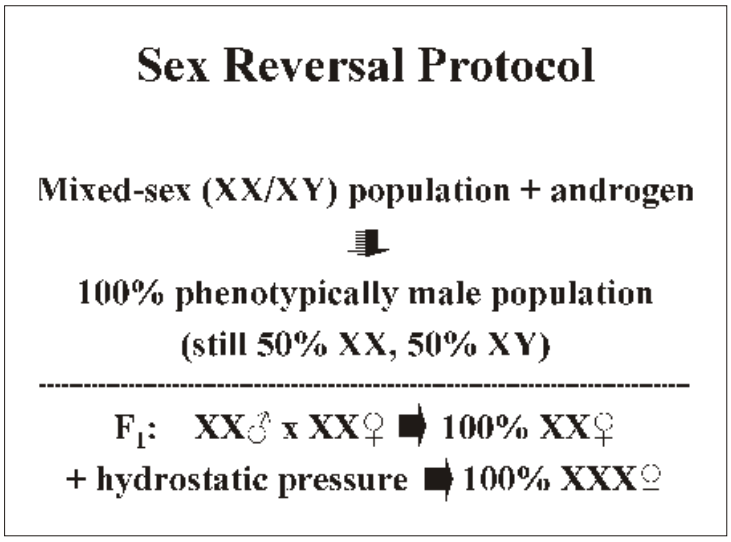

Figure 4

Protocol for producing all-female diploid or triploid populations in species having female homogamety (equivalent to XX-female and XY-male). This protocol is already used for commercial production of rainbow trout and Atlantic halibut, and is currently being developed for Atlantic cod. Note that androgen treatment is applied to the broodstock population and that production fish (their F_1 offspring) are not treated with any hormones. (XX male = diploid neomale, XX female = normal diploid female, XXX female = triploid female)

Disease Resistance in Triploids

Disease resistance in triploids could conceivably be affected for a number

of reasons related to their basic genetic and physiological differences compared to diploids.[3,17,18] These include their doubled maternal genome (resulting in increased heterozygosity but also unbalanced gene dosage), diminished ovarian development (resulting in altered energy allocation among tissues and reduced sex steroid levels in females), and increased nuclear volume and concomitant increase in cell volume and decrease in cell number in a variety of cells, including circulating leukocytes. Triploids also appear to be less tolerant of chronic stress[3,17,18] and have a lower thermal optimum.[19] It has also been reported that gill surface area is reduced in triploid Atlantic salmon (*Salmo salar*).[20] However, the limited information currently available suggests that triploids are not remarkably different from diploids with respect to immunocompetence, disease resistance or vaccine efficacy when exposed to viral or bacterial pathogens.[17]

Within the context of this workshop, we are currently examining genetic resistance and susceptibility to *Loma morhua* infection in both diploid and triploid Atlantic cod. This microsporan parasite is common in both wild and farmed Atlantic cod, and is known to cause emaciation and mortality in cultured populations.[21-23] Infection results in a dramatic reduction in circulating lymphocyte numbers and blood oxygen carrying capacity,[21] as well as the formation of xenomas in tissues associated with respiration (i.e., gill lamellae), circulation (i.e., heart) and blood cell mobilization (i.e., spleen). These characteristics are of particular relevance to triploids, given that their aerobic and immune functions may already be limited by reduced erythrocyte and leukocyte numbers compared to diploids. We are therefore predicting that triploids will be even less tolerant of *L. morhua* infection than diploids, with obvious ramifications for their use in aquaculture. Our current study is testing this prediction.

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