AN ABSTRACT OF THE THESIS OF

Lauren Jean Norris for the degree of Master of Science in Microbiology presented on September 1, 2017.

Title: Controlling Important Pathogens in Zebrafish (*Danio rerio*): Assessing Cryopreservation Survival of Bacteria and Parasites and Clinical Sensitivity of Mycobacterial qPCR Assays

Abstract approved:

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Michael L. Kent

Zebrafish (*Danio* rerio) are one of the most commonly used animal models in biomedical research. Zebrafish resource facilities, like the Zebrafish International Resource Center (ZIRC) in Eugene, Oregon, are the main providers and keepers of numerous zebrafish wild-type, mutant, and transgenic lines. Although ZIRC maintains live zebrafish at various life stages, sperm cryopreservation allows them to maintain the vast array of zebrafish lines that they receive from outside facilities. Hence, there is a concern about the potential of vertical transmission of pathogens capable of surviving the freezing and thawing process.

My first study was to determine whether zebrafish pathogens are capable of surviving the sperm cryopreservation process used by ZIRC (i.e., the ZIRC method). I assessed the survival of two strains of *Mycobacterium chelonae* (H1E1 and H1E2), one strain of *Mycobacterium marinum* (OSU 214), one strain of *Edwardsiella ictaluri, Pseudocapillaria tomentosa* eggs and *Pseudoloma neurophilia* spores, which are all

pathogens of concern in zebrafish research facilities. These pathogens were also frozen and thawed without cryopreservant, and the pathogens were frozen at either -80^{o}C or -20^{o}C with only a small amount of Phosphate Buffered Saline (PBS).

Each bacterial species survived both freezing and thawing methods, however the samples subjected to the ZIRC method had the higher percentages of bacterial survival compared to the freezing without cryopreservant samples. The mycobacteria had higher survival rates compared to Gram-negative *E. ictaluri* in both freezing methods. *E. ictaluri* exhibited a 1-2 log decrease in concentration following the freezing without cryopreservant.

For the *P. tomentosa* eggs, survival was based on larvation. Eggs were examined at Day 0 (immediately after collecting or thawing) and at Day 7 (a week after being collected or thawed). No larvation was observed on Day 7 with eggs processed by the ZIRC method or simple freezing (-80^{o}C in 1X PBS and no cryopreservant). In contrast, the positive controls, kept at 28^{o}C, showed 80-93% larvation at Day 7. Most of the eggs observed in either freezing method were unlarvated and intact, however some, exhibited signs of internal deformation of the egg contents.

In 2014, our lab conducted a similar cryopreservation study on the ZIRC cryopreservation method in place at that time. In that study *P. neurophilia* spores were tested for their ability to survive cryopreservation. I repeated this study in 2017 using the 2017 ZIRC cryopreservation protocol. In both experiments, two fluorescent stains, SYTOX and Fungi-Fluor, and presence of a spore vacuole were used to determine spore viability. SYTOX green is a fluorescent nucleic acid stain, and cells are scored as dead when the dye enters the cells and results in green fluorescence. *P. neurophilia* spores also

contain a long, coiled, polar filament or tube that is thought to aid in infecting hosts cells when extruded. Spores are scored as alive if they are stained with Fungi-Fluor, exposed to ultra violet light, and then expel their polar tubes. Presence of a vacuole observed by light microscopy indicated that spores were alive.

The 2014 and 2017 experiments yield very similar results, and some spores were able to survive the ZIRC cryopreservation method. Spore survival varied depending on the fluorescent stain used. Samples stained with SYTOX yielded higher percentages of survival than those stained with Fungi-Fluor or quantified using vacuole presence. Nevertheless, in both the 2014 and 2017 experiments, about 10% of the spores were scored as alive using the more conservative Fungi-Fluor and vacuole presence tests following the ZIRC cryopreservation method. Very few spores were scored as alive following freezing without cryopreservant with any method.

The second study I conducted entailed working with the Oregon State University Veterinary Diagnostics Laboratory (OSU VDL) to evaluate the clinical sensitivity of their real-time quantitative PCR (qPCR) assays for three *Mycobacterium* spp: *M. chelonae, M. marinum,* and *M. haemophilum.* To test the clinical sensitivity of these assays, we spiked actual zebrafish tissue samples with known concentrations of diluted bacterial samples and determined the lowest detectable bacterial concentration. For this study *M. chelonae* (H1E2) and *M. marinum* (OSU 214) were diluted and spiked into minced zebrafish tissue. These samples were then assayed using qPCR. *M. haemophilum* was not used in this study due to difficulties with culturing. For *M. chelonae*, 61,000 colony forming units (CFUs)/mL and 437 CFUs per PCR reaction was the lowest detectable

concentration. On the other hand, 3,700 CFU/mL and 27 CFUs per PCR reaction was the lowest detectable concentration for *M. marinum*.

In this thesis research, I showed that *M. chelonae*, *M. marinum*, *E. ictaluri*, and *P. neurophilia* spores can survive the ZIRC cryopreservation method and in some cases freezing without a cryopreservant, but *P. tomentosa* eggs did not survive freezing. Given these results, I recommend that zebrafish and fish facilities that implement sperm cryopreservation consider testing sperm samples prior to freezing them or using them for *in vitro* fertilization. We also determined the clinical sensitivity of the *Mycobacterium* qPCR assay used by the OSU VDL. Although this assay can identify *Mycobacterium* species in fish tissue, specifically *M. chelonae* and *M. marinum*, it showed rather moderate sensitivity. I therefore recommend these tests for species identification of mycobacteria in fish in which mycobacteria are first detected by other methods (e.g., acid fast staining) rather than for screening zebrafish for the presence of bacteria in fish with no other indications of infection.

Controlling Important Pathogens in Zebrafish (*Danio rerio*): Assessing Cryopreservation Survival of Bacteria and Parasites and Clinical Sensitivity of Mycobacterial qPCR Assays

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APPROVED:

Major Professor, representing Microbiology

Chair of the Department of Microbiology

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Lauren Jean Norris, Author

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CONTRIBUTION OF AUTHORS

Virginia Watral was the primary researcher for the 2014 *Pseudoloma neurophilia* cryopreservation study and provided technical support and advice. Andree Hunkapiller provided technical support and performed the PCR assays. Donna Mulrooney assisted in the experimental design of the PCR validation project.

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Chapter 1: Introduction

The zebrafish (*Danio rerio*) animal model

Zebrafish (*Danio rerio*) are naturally found in waters throughout Central Asia,[1] but they may also be found in household aquariums all around the world. These fish have quickly become one of the most commonly used, non-rodent, animal models in biomedical research.[2,3,4] The NIH Zebrafish Information Network (ZFIN) summarized the records from their clients, and they list 2,474 academic and 86 commercial users of zebrafish between 2009 and 2014. The rate of funding R01 grants using zebrafish has almost doubled since 2008.[5] According to Howe *et al*. (2013), 71.4% of human genes have at least one zebrafish orthologue and 69% of zebrafish have at least one human orthologue.[6] The size, transparency, internal physiological similarities to humans and availability of numerous wild-type, mutant and transgenic lines have made zebrafish ideal models for a wide range of experimental studies.

Cancer research is one area that is utilizing the zebrafish animal model, especially zebrafish embryos. Their transparency allows researchers to visually observe the development and dissemination of cancerous tumors.[7,8] In one study, the physical interaction between macrophages and tumor cells in zebrafish embryos was observable and this direct interaction is thought to assist in the spread of tumor cells to nearby tissues and organs.[9] Certain strains of these zebrafish have also proven to be useful in muscular dystrophy research as they will not only exhibit the genetic severities of this disease (e.g.

muscle disorganization, progressive motor dysfunction), but they will also express the extreme clinical signs of this disease, which is lacking in current mouse models.[10] Zebrafish are also being used to study various brain disorders and even sleep deprivation.[11,12]

The above are only a few examples of a wide range of biomedical research using the zebrafish model. As more information about their physiological responses to human diseases is explored and as more work is done to develop zebrafish mutant and transgenic lines, the role of zebrafish in research will only continue to expand. Some research institutions will breed their own zebrafish for their studies but also common is to obtain these fish from zebrafish facilities. One of the main suppliers of zebrafish to the research community is the NIH Zebrafish International Resource Center.

The Zebrafish International Resource Center (ZIRC)

The Zebrafish International Resource Center (ZIRC) is a zebrafish facility located in Eugene, Oregon on the University of Oregon campus and was established in 1999. The overall goal of ZIRC is to acquire, maintain and redistribute zebrafish resources to the research community worldwide.[13,14] ZIRC is the main distributor of wild-type, mutant and transgenic zebrafish adults, embryos and sperm to research facilities all around the world. They provide diagnostic pathology services, as well as information about zebrafish husbandry, health, potential pathogens, sperm cryopreservation and *in vitro* fertilization.[13] ZIRC utilizes sperm cryopreservation in order maintain such a vast array

of zebrafish lines. While zebrafish are still reared and housed in tanks at ZIRC, sperm cryopreservation allows numerous zebrafish lines to be stored without occupying a lot of space, time and resource, with an overall reduction in expenses. Through sperm cryopreservation ZIRC has preserved more than 10,500 zebrafish lines, which make up 36,000 genetic modifications and alleles.

Gamete cryopreservation

Cryopreservation is the preservation of live cells and tissues, using extremely low temperatures [-80^{o}C and -196^{o}C (liquid nitrogen)]. This technique is often used in research science, agriculture production, and human fertility. It is commonly used to preserve bacterial samples, animal tissues, and even human tissues, such as sperm. Research is being done to assess the potential use of this technique to conserve the sperm of endangered animal species.[15,16,17] The cryopreservation of gametes is common in animal agricultural industries, most notably being the cattle industry,[18] as two of the main methods used for animal reproduction are artificial insemination (AI) and *in vitro* fertilization (IVF). The cryopreservation of human gametes is also a common practice. Sperm cryo-bank facilities and fertility clinics preserve human sperm, testicular tissue, and embryos through cryopreservation, so that they may later be used for AI and IVF. Sperm cryopreservation continues to be explored in many other mammal species such as bison,[19] boar,[20] and horses.[21]

Aquatic gamete cryopreservation

With the rapid increase in aquaculture, there has been a concurrent increase in the use of cryopreserved sperm in these endeavors.[22] As mentioned above, ZIRC employs sperm cryopreservation in order to store each zebrafish line that they receive. Other fish models like the medaka (*Oryzias latipes*) and *Xiphophorus* are also preserved in this manner.[23] However it is with large food fishes, such as salmonids, cyprinids and sturgeons where most of the research and application of cryopreservation is carried out.[22] Recently, many aquatic species have begun to decline in population due to changes in the environment or in some cases overfishing.[24] These downward population trends have, in turn, increased efforts in aquatic cryopreservation research. A recent study done by Dietrich *et al*. (2016), examined the effect of cryopreservation on the semen of whitefish (*Coregonus lavaretus*) and northern pike (*Esox lucius*) and found that thawed sperm samples had similar capacities for successful fertilization as fresh sperm samples.[24] Cryopreservation of gametes of commercially-important shellfish is also used, and a study done by Riesco *et al*. (2017) demonstrated the first successful sperm cryopreservation of the Portuguese oyster, *Crassostrea angulata*.[25]

Concern of pathogen transmission

One concern with sperm cryopreservation is the possibility of pathogen transmission. Whether the sperm are naturally infected with a pathogen or contaminated with a pathogen during the sperm collection process, there remains the potential for

pathogens to survive cryopreservation and remain sufficiently viable to infect the progeny or females if artificial insemination is employed. In cattle *Tritrichomonas foetus* and *Campylobacter fetus veneralis* can be transmitted through semen and are capable of surviving cryopreservation.[26] Both of these pathogens don't cause significant disease in infected bulls, but in female cows they can cause vaginitis, infertility, and early embryonic death.

Regarding fish, certain deadly pathogens have been associated with maternal transmission in fishes. One of the most notable is *Renibacterium salmoninarum,* a gram-positive that can cause bacterial kidney disease (BKD) in both wild and farmed salmonids.[27] This bacteria can be vertically transmitted to salmonids eggs through ovarian fluid.[28,29]*Flavobacterium psychrophilum*, which can be fatal to infected fish, has been found in the ovarian fluids and in the sperm of various species of salmon and trout.[30] In a recent study by T.P Loch and M. Faisal (2016), *Flavobacterium columnare* was found in a sperm sample taken from a Chinook salmon *Oncorhynchus tshawytscha*.[31]

Several viruses can be maternally transmitted. For instance in humans there are many sexually transmitted diseases caused by viruses that can be spread through semen or vaginal fluid, one of the most common being human immunodeficiency virus (HIV). Another emerging sexually transmitted infection that is on the rise is caused by the protozoan *Trichomonas vaginalis. T. vaginalis* can be spread from men to women and from women to men during sexual intercourse and is often associated with infertility in men.[32] Pig semen is known to contain various bacterial and in some cases viral

pathogens.[33] Porcine parvovirus (PPV) has been found in the sperm of naturally infected boar and PPV causes reproductive problems in infected females.

Zebrafish pathogens:

The following are the most common bacterial and parasitic species afflicting zebrafish in research facilities.[14] Moreover, they all may infect gonads and some have been documented or are suspected to be maternally transmitted.

***Mycobacterium*:** Most *Mycobacterium* spp. are found in the soil and in water.[34] A few species, like *Mycobacterium tuberculosis*, are naturally found in vertebrates.[35] Mycobacteriosis is the second most common infection of zebrafish in research facilities[36] One unique feature of these bacteria is their thick waxy cellular wall. This wall consists of a thick peptidoglycan layer, not unlike most Gram-positive bacteria, however this layer is also surrounded by a layer composed of arabinogalactan ligated to long-chain mycolic acids.[37] The species that are of most concern to ZIRC, are *Mycobacterium chelonae*, *Mycobacterium marinum*, and *Mycobacterium haemophilum*.[14] *M. chelonae* is frequently found in zebrafish facilities and infected fish often show no physiological symptoms and experience low mortalities.[38] *M. marinum* causes more severe infections in zebrafish and can cause what is known as “fish handlers disease ” or “fish tank granuloma” in humans.[39] Fish infected with *M. marinum* may exhibit ulcerative lesions and granulomas in the spleen, kidneys and liver.[40,41] *M. haemophilum* also causes severe infections with

remarkably high numbers of bacteria and chronic inflammation in organs like the kidneys, spleen, liver and pancrease.[42] Zebrafish infected with *mycobacterium* experience high rates of mortality with both *M. marinum* and *M. haemophilum*. Presently there is no direct evidence that the bacterium is vertically transmitted, but the ovaries are frequently infected, [38,43] and it has been found in the testes and the mesonephric duct of zebrafish.[14]

***Edwardsiella ictaluri*:** This is a Gram-negative bacterium most known for causing devastating enteric septicemia in channel catfish*, Ictalurus punctatus.* Outbreaks occur in other warm water fishes, including zebrafish.[44] In zebrafish it causes rapid, high mortality, associated with hemorrhaging of the skin by the eyes, base of the fins, and of the abdomen and can infect multiple organs.[44] Vertical transmission is unknown.

***Pseudocapillaria tomentosa*:** These capillarid nematodes have a wide range of freshwater fish hosts,[45] including zebrafish. Infections with *P. tomentosa* nematodes prevent their hosts from absorbing any nutrients due to parasitic infection of the intestines,[36] causing the host to experience severe emaciation and eventual death.[38] The life cycle of this nematodes include eggs being released in the feces of infected fish.[46]

***Pseudoloma neurophilia*:** This microsporidian parasite is the cause of neural and musculature microsporidiosis in infected fish. It is the most common pathogen in zebrafish research facilities, and about 50% of research facilities are positive for this

infection based on ZIRC diagnostic service.[47] Infected fish can become emaciated and lethargic, and in some cases will develop a curved spine.[36,48] *P. neurophilia* are commonly found in the brain, hindbrain and spine of infected fish, but spores have been found in the testes and squeezed sperm samples of zebrafish.[48,47] This pathogen is common in ovaries and also develops within fish ova. Sanders *et al*. (2013), provided unequivocal results that the parasite undergoes true vertical transmission within embryos and persists in larvae and juveniles after hatching.[49]

Current biosecurity measures

There are various protocols in place at ZIRC to prevent the entrance and spread of such virulent pathogens. Murray *et al*. (2016), describes the biosecurity measures established at ZIRC and these same procedures have been implemented in other zebrafish facilities as well.[14,50] Some of the biosecurity protocols utilized by ZIRC include the following:[14]

- Live fish that are imported from outside facilities will reside in the quarantine room for at least two weeks before sperm are collected and cryopreserved.
- All embryos are surfaced-sanitized with sodium hypochlorite whether they remain in the quarantine room or are moved to the main fish room.
- All mutant and transgenic sperm lines are cryopreserved and the males from which the sperm were collected are euthanized and processed for histopathology. If these samples are pathogen free, the sperm can be used for *in vitro* fertilization

(IVF) and the progeny are kept the main fish room. If the males sampled test positive for a virulent pathogen or if the sperm samples are received from an outside facility that has an unknown or known pathogen status, samples will be submitted for histopathology. Depending on the type of pathogens detected, if any, more testing, such as polymerase chain reaction (PCR) to test for the presence of *mycobacterium* DNA, may be performed. These sperm samples may still be used for IVF, but the sperm samples will be marked accordingly in the ZIRC database, and any resulting embryos will be kept in the quarantine room or occasionally in the main room.

PCR testing for pathogens that may be vertically transmitted should be incorporated into a biosecurity plan. Many PCR tests are validated for sensitivity by adding bacteria to water or saline followed by serial dilution, as opposed to dilution in the target tissue.[51] I suspect that simple dilution of pathogens in water may result in inaccurate estimates of the true sensitivity of these tests when applied to tissues. This could mean that the PCR tests used on the tissue samples may not be as sensitive as presumed.

Aim of research

For the first part of my thesis research, the aim was to assess the ability of the most important zebrafish pathogens to survive freezing and thawing and to quantitate the

relative survival of these pathogens. I specifically wanted to determine whether these pathogens could survive the ZIRC sperm cryopreservation method as well as freezing methods in which pathogens are stored at subzero temperatures without a cryoprotectant. The same solutions and freezing protocols used by ZIRC to cryopreserve zebrafish sperm were used to evaluate two strains of *M. chelonae*, one strain of *M. marinum*, the zebrafish strain of *E. ictaluri*, the eggs of *P. tomentosa*, and the spores of *P. neurophilia*.

My second aim was to clinically validate commercially-available PCR tests for mycobacteria of importance to zebrafish. The Oregon State University Veterinary Diagnostics Laboratory, in Corvallis, Oregon, provides PCR testing through a panel for *M. marinum*, *M. chelonae*, and *M. haemophilum*, which were developed from Meritet *et al.* (2017) and use the same primers and probes.[52] Here I evaluated the sensitivity of these tests by diluting known numbers of these bacteria in fish tissues.

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Chapter 2

Survival of bacterial and parasitic pathogens from zebrafish (*Danio rerio*) following cryopreservation and thawing

Lauren J. Norris,[1] Virginia Watral,[1] and Michael L. Kent[1,2]

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[1]Department of Microbiology, Oregon State University, Corvallis Oregon
[2]Department of Biomedical Science, Oregon State University, Corvallis, Oregon

Abstract

Cryopreservation is a common method used to preserve the sperm of various animal species, and is widely used with zebrafish (*Danio rerio*). As with other animals, there is a possibility of paternal pathogen transmission through sperm. We evaluated the ability of five common and important pathogens of zebrafish to survive cryopreservation as used with zebrafish sperm and freezing without cryopreservant. This study evaluated *Mycobacterium chelonae*, *Mycobacterium marinum*, and *Edwardsiella ictaluri*, each originally isolated from zebrafish, eggs of *Pseuodcapillaria tomentosa* and spores of *Pseudoloma neurophilia*. Each mycobacterial isolate showed relatively minimal reduction in survival following freezing and thawing, particularly when subjected to cryopreservation. *Edwardsiella ictaluri* also showed survival following cryopreservation, but exhibited several log reduction following freezing at -80^{o}C without cryopreservant. With *Pseudoloma neurophilia*, two separate experiments conducted three years apart yielded very similar results, showing some, but reduced survival of spores using three different viability assays: SYTOX stain, Fungi-Fluor stain, and presence of a spore vacuole. Eggs of *P. tomentosa* showed no survival based on larvation of eggs when subjected to either freezing method. Given that four of the five pathogens exhibited survival following cryopreservation, we recommend that sperm samples or donor male zebrafish fish be tested for pathogens when sperm are to be stored using cryopreservation.

Introduction

Zebrafish (*Danio rerio*) are becoming one of the most commonly used animal models in research. Their transparency and internal physiological similarities to humans[1,2] make them ideal models for a variety of research areas including immunological and cancer development studies.[2,3] There are numerous wild-type, mutant and transgenic zebrafish lines in existence and the NIH Zebrafish International Resource Center (ZIRC), Eugene, Oregon is a principle supplier of such lines.

ZIRC provides the research community with a place to store and obtain various zebrafish strains.[4] In 2015 alone, ZIRC received 10,950 zebrafish lines and shipped a total of 79,561 zebrafish embryo and adult strains (Z. Varga, Zebrafish International Resource Center, Eugene Oregon). They supply the research community with live fish, embryos and frozen sperm samples, as well as provide diagnostic pathology services and knowledge about zebrafish health, husbandry practices, sperm cryopreservation and *in vitro* fertilization (IVF).[5] Cryopreservation of zebrafish sperm enables ZIRC to store the vast array of zebrafish strains they receive. By using this technique they have been able to preserve more than 10,500 zebrafish strains making up about 36,000 genetic modifications and alleles.[5]

Implementation of sperm cryopreservation and IVF has proven to be as beneficial in the zebrafish research community and at ZIRC[6] as it has been with food fish aquaculture. Numerous microorganisms detected in semen of domestic animals pose risks of maternal transmission.[7,8] These microbes include both obligate pathogens such as

viruses, parasites and certain bacteria, as well as opportunistic bacterial contaminants. Hence, the risk of transmission of certain pathogens with cryopreserved fish sperm should be considered.[7] There are several maternally transmitted pathogens in salmonid fishes, particularly via eggs and ovarian fluid.[8] Therefore, knowledge of the pathogen history of brood fish providing gametes, is a key element in avoidance of transmission in the aquaculture industry.[8,9] In zebrafish, the common pathogen, *Pseudoloma neurophilia*, is maternally transmitted.[10] This parasite has been detected using PCR associated on sperm squeezed from intact fish and dissected testes,[11] and *Mycobacterium chelonae* is often observed in ovaries and testes of zebrafish.[12,13] Moreover, knowledge of the disease and pathogen history for adult zebrafish providing sperm for cryopreservation is often lacking, and hence there is concern that the sperm from these fish may contain pathogens.

The objective of this study was to evaluate the survival potential of zebrafish pathogens that were subjected to the freezing and thawing solutions and procedures utilized by ZIRC to cryopreserve sperm samples. We evaluated the survival potential of the following pathogens in the cryopreservant[14] and without cryopreservant: *Mycobacterium chelonae, M. marinum, Edwardsiella ictaluri*, eggs of *Pseudocapillaria tomentosa*, and spores of *Pseudoloma neurophilia.* These are five of the six most common pathogens associated with disease in zebrafish research facilities.[4] *Mycobacterium haemophilum* is also recognized as a serious pathogen of zebrafish[15] but this was not included in our study due to its extremely slow in vitro growth and other difficulties with culture.

Materials and methods

BACTERIA

Pathogen preparation

Two strains of *Mycobacterium chelonae* (H1E1 ZF55 and H1E2 2F60)[12] and one strain of *M. marinum* (OSU 214),[16] all which were originally isolated from zebrafish, were used in this study. These bacteria were cultured on Middlebrook 7H10 plates supplemented with 0.5% glycerol and 10% OADC enrichment. Colonies selected from plates were used to inoculate Middlebrook 7H9 broths supplemented with 0.2% glycerol, 0.05% Tween and 10% ADC enrichment (Remel) and incubated at 28^{o}C with gentle shaking. The broths were incubated for 3 days (*M. chelonae*) and 7 days (*M. marinum*), after which they were used to inoculate new broths that were then allowed to incubate for about 2-5 days, depending on the species, to obtain exponentially growing cells to subject to the cryopreservation protocol. This was done so that the bacteria would be in a log phase of growth when they were frozen.

An isolate of *Edwardsiella ictaluri* originally obtained from an outbreak in zebrafish[17] was employed in this study. The bacterium was cultured on Blood agar plates (TSA with 5% sheep blood) (Remel, Lenexa, Kansas). Brain Heart Infusion (BHI) porcine broth (BD Bacto) was then inoculated with a colony from the cultured *E. ictaluri* plates, and incubated at 28^{o}C with gentle shaking. Again, the same procedure that was used for the *Mycobacterium* samples to ensure the bacteria were in a log phase of growth when frozen was also used with *E. ictaluri*.

Bacterial cryopreservation

ZIRC cryopreservation

We used the ZIRC protocol for cryopreserving zebrafish sperm with only a few modifications.[14] Before freezing the bacterial samples, two McFarland standards No.1 and No. 3 ($3x10^8$ and $9x10^8$ bacterial colony forming units (CFU)/mL respectively) and a spectrophotometer were used to estimate the density of the cultured broths. We aimed for an absorbance between 0.2-0.4 nm, which is estimated to be about $3x10^8 – 6x10^8$ CFU/mL. The bacterial broths were then diluted in 1X Phosphate Buffered Saline (PBS) using a 1:10 serial dilution. ZIRC cryopreserves 20 µL samples, taken from solutions consisting of 5 µL of sperm, 1 µL E400 and 15 µL Raffinose freezing medium (RMMB). E400 is a high potassium, buffered salt solution that has an osmolality of 400mmol/kg that is used after the sperm from the zebrafish have been collected, in order to keep urine from activating the sperm cells.[14] E400 consists of 130 mM KCl, 50 mM NaCl, 2 mM $CaCl_2$, 1mM $MgSO_4$, 10 mM D-(+)-Glucose, and 30 mM HEPES-KOH (7.9).[14] RMMB is the cryopreservant and consists of 20% (w/v) D-(+)-Raffinose pentahydrate (Sigma R7630), 2.5% (w/v) Difco Skim Milk (Difco #232100), 6.67% (v/v) Methanol (Acetone-free, Absolute, Certified ACS Reagent Grade, Fisher Scientific A412), and 30 mM Bicine-NaOH (ph 8.0).[14] The same ratio of solutions and sample volume were used in this experiment except that the bacteria took the place of the sperm. A total of 90 µL of RMMB solution was added to a sterile 2 mL cryogenic vial (Corning). To that same vial,

6 μL of E400 and 30 μL of the 10^4 bacterial concentration were then added and mixed by pipetting. Then 20 μL of this mixture was transferred into cryo-vials in triplicate. These cryo-vials were then capped and placed onto another empty cryo-vial without a cap and both were then placed into 15 mL conical tubes (Falcon). These 15 mL tubes were then capped and placed into a container filled with powdered-dry until the caps were flush with the surface of the ice. The samples remained in the powdered-dry ice for at least 20 minutes. This configuration yields a freeze rate of about -20^{o}C/min. After 20 minutes, each sample was removed from the conical tubes, placed on a cane, and quickly placed in a liquid nitrogen (LN_2) dewar. This same procedure was also done using the starting broth which had the high bacterial concentration, (10^8).

Freezing without cryopreservant

Bacterial samples were diluted in 900 μL of 1X PBS. 100 μL of the same concentrations used in the ZIRC method (10^4 and 10^8) were pipetted into 2 mL cryogenic vials (Corning), and then put directly in a -80^{o}C freezer. There was no cryoprotectant or freezing medium added to these samples. This was done in triplicate for each dilution.

Calculating starting bacteria concentrations

Using the bacterial concentrations generated from the 1:10 serial dilution, 100 μL concentrations of 10^4, 10^3, 10^2, 10^1 were plated to estimate the number of bacterial colony forming units (CFU) in each sample. Each concentration was plated in triplicate on the appropriate media for the corresponding bacteria, and plates were incubated at

28°C. The number of colonies on each plate were counted and averaged and then used to calculate the amount of CFU in each vial before freezing.

Thawing bacterial samples

ZIRC thawing method

For the ZIRC method, samples were removed from the liquid nitrogen dewar and placed in a 38°C water bath for 10-15 seconds and then immediately removed. Once thawed, 150 μL of Sperm Solution 300 (SS300) was added to each cryo-vial and mixed by pipetting. SS300 is a sperm solution with an osmolality of 300 mmol/kg[14] and is used to activate thawed sperm samples. It consists of 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM D-(+)-Glucose, and 20 mM Tris-Cl (8.0).[14] The 10^4 samples were then plated in triplicate and incubated at 28°C. The 10^8 samples were diluted out in PBS in a 1:10 dilution series. Each of the four lowest concentrations in the series (10^4, 10^3, 10^2, 10^1) were plated in triplicate and then incubated at 28°C. Once colonies on the plates began to appear, they were counted and used to determine the amount of bacteria in each sample that survived the freezing process.

Thawing method without cryopreservant

The bacterial samples that were stored in the -80°C freezer with just 1X PBS, were removed from the freezer and placed into a 38°C water bath for 10-15 seconds or until the solution was no longer frozen, and then quickly removed. Once thawed, the 10^4

samples were plated in triplicate and incubated at 28°C. The 10^8 samples were diluted out in PBS using a 1:10 dilution series, and as was done with the ZIRC samples, each of the four lowest concentrations in the series were plated in triplicate and incubated at 28°C. Once colonies on the plates began to appear, they were counted and used to determine the amount of bacteria survived the freezing process.

PARASITES

Pseudocapillaria tomentosa egg collection

A 16 L mouse cage, converted to a spawning tank with a stainless steel screen, was filled with system water at 28°C and was established with 20 *Pseudocapillaria tomentosa* infected zebrafish. To obtain freshly released unlarvated eggs, the fish were kept in the tank overnight and then the following morning, they were removed and the tank water containing any shed nematode eggs was allowed to settle for a few hours. Approximately 90 % of the tank water was removed using a vacuum pump (Barnant Company) and the remaining water and eggs were divided into 300 mL Nalgene bottles. These were then centrifuged for 45 minutes at 1,500 g. After that, about 90% of the supernatant was removed from the Nalgene bottles and the remaining water and eggs were divided into 50 mL conical tubes and centrifuged for 30 minutes at 900 g. After centrifugation, most of the supernatant was carefully removed, leaving about 1-5 mL of solution. Three 25 μL drops of the egg solution were examined at X10 with a compound

microscope, and the number of eggs observed were counted. This was done in order to calculate the total number of eggs in the 1-5mL solution.

Pseudoloma neurophilia spore collection

Pseudoloma neurophilia spores were collected from 20 known infected adult zebrafish that were euthanized by rapid cooling.[18] The brain, hindbrain and spinal cord from these 20 fish were collected and divided into two small petri dishes containing a 1X penicillin-streptomycin solution. Each dish contained 10 brains, 10 hindbrains, and 10 spinal cords. These solutions were then homogenized by continuously passing them through successively smaller gauges of needles (18, 23, 26 g). Once homogenized, the solutions were placed into 50 mL conical tubes and filled completely using ddH_2O. The tubes were then centrifuged for 20 minutes at 1,400 g. The pellets were collected, re-diluted to 50 mL with ddH_2O and placed on a shaker overnight at room temperature to enhance host cell lysis and liberation of spores from tissue. The next day the tubes were centrifuged for 20 min. at 100 g. The supernatant was removed and the pellets were then re-suspended in about 1 mL of ddH_2O. The number of spores in the final solutions, were estimated using a hemocytometer.

Parasite cryopreservation

ZIRC method

As with bacteria, the ZIRC protocol for cryopreserving zebrafish sperm was used but higher sample volumes were frozen down in order to ensure that an adequate amount of *Pseudocapillaria tomentosa eggs* and *Pseudoloma neurophilia* spores were in each sample. The same solution ratios were used. Each sample in the trial 1 freezing of *P. tomentosa* eggs contained a total volume of 200 µL consisting of 48 µL of eggs. In trial 2 each sample had a total volume of 252 µL, 60 µL being *P. tomentosa* eggs. The 2017 *P. neurophilia* samples also had a total volumes of 200 µL, of which 48 µL were the spores.

2014 ZIRC method

One experiment with *P. neurophilia* was conducted in 2014, and used a slightly different protocol based on ZIRC's protocol at that time.[19,20] The freezing mediums used at this time were two solutions of Ginsburg Fish Ringers, one with methanol and one without methanol.[19,20] 5 µL of *P. neurophilia* spores was put into 2 mL cryo vials (Corning), in triplicate. Added to each vial were 1.5 µL of Ginsburg Fish Ringers without methanol and 8.5 µL of Ginsburg Fish Ringers with methanol. The cryo-vials were then capped and placed directly into 15 mL falcon tubes, one cryo-vial per tube. The falcon tubes were capped and plunged into finely crushed dry ice for 20 minutes. The cryo-vials were then transferred to canes and put in liquid nitrogen.

In addition, some samples from the 2014 study also contained zebrafish sperm. Sperm from 2 male zebrafish was collected in a glass capillary, and normalized to 3.3 µL with Ginsburg Fish Ringers without methanol. Next was the addition Ginsburg Fish

Ringers with methanol, brining the mixture up to 20 µL. This sperm and freezing medium mixture was then expelled onto a watch glass and 10 µL of *P. neurophilia* spores were mixed into it. 15 µL of this final mixture was then pipetted into a 2 mL cryo-vial (Corning) and the same freezing procedure that was implemented on the samples without sperm, was followed. This was done in triplicate.

Freezing without cryopreservant

For the trial 1 *P. tomentosa* freezing, 50 µL of eggs were put into 2 mL cryo-vials, in triplicate, and to each vial 100 µL 1X PBS was added. For trial 2, 60 µL of eggs were placed into cryo-vials containing 110 µL of 1X PBS. This was done in triplicate. All vials were then stored in a -80^{o}C freezer.

In the 2014 experiment, 10 µL of spores in dH_2O were placed into 2 mL cryo-vials in triplicate, placed on canes and stored in liquid nitrogen. 10 µL of spores in dH_2O were also put into 1.5 mL Eppendorf tubes in triplicate, capped and placed in a -20^{o}C freezer. For the recent freezing of *P. neurophilia*, 125 µL of spores kept in sterilized fish water were put into 2 mL cryo-vials, in triplicate, and placed in a -20^{o}C freezer.

Thawing parasite samples

ZIRC method

For the ZIRC methods**,** samples were thawed in a 38^{o}C water bath for 15-20 sec. or until there were no longer ice crystals and then immediately removed. To each thawed

vial, SS300 was added. 150 µL is the general amount of SS300 that is added to the 20 µL frozen sperm samples. In our case, because the volume frozen down in our samples was higher than 20 µL, the amount of SS300 that was added to these samples was increased. With trial 1 *P. tomentosa* eggs we added 1.5 mL of SS300. Three of these samples were incubated at 28^{o}C for 7 days with the SS300 left in each vial (Table 2.1, ZIRC^{B}). For the other two samples (Table 2.1, ZIRC^{A}), after the addition of SS300, the vials were then centrifuged for 5 min. at 100 g. The supernatant was removed and 300 µL of sterilized water from our system was added to each vial. The samples were then incubated at 28^{o}C for 7 days. For trial 2 (Table 2.1), 1.9 mL of SS300 was added to the *P. tomentosa* samples. They were then centrifuged for 5 min. at 100 g. As with trial 1, the supernatant was removed and 300 µL of sterilized fish water was added to each vial. Half of the eggs were counted immediately after thawing (Table 2.1, Day 0) while the rest were incubated at 28^{o}C for 7 days (Table 2.1, Day 7).

The same thawing procedure was employed with *P. neurophilia* spores, with 1.5 mL of SS300 added to each thawed cryo-vial. They were then centrifuged, the supernatant was removed and 100 µL of sterilized fish water was added. They were kept at room temperature (23-25^{o}C).

2014 ZIRC method

The 2014 *P. neurophilia* experiment used the ZIRC thawing protocol at that time.[19,20] The cryo-vials were taken out of the liquid nitrogen, the caps were removed,

and each vial was placed half way into a 33°C water bath for 8-10 sec. To this, 10 µL of Hank's Balanced Salt Solution (HBSS) was added. The spores were then examined for viability.

Thawing without cryopreservant

P. neurophilia spores and *P. tomentosa* eggs were both thawed in a 38°C water bath for 10-15 sec. As in the ZIRC method, half of the *P. tomentosa* eggs were examined immediately after being thawed and the rest were incubated for 7 days at 28°C. For the 2014 experiment, spores were thawed in a 33°C water bath.

Viability of P. tomentosa eggs

Eggs of *Pseudocapillaria tomentosa* exhibit fully formed, vermiform larvae after about 5-6 d,[21] and larvation was used as an indicator of parasite survival. The number of larvated, unlarvated and obviously dead eggs in each vial were determined after the samples were thawed out (Day 0) and these numbers were compared to the same endpoints at (Day 7). The eggs were viewed on a microscope using X10 and X20 lenses.

Viability of P. neurophilia spores

The viability of *P. neurophilia* spores was assessed using two fluorescent stains: SYTOX Green nucleic acid stain (Molecular Probes) and Fungi-Fluor stain (Polysciences). We followed the same procedure described by Ferguson *et al*. (2007), in

which positive staining with SYTOX indicates dead spores, whereas extrusion of the polar filament following incubation in Fungi-Flour indicates that the spores are viable.[22] On a microscope slide, 5 µL of 100 µM SYTOX Green was added to 5 µL of spore solution. These spores were then viewed under oil (X1,000) using a Lecia DMR fluorescent microscope with a FITC green filter (480 to 490 nm excitation, 527/30 emission). Spores that fluoresced bright green were counted as dead (Fig. 2.3c) whereas spores that showed no signs of fluorescence were considered alive.

For our Fungi-Fluor stained spores, 5 µL of solution A of the Fungi-Fluor stain was added to 5 µL of spores on a microscope slide. They were then viewed under oil with at X1,000 with a DAPI filter (340 to 380 nm excitation, 425 nm emission). Spores were considered alive if they extruded their polar filament when exposed to UV light within 20 sec. (Fig. 2.3d). The presence or absence of a posterior vacuole was recorded for all spores from both the SYTOX and Fungi-Fluor assays (Fig. 2.3a).

Statistical analysis

To compare the differences between the initial bacterial concentrations and the post-thaw bacterial concentration among each strain of bacteria a one-sample z-test was used with the level of significance set at a p-value of < 0.05.

For the *P. tomentosa* Day 7 ZIRC method results, we wanted to determine the minimum number of larvated eggs that would be expected if the population size was set at 100,000 eggs. We used the following equation:

$$n = [1-(1-p)^{1/d}] \times [N-(d/2)] +1$$

where, n = the required samples size, N = total population size, p = probability of detecting one infected fish (in our case one larvated egg) ($p = 0.95$), d = maximum number of infected fish (in our case larvated eggs at Day 7) expected given a presumed prevalence (P) so that d = P x N. This equation is commonly used to calculate how many fish should be sampled (*n)* in order to detect at least one diseased fish presuming that 1% of the total population is infected with a disease.[23,24] In our case we already had *n,* which was the total number of eggs examined in all ZIRC Day 7 samples, so we used this equation to first solve for *d.* Solving for *d*, would give us an estimate of the maximum number of larvated eggs at Day 7 that would be predicted to occur out of a total population of 100,000 eggs, subjected to the ZIRC cryopreservation method, with a 95% confidence interval. Then we used the calculated value for *d* to solve for P, which is the percentage of the total population of eggs at Day 7 that we can expect to be larvated.

For the 2014 and 2017 *P. neurophilia* spore results, to assess the difference between the control and ZIRC method samples, we used a two-sample t-test with the significance level set at a p-value of < 0.05.

Results

All of the pathogens tested, with the exception of *Pseudocapillaria tomentosa*, showed survival following cryopreservation.

Edwardsiella ictaluri

Processing samples of this bacterium through the ZIRC cryopreservation method resulted in substantial survival (about 73%) compared to unfrozen controls (Fig. 2.1). In contrast, only 2-6% of the *E. ictaluri* subject to freezing at -80^{o}C without a cryopreservant, survived compared to the unfrozen control.

Mycobacterium spp.

The mycobacteria (two strains of *M. chelonae* and one strain of *M. marinum*) showed minimal reduction in survival following freezing with the ZIRC method (Fig. 2.1). Some samples showed evidence of greater growth following cryopreservation compared to the unfrozen controls. However, this increase was not statistically significant (*M. chelonae* H1E1: $p = 0.89$, H1E2: $p = 1.00$, *M. marinum* OSU 214: $p = 0.81$). For the -80^{o}C samples, each mycobacterial isolate experienced a decline in bacterial concentration, but exhibited higher bacterial concentrations compared to *E. ictaluri* (Fig. 2.1). *Mycobacteria* survival for the three strains frozen with the ZIRC method, ranged between 30-70% compared to the -80^{o}C controls (Fig. 2.1).

Pseudocapillaria tomentosa

Egg larvation was used as an indication of viability for the *P. tomentosa* eggs. Figure 2.2 shows the typical appearance of a larvated egg compared to an undeveloped

(unlarvated) egg and an egg scored as dead. In trial 1, no eggs showed larvation after the samples were thawed and allowed to incubate for 7 days at 28^0C (Table 2.1, trial 1), while 80% - 93% of the control eggs larvated. However, there was one instance of a larval worm partially hatched out of its shell at Day 0 in one of the ZIRC method samples (Fig. 2.2e) (Table 2.1, trial 1). As this fully developed larva was observed at time zero, it was likely present in the egg before freezing.

Many of the unlarvated eggs in either freezing method appeared intact as shown in Figure 2.2a. But, there were instances where the internal material of the eggs was concentrated to the middle of the shell and had more of a prominent granular appearance (Fig 2.2d). Eggs that were scored as dead were devoid of internal material (Fig. 2.2c), and these were the second most commonly seen eggs in our frozen samples, the first most common being unlarvated eggs. The percentage of these eggs scored as "dead" increased slightly from Day 0 to Day 7 in each sample (Table 2.1).

Results from trial 2 were similar to those from the first trial. No larvation was observed on Day 7 using either freezing method (Table 2.1, trial 2). In the ZIRC samples 100% of the eggs observed at Day 7 were unlarvated.

In the -80^{o}C samples, at Day 7 the number of unlarvated eggs was about 95 - 100%, with the remaining scored as dead (Table 2.1). In contrast, control eggs showed between 66 - 74% larvation at Day 7. There was one larvated egg that was seen at Day 0 in one of our control samples, but again, this was likely an egg that had already larvated prior to being collected. In other words, while eggs were collected from fresh feces

released within 24 hours, this egg was likely an older egg that had been ingested by a fish and released in a larvated state.

The probability of larvation in ZIRC method

Because the results of freezing *P. tomentosa* yielded 0% larvation, we used the probability of detecting a positive sample[23] in a given population to assess the power of this negative result. We only examined 215 eggs, but if we were to examine 100,000 *P. tomentosa* eggs that were subjected to the ZIRC cryopreservation method, we asked what would be the maximum number of larvated eggs that we would predict to observe on Day 7, based on our negative result with 215 eggs with a 95% confidence level? Using the formula described in the Methods, and solving for *d,* we obtained a value of about 1,400. We then solved for P and obtained a value of 1.4%:

$$n = [1-(1-p)^{1/d}] * [N-(d/2)] +1$$
$$215 = [1-(1-0.95)^{1/d}] * [100{,}000-(d/2)] +1$$
$$d = 1{,}400$$
$$d = \mathrm{P}\ x\ N$$
$$\mathrm{P} = 1400/100{,}000$$
$$\mathrm{P} = 1.4\%$$

Therefore, if we had a large population of eggs (e.g. 10^5), that were subjected to the ZIRC cryopreservation method, and having examined 215 of these eggs and observing no larvation, we can only conclude with 95% confidence, that no more than 1.4% of the total population of eggs would be larvated on Day 7.

Pseudoloma neurophilia

We conducted two separate trials, three years apart and using slightly different cryopreservants. These two trials yielded similar results, with some spores surviving the ZIRC protocols, regardless of the scoring method.

In the 2014 trial, control spores (held at 4^{o}C in dH_2O) showed more than 80% survival using the two vital stains, SYTOX and Fungi-Fluor, and about 60% had vacuoles (Fig. 2.4). Likewise, spores held with cryopreservant and sperm but not frozen also showed high survival. Other controls (boiling or holding spores at – 20 or – 196^{o}C), showed no survival using all three viability methods, except about 50% of the spores evaluated by SYTOX and 2% of the spores evaluated by Fungi-Fluor were scored as positive when held at -196^{o}C. With both ZIRC-S and ZIRC cryopreservation samples, spores stained with SYTOX displayed a much higher percentage of survival than those scored by Fungi-Fluor and presence of vacuoles (Fig. 2.4). However, there was considerable variability between replicate samples for each exposure method. Using a two-sample t-test to compare the 4^{o}C samples to the ZIRC-S and ZIRC groups, the Fungi-Fluor and vacuole results were significantly different (Fungi-Fluor: 4^{o}C vs. ZIRC-S, $p = 0.003$; 4^{o}C vs. ZIRC, $p = 0.002$. Vacuoles: 4^{o}C vs. ZIRC-S and ZIRC, $p = 0.03$).

The same endpoints were used to assess spore survival in the 2017 trial, but here we used the current ZIRC sperm cryopreservation freezing and thawing methods and solutions. We observed similar spore survival results as in the 2014 assay (Fig. 2.5). In

this trial, the controls were held at 20°C, and again viability of spores was > 50 % using all three methods for scoring spore survival. There was slightly less survival observed with the spores in the ZIRC group stained with SYTOX compared to unfrozen controls (66% vs. 80%). Fungi-Fluor showed only 19% survival. There was a significant decrease in the number of spores with vacuoles in the ZIRC samples compared to the positive control ($p = 0.005$), with only 8% of spores in the ZIRC samples showing vacuoles (Fig. 2.5). Overall, both vital stain procedures and the vacuole presence method revealed that the number of live spores in the samples that were frozen was less than the number of spores that were kept under unfrozen conditions (4°C or 20°C).

Discussion

Both species of mycobacteria showed relatively minimal reduction in survival when subjected to either freezing conditions. Research involving the cryopreservation and freezing of mycobacteria has been conducted since the 1960's,[25] and these studies have consistently demonstrated the ability of mycobacteria to withstand subzero temperatures with minimal loss in viability.[26,27] *Mycobacteria* are naturally found in soils, water and only a few species are restricted to their vertebrate host (e.g., *Mycobacterium tuberculosis*).[28,29] Dwelling in what are often very harsh and variable environments, these organisms have evolved to survive various environmental stressors. One characteristic unique to *Mycobacterium* is their cellular wall that is comprised of long-chain mycolic acids ligated to arabinogalactan that surrounds a thick peptidoglycan layer.[30] This thick,

waxy, impermeable barrier[30] likely played a significant role in the survival of each *Mycobacteria* species in both the ZIRC and -80°C samples (Fig. 2.1).

During freezing the extracellular medium freezes first, causing the external osmolality of the cells to increase and the internal water of the cells to cool but remain transiently unfrozen.[31,32] This imbalance in which external osmolality of the cells is higher than the internal osmolality of the cells, causes the cooled water inside the cells to travel across the cell membrane and cell wall where it subsequently freezes with the extracellular medium.[31,32] If the cells are unable to dehydrate quick enough, internal ice-crystals may form (often the case with rapid cooling), which can cause significant damage to the cells.[32] Cryoprotectants are used to reduce the development of ice crystals inside of the cells.[31] Cryoprotectants increase the solute concentration in the solution, which in turn decrease the freezing point of the cells.[33] Therefore, whereas mycobacteria survived well following freezing without a cryopreservant, survival was even better with the cryopreservant.

Sample freezing rate can also impact pathogen survival. The freezing rate generated by the ZIRC method lies between what some would consider fast (-100 to -400°C/ min.) and slow cooling rates (-2 to -4°C/min.).[31,34] The configuration of the ZIRC samples in the 15 mL conical tubes placed in powdered dry-ice, created a cooling rate of about -20°C/min. Another study found the cooling rate of vials placed directly in a -80°C freezer to be similar.[31] It's plausible that the mycobacteria were able to generate internal and external osmotic balance because their thick cell walls slow the internal cooling rate

of the cells, therefore giving them ample time to dehydrate and remain internally unfrozen. In turn, the bacterial cells that did not survive freezing, likely developed intracellular crystals that caused damage to the cells' structure either during freezing or when the cells were thawed. Although not statistically significant, we surprisingly observed increased bacterial counts following cryopreservation compared to unfrozen controls for some mycobacteria samples. Iivanainen *et al*. (1995),[28] also reported this phenomenon with environmental samples, and attributed an apparent increase in mycobacteria counts to disruption of bacterial aggregates or a decrease of other bacterial organisms in the following freeze/thaw process. It's possible that the freezing and thawing procedure used on our mycobacteria samples, caused the bacteria to spread out in our samples, and therefore, when we plated the bacteria after thawing, more bacteria were being plated.

Edwardsiella ictaluri also showed survival under both freezing conditions, but not to the extent seen with the mycobacteria. Studies involving the freezing of *E. ictaluri* as well as other members of the *Enterobacteriaceae*, have shown that these bacteria can survive freezing at -20^{o}C and -80^{o}C, but a decrease in bacterial concentrations is expected.[35,36,37] In many of these studies, there was no cryoprotectant utilized and instead the bacteria were frozen while in fish tissue. In our study the ZIRC method samples contained a specific cryoprotectant (Raffinose Freezing Medium) and was probably an important contributing factor for the survival of *E. ictaluri* compared to being frozen at -80^{o}C.

Pseudocapillaria tomentosa eggs showed no survival with either freezing protocol. Nematodes and their eggs are reportedly capable of surviving these types of freezing conditions. However, these studies have been conducted with terrestrial nematodes, which have evolved to survive freezing temperatures.[38,39,40] *Psueodcapillaria tomentosa* has a very broad host and geographic range in aquatic environments,[41] but has unlikely evolved mechanisms for its eggs to survive freezing in water as they would not regularly experience extreme temperature fluctuations. In our study, observation of larvation within eggs at 7 d (seven days after being shed or thawed) was a direct indication of survival, and most of the *P. tomentosa* eggs in the control samples were larvated by this time (66% – 93%). Based on these results, we conclude that *P. tomentosa* eggs cannot survive freezing, even in the presence of a cryoprotectant. Nematode eggs, with their thick shell walls, are generally quite resistant to external agents. Whereas the shells of the eggs provide a strong barrier to factors detrimental to the developing worms, this same barrier probably prevents the transport of the cryoprotectant into the egg, causing the eggs to be more vulnerable to the subzero temperatures.

In previous studies, nematode eggs were considered to be non-viable if damage to the eggs was observed or if the eggs were not intact.[40,42] In our study, unlarvated and dead (empty shell) eggs were intact and showed no signs of damage. It is possible that some of the eggs designated unlarvated but still with intact contents were still viable and delayed in their development as a result of the cryopreservation protocol. This is unlikely because we conducted later observations of these eggs (e.g., 10-14 d after thawing) and

larvation was never observed. Moreover, it is very unlikely that the empty eggs scored as dead following freezing are hatched eggs, as we saw no larvae free or within eggs at 7 d.

The survival of microsporidian spores at subzero temperatures has previously been examined and certain species have shown the ability to survive in liquid nitrogen, without a cryoprotectant, for up to 25 years.[43] Some species of microsporidia maintain infectivity following freezing.[44,45]

These obligate intracellular parasites infect a wide range of organisms including numerous fish species.[46,47]

To evaluate the survival of *P. neurophilia* spores after freezing, we followed the same procedure used by Ferguson *et al*. (2007), which involved the use of two fluorescent stains: Fungi-Fluor and SYTOX.[22] The presence of a vacuole was also recorded for all of the spores examined. A few *P. neurophilia* spores in both of our assays (2014 and 2017) were able to survive the ZIRC method of freezing as well as freezing at -196^{o}C and -20^{o}C without cryopreservant. This supports what was observed in the Maddox and Sotlter (1996) study, in which the spores of various microsporidian survived freezing in liquid nitrogen. Interestingly, *Nosema* spp. from terrestrial insects survived better than *Nosema algerae* from aquatic stages of mosquitos.[43]

One unique feature of these parasites is the presence of a large, conspicuous posterior vacuole inside their spores[48]. The presence of this vacuole is an indicator that the internal structure of the spore is intact, and hence is an indicator of spore viability. Microsporidian spores also contain a long, coiled, polar filament or tube. This tube can

form between 4-30 coils inside the spore and upon excitation, this polar tube is expelled and this is believed to be the primary mechanism the spores use to infect the cytoplasm of host cells.[49] Therefore, the ability of a spore to extrude its polar tube also indicates that a spore is viable and infectious. Last, exclusion of the SYTOX dye suggests that the spore wall is intact. There was some variation in our results between the three viability methods. We observed similar results as reported by Ferguson et al.[22], in which spores from the same treatment consistently showed higher survival with SYTOX compared to those with Fungi-Fluor. And our study showed similar results with the latter stain and the presence of a spore vacuole. With SYTOX, spores that are scored as dead should exhibit internal green fluorescence. As with other vital dyes that rely on cell permeability, in order for the SYTOX stain to reach the internal area of the spore there has to have been damage to the spore wall. In other words, some intact spores could still be dead, or otherwise non-viable, but would be scored as alive with SYTOX. Amigó *et al*. (1996) observed a very similar situation with spores of the fish microsporidian *Glugea stephani*, where spores subjected to freezing showed 58-97 % viability based on exclusion of a propidium iodide, while the same samples showed only 9- 48% viability based on polar tube extrusion.[45] Similar challenges regarding interpretation of results with vital dyes that rely on permeability have been reported with other eukaryotic microorganisms with thick protective outer walls, such as *Cryptosporidium* spp.[50,51]

Although there were slight differences with a few samples, overall the results for Fungi-Fluor and vacuole presence, scoring spores as alive were more similar to each

other than they were to the SYTOX method. Even with the SYTOX results, which may erroneously score spores as alive, we still observed spore survival with both ZIRC cryopreservation tests. Albeit considerably reduced compared to non-frozen controls, there was generally higher percentages of survival in the ZIRC cryopreserved samples compared to freezing at either -196^{o}C, and -20^{o}C. It should also be noted that we observed few differences amongst all three viability methods with unfrozen control samples, where most of the spores were scored as alive.

Overall our results demonstrated the ability of certain zebrafish pathogens to survive subzero temperatures and more specifically the sperm cryopreservation method utilized by ZIRC. Although we visually determined the survival of these pathogens, the next step would be to examine their infectivity after freezing with *in vivo* transmission studies. That being said, regarding sperm cryopreservation protocols it is recommended that sperm samples or donor fish be tested for pathogens either prior to or during the time at which they are used for cryopreservation.[52,53]

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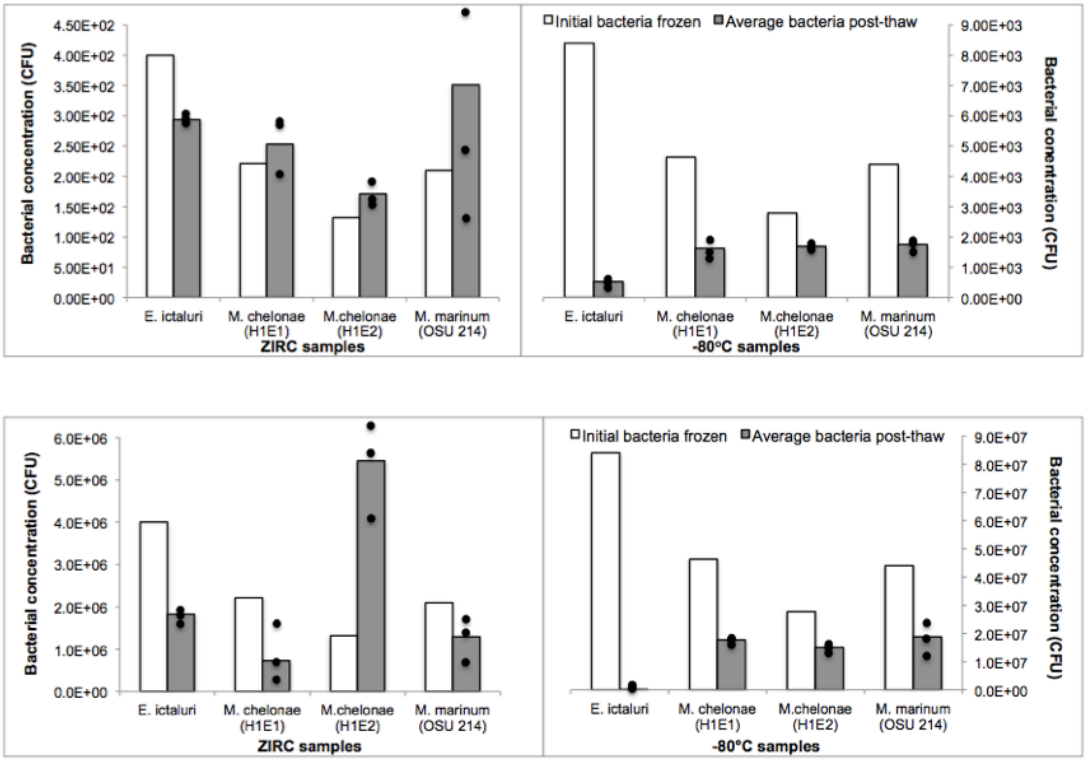

Figure 2.1 Bacterial survival following freezing by ZIRC cryopreservation and -80°C. Low bacterial concentrations (10^4) represented by top graphs and high concentration (10^8) represented in bottom graphs. White bars are the starting bacterial concentrations and the grey bars are the average bacterial concentrations calculated after thawing. Black dots represent individual plate counts and show the range of data. One sample z-test was performed, for the high concentrations the H1E1, H1E2, and OSU 214 ZIRC samples were not statistically significant (One-sample z-test, $p > 0.05$). For low concentrations, and H1E2 ZIRC sample was not statistically significant ($p = 1.00$). All of the other samples were found to be statistically significant (One-sample z-test, $p < 0.05$).

Table 2.1 Viability of *Pseudocapillaria* eggs following cryopreservation and freezing without cryopreservant. Two separate trials were conducted. In trial 1, ZIRC[A] are samples where the SS300 thawing solution was added to the vials after thawing and then removed before they were incubated. ZIRC[B] are samples where the SS300 thawing solution was added to the vials after thawing, and not removed.

	Viability by larvation					
Trial	Method	Replicate	n	Larvated	Unlarvated	Dead
1	ZIRC[A] Day 0	1	28	0%	92.9%	7.1%
		2	55	0%	98.2%	1.8%
		3	-	-	-	-
	ZIRC[A] Day 7	1	33	0%	81.8%	18.2%
		2	38	0%	92.1%	7.9%
		3	-	-	-	-
	ZIRC[B] Day 0	1	55	0%	100%	0%
		2	47	2.1%	93.6%	4.3%
		3	39	0%	100%	0%
	ZIRC[B] Day 7	1	28	0%	85.7%	14.3%
		2	60	0%	95%	5%
		3	29	0%	89.7%	10.3%
	-80°C Day 0	1	58	0%	96.6%	3.4%
		2	87	0%	97.7%	2.3%
		3	76	0%	97.4%	2.6%
	-80°C Day 7	1	18	0%	100%	0%
		2	41	0%	95.1%	4.9%
		3	30	0%	90%	10%
	28°C Day 0	1	208	0%	89.9%	10.1%
		2	215	0%	96.7%	3.3%
		3	378	0%	96.6%	3.4%
	28°C Day 7	1	56	82.1%	12.5%	5.4%
		2	101	93.1%	5%	1.9%
		3	91	79.1%	18.7%	2.2%
2	ZIRC Day 0	1	22	0%	100%	0%
		2	19	0%	94.7%	5.3%
		3	15	0%	100%	0%
	ZIRC Day 7	1	3	0%	100%	0%
		2	7	0%	100%	0%
		3	17	0%	100%	0%
	-80°C Day 0	1	61	0%	88.5%	11.5%
		2	86	0%	94.2%	5.8%
		3	46	0%	100%	0%
	-80°C Day 7	1	7	0%	100%	0%
		2	15	0%	100%	0%
		3	19	0%	94.7%	5.3%
	28°C Day 0	1	172	0.6%	94.2%	5.2%
		2	134	0%	92.5%	7.5%
		3	132	0%	93.2%	6.8%
	28°C Day 7	1	54	66.7%	16.7%	16.6%
		2	92	73.9%	15.2%	10.9%
		3	76	65.8%	27.6%	6.6%

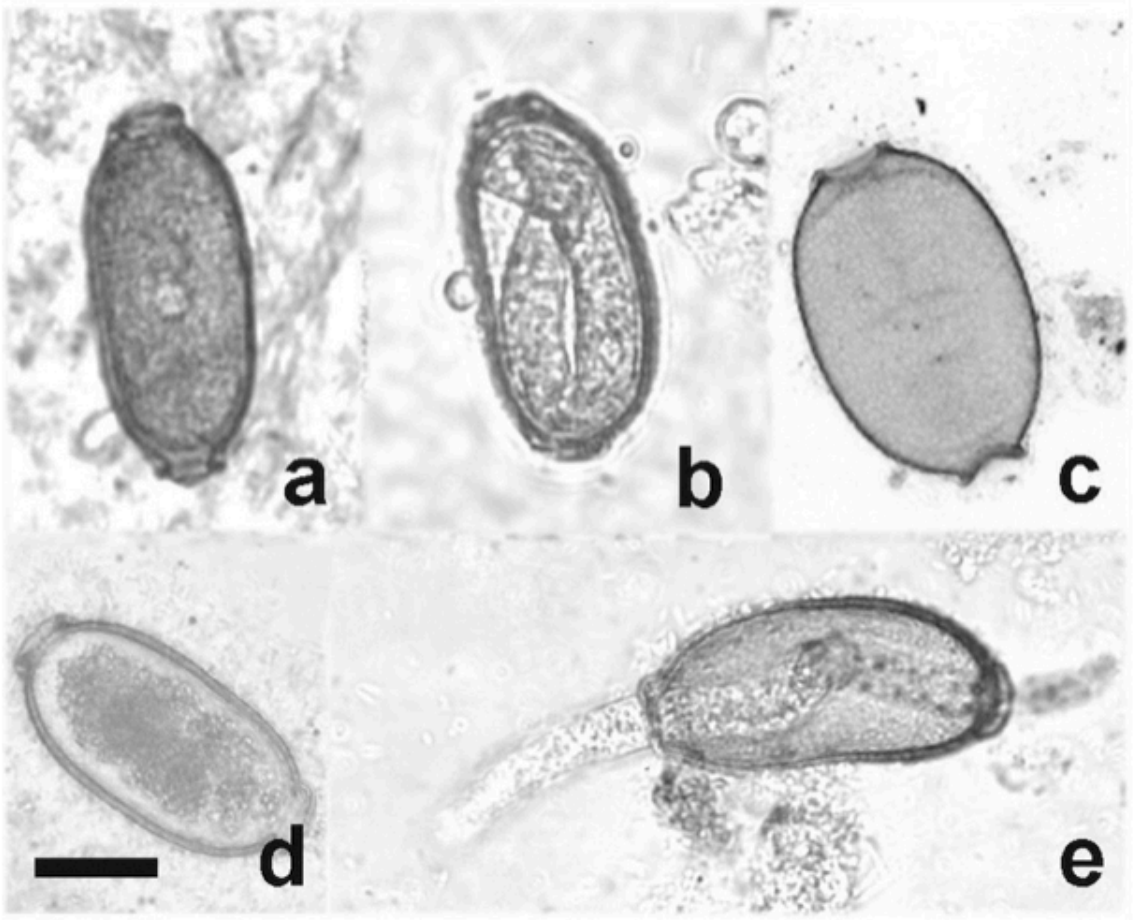

Figure 2.2 *Pseudocapillaria tomentosa* eggs. a) An unlarvated egg. b) A larvated egg. c) Egg devoid of contents, scored as dead. d) Egg 7 d after thawing from freezing using the ZIRC method. These eggs typically exhibited contracted internal material with more prominent granulation. e) Partially hatched *P. tomentosa* eggs observed in trial 1 on day zero. Bar 10 μm.

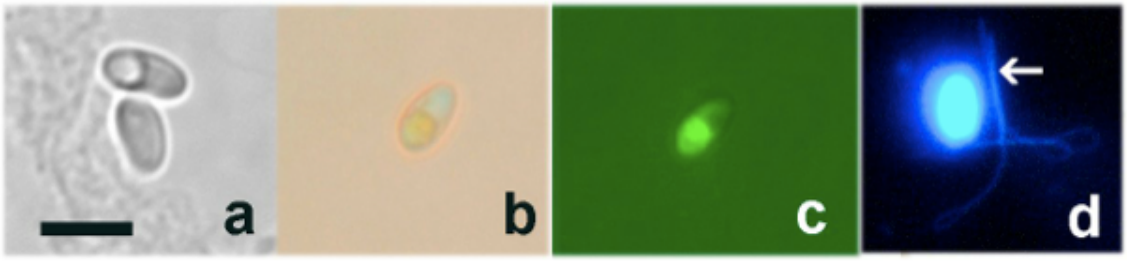

Figure 2.3 Spores of *Pseudoloma neurophilia*. a) The top spore has a vacuole, this is absent in the lower spore. b,c) A spore stained with SYTOX. b shows the spore under brightfield and c is the same spore but under fluorescence. This spore would be scored as dead. d) Spore that expelled its polar tube (white arrow) following staining with Fungi-Fluor and exposure to UV light. This spore would be marked as alive. Bar = 5 μm.

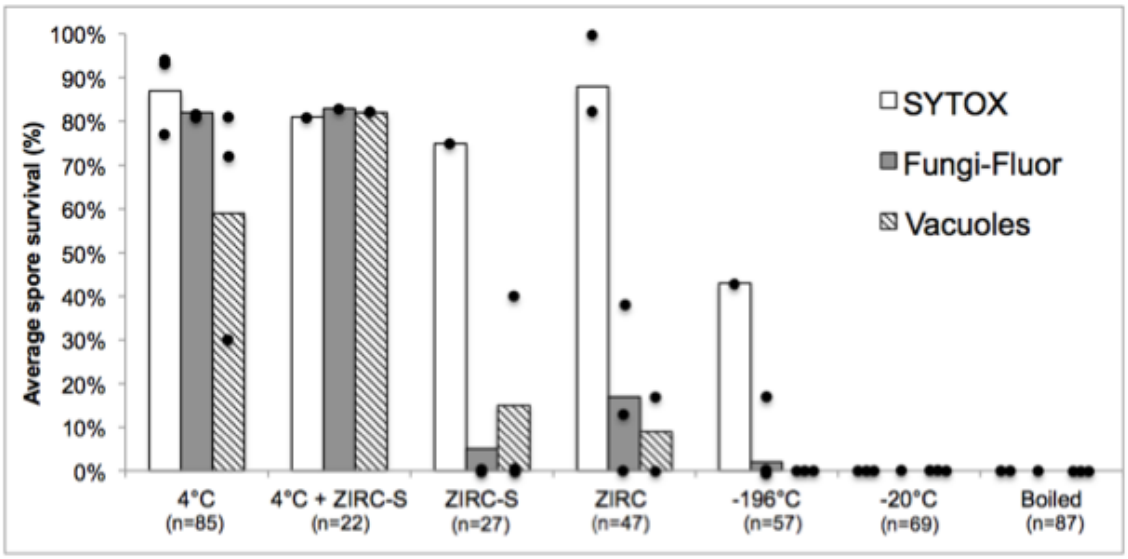

Figure 2.4 *Pseudoloma neurophilia* 2014 cryopreservation results. The ZIRC sperm cryopreservation method used at that time was used in this assay. Each bar is the average spore survival based on SYTOX (white bars), Fungi-Fluor (grey bars), or presence of spore vacuoles (striped bars). Black dots represent each of the replicate samples. ZIRC-S = cryopreservation with sperm, ZIRC = without sperm. All of the other samples contained only spores with dH_2O. The 4^oC + ZIRC-S samples contained spores, zebrafish sperm, and the freezing medium, (Ginsburg Fish Ringers), but kept at 4^oC.

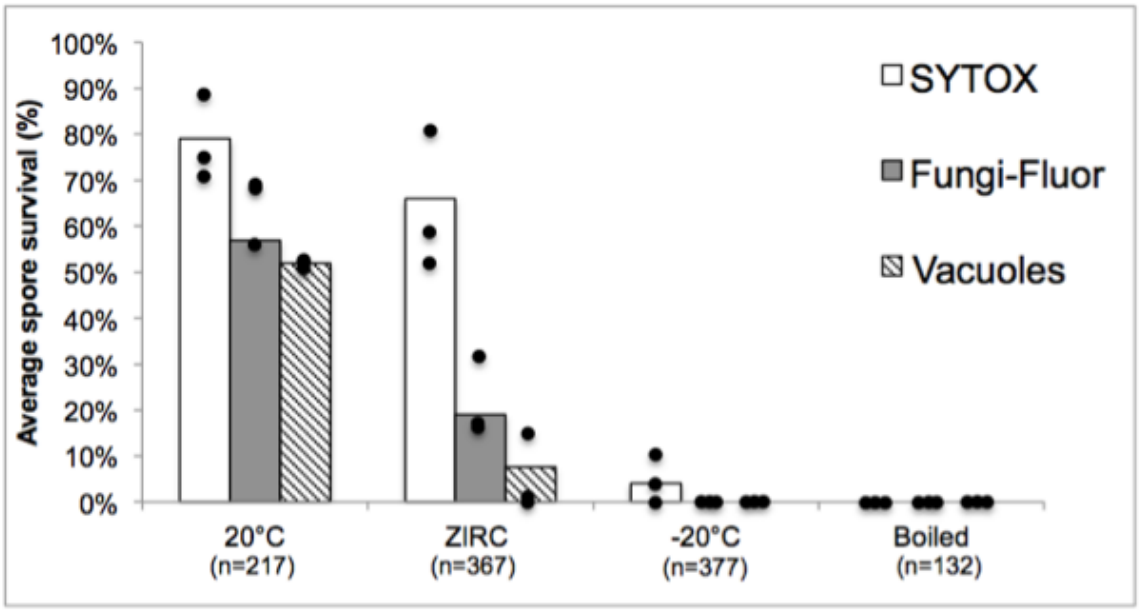

Figure 2.5 *Pseudoloma neurophilia* 2017 cryopreservation results. The current ZIRC sperm cryopreservation method (2017) was used in this assay. Each bar is the average spore survival based on SYTOX (white bars), Fungi-Fluor (grey bars), or presence of spore vacuoles (striped bars). Each black dot = one of three replicate samples. There was a significant decrease in the percentage of spores scored alive by the presence of vacuoles or by Fungi-Fluor in the ZIRC samples compared to the 20^{o}C samples. (Two-sample t-test, p = 0.005).

Chapter 3

Clinical validation of a real-time PCR test for *Mycobacterium chelonae and M. marinum* using zebrafish (*Danio rerio*) tissue

Lauren Norris,[1] Andree Hunkapiller,[2] Donna Mulrooney,[2]
and Michael L. Kent[1-3]

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[1]Department of Microbiology, Oregon State University, Corvallis, Oregon
[2]Oregon Veterinary Diagnostic Laboratory, Oregon State University, Corvallis, Oregon
[3]Department of Biomedical Sciences, Oregon State University, Corvallis, Oregon

Mycobacteriosis is one of the most common infections of zebrafish (*Danio rerio*) in research laboratories[1] and is caused by various species of *Mycobacterium*, including *Mycobacterium chelonae*, *Mycobacterium marinum*, and *Mycobacterium haemophilum.* Of these mycobacteria*,* zebrafish are most commonly infected with *M. chelonae* but often experience low levels of mortality[2] and do not display external signs of infection. Fish with *M. marinum* develop severe infections exhibited by the development of ulcerative lesions as well as granuloma formation in multiple organs and acute mortalities[3,4]. *M. haemophilum* is linked to chronic mortality and inflammation of organs including the kidneys, spleen and liver.[5]

Histology is the first and most common method used for diagnosing disease and identifying pathogens in zebrafish in research facilities.[2] Acid-fast staining of histological sections is the primary method used to diagnose mycobacteriosis, but only allows for identification to the genus level.[3] It is important to identify these mycobacteria at a species level given the significant differences in pathogenesis and morbidity caused by the different species that commonly infect zebrafish.

One method to identify mycobacteria on a species level is through culturing and performing biochemical tests on isolated bacteria.[2] For example, when cultured, *M. chelonae* displays plate colony formation after about a week and the colonies are smooth and white. In contrast, *M. marinum* colonies form after two weeks and will turn yellow to orange in color typically after about a week. Rapid and sensitive diagnostic methods have expanded mycobacterial identification at the species level following culturing by testing

samples with specific PCR assays or obtaining taxonomically relevant genomic sequences from bacteria isolated from samples.[6] However, certain mycobacteria that infect fish, such as *M. haemophilum* in zebrafish[5] or *M. triplex*-like bacteria from swordtails and mollies,[7] grow very slowly in culture and hence it may be more appropriate to attempt to identify mycobacteria infections to the species level using PCR directly on tissue samples. Culturing limits detection to viable bacterial cells, potentially leading to variation in bacterial detection,[8] whereas PCR detects both viable and non-viable bacterial cells.

Meritet *et al.* (2017) developed real-time quantitative PCR (qPCR) assays for each of the three *Mycobacterium* species previously mentioned: *M. chelonae*, *M. marinum*, and *M. haemophilum* based on the heat-shock protein 65 gene and they are presently used in commercial diagnostic tests offered by the Oregon Veterinary Diagnostic Laboratory (OVDL), Oregon State University, Corvallis, Oregon in collaboration with the Zebrafish International Resource Center (http://vetmed.oregonstate.edu/diagnostic). The analytical sensitivity of these assays was determined by using 1:10 dilution series made from stock solutions of each bacterial isolate, and ranged from about $3x10^4$ - $3x10^6$ bacteria/mL, depending on strain and species.[9]

Analytical sensitivity is usually based on diluting DNA or bacteria in media or water, and may not accurately represent the sensitivity when these assays are used on biological samples such as tissues, blood, or feces (clinical sensitivity). There are

numerous reports that have shown that PCR tests on such samples have reduced sensitivity due to lower DNA extraction efficiencies or chemical inhibition of the assays.[10,11] The purpose of this study was to determine the clinical sensitivity of the mycobacteria real-time qPCR assays offered to the zebrafish community by the OVDL. These were developed and modified from Meritet *et al*.[9], but used the same primers and probes (Table 3.1). Known concentrations of mycobacteria were used to spike chopped up zebrafish tissue, which were then tested using the real-time qPCR assays (Fig 3.1). Due to difficulties in culturing and enumerating *M. haemophilum*, only *M. chelonae* and *M. marinum* were evaluated in the present study.

One strain of *M. chelonae* (H1E2 2F60)[12] and one strain of *M. marinum* (OSU 214),[3] were cultured on Middlebrook 7H10 plates supplemented with 0.5% glycerol and 10% OADC enrichment. Colonies selected from each plate were then used to inoculate Middlebrook 7H9 broths supplemented with 0.2% glycerol, 0.05% Tween and 10% ADC enrichment (Remel) and incubated at 28^{o}C with light shaking. The broths were incubated for 3 days (*M. chelonae*) and 7 days (*M. marinum*). To obtain bacteria in a log phase of growth, the previous broth cultures were used to inoculate new broths. These were then allowed to incubate for about 2-5 days, depending on the bacterial species.

Zebrafish used for bacterial dilutions were obtained from the Sinhubber Aquatic Resource Laboratory at Oregon State University (SARL). This Specific Pathogen Free (SPF) facility has been shown to be pathogen-free for *M. marinum*, *M. haemophilum*, but occasionally older fish may be infected with *M. chelonae*.[13] For *M. chelonae*, fish were

obtained directly from the SARL, whereas fish used for the *M. marinum* study where maintained in our laboratory for about 9 months after transfer from SARL. For the *M. chelonae* (H1E2) sample preparations, four frozen zebrafish, negative for *M. chelonae* as determined by the qPCR before use, were minced together and divided into seven 2 mL tubes containing 2.3 mm beads and 1 mL of 1X Phosphate Buffered Saline (PBS). Each tube contained about 0.21 g of whole body fish tissue. A 1:10 serial dilution using 1X PBS was performed on the inoculated broth. Then 100 µL of 10^6-10^1 concentrations were used to inoculate six of the 2 mL tubes containing the zebrafish tissue. For a control, one 2 mL tube containing only zebrafish tissue was not inoculated with the bacteria. Another 2 mL tube with 2.3 mm beads and 1X PBS was inoculated with 100 µL of the undiluted broth (10^8) and contained no fish tissue. The lowest four concentrations (10^4-10^1) were then plated in triplicate and later used to calculate the number of colony forming units (CFUs) in each sample. The same procedure described above was used to prepare the *M. marinum* (OSU 214) samples. Here 5 frozen fish were used and 0.18 g of fish tissue was placed into the tubes.

Samples were then delivered to the OVDL for further analysis, and were processed further by their staff with no prior knowledge of bacterial concentrations in each sample. The OVDL procedures for processing samples for PCR analyses are outlined in Figure 3.1. The procedure is available from the OVDL as SOP# MOL.P.191 Revision 03. The total time to run the assays was 1 hr. and 43 min. We used the PCR primers and probes (Table 3.1) and amplification parameters described in Meritet *et al*.

(2017), except 45 cycles of denaturation at 95°C for 15 s was used for the present study. Calculations for determining the amount of bacteria in each 5µL real-time qPCR reaction using, 10^5 dilution for *M. chelonae* as an example, are as follows: 1) Starting concentration in the 10^5 dilution was 6.1 x 10^4 CFU/mL according to our plate counts; 2) ($6.1x10^4$)/1000 µL X 175 µL = 10,675 CFU in 175 µL. 3) The 175 µL bacteria solution was added to 300 µL of Special Lysis Solution = 10,675 CFU in 475 µL 4) 400 µL of solution from step 3 = 8,989.5 CFU in the 400 µL, this was placed in a new microcentrifuge tube 5) 350 µL of solution from step 4 (containing 7,866 CFU) is subjected to DNA extraction; 6) One PCR reaction uses 5 µL of step 5 = 437 CFU in each 5 µL PCR reaction. The OVDL ran each PCR reaction 3 times using the single final extraction preparation for each concentration.

Overall, both PCR tests were specific and showed a dose-dependent trend in sensitivity, but there were a few anomalous results (Table 3.2). For *M. chelonae,* the lowest concentration detected was the 10^5 sample, 61,000 CFUs/mL, with all three replicates showing a threshold cycle (Ct) in the designated "positive to weak positive" range. This equates to 437 CFUs per 5 µL PCR reaction as only 0.825% of the fish tissue was estimated to ultimately be included in the PCR reaction based on dilutions that occurred as part of the preparations for PCR testing (Fig 3.1). Based on this we determined that only about 1.7 mg of fish tissue was in each 5 µL PCR reaction (Calculations not shown). Zebrafish weigh about 500 mg, and hence only 0.34% of the fish is evaluated in each reaction. Therefore, these results suggest a threshold for

detection at about 128,000 bacteria/fish. Interestingly, the 10^1 concentration had Ct values within the range of "weak positive or suspect" (Table 3.2). We considered this negative as all three replicates were in this range. Regarding specificity, no positive results occurred with either the *M. haemophilum* or *M. marinum* PCR tests. The Ct of 41.15 with the *M. marinum* test for the 10^2 concentration was scored as negative as the other replicates showed no Ct values. Tissues in which no bacteria were added were all negative.

For *M. marinum* the lowest concentration that was detected was 10^4, which had 3,700 CFUs/mL and 27 CFUs per PCR reaction (Table 3.2). The actual amount of fish tissue calculated to be in each PCR reaction for *M. marinum* samples was about 1.5 mg, indicating a detection of about 9,000 bacteria/fish. As with *M. chelonae*, these three replicates at this threshold point showed Ct values in the "positive to weak positive" range. The 10^3 scores ranged from 37-41, and hence we conservatively scored this concentration as negative. Two concerning results occurred with the *M. marinum* study. First, the tissue without bacteria added was "positive to weak positive" (Ct = 36.62) for one reaction, but the other two replicates were negative. Therefore, one may score this as negative. Nevertheless, this is concerning, and it is possible the fish used in this study were contaminated in our laboratory either as live fish or during processing as we work extensively with this bacterium, and the SARL laboratory is negative for this infection. Likewise, the 10^6 sample `showed one of three samples to be positive (33.43) for *M.*

chelonae. Whereas the other replicates were negative, it is conceivable that there was a background *M. chelonae* infection in the donor fish.

Analytical sensitivity and specificity is important to define for a PCR test during development, but the clinical sensitivity and specificity is more informative to veterinarians and other clients. Our results indicated that the *M. chelonae* and *M. marinum* qPCR assays developed by the OVDL have a greater sensitivity when compared to the analytical sensitivity of the *Mycobacterium* qPCR assays developed by Meritet *et al.* (2017). Perhaps the increased sensitivity in the present study is due to subtle refinements in testing at the OVDL compered to the original study.

In conclusion, the moderate sensitivity of these tests indicates that there may be some limitations using these qPCR assays to screen clinically normal fish for the presence of these bacteria. However, it is clear that these tests are still very useful for identification of mycobacteria within fish with active infections. Moreover, these same tests have even been used effectively for species identification in zebrafish tissues from paraffin blocks with obvious granulomas and acid- fast bacteria.[4,9]

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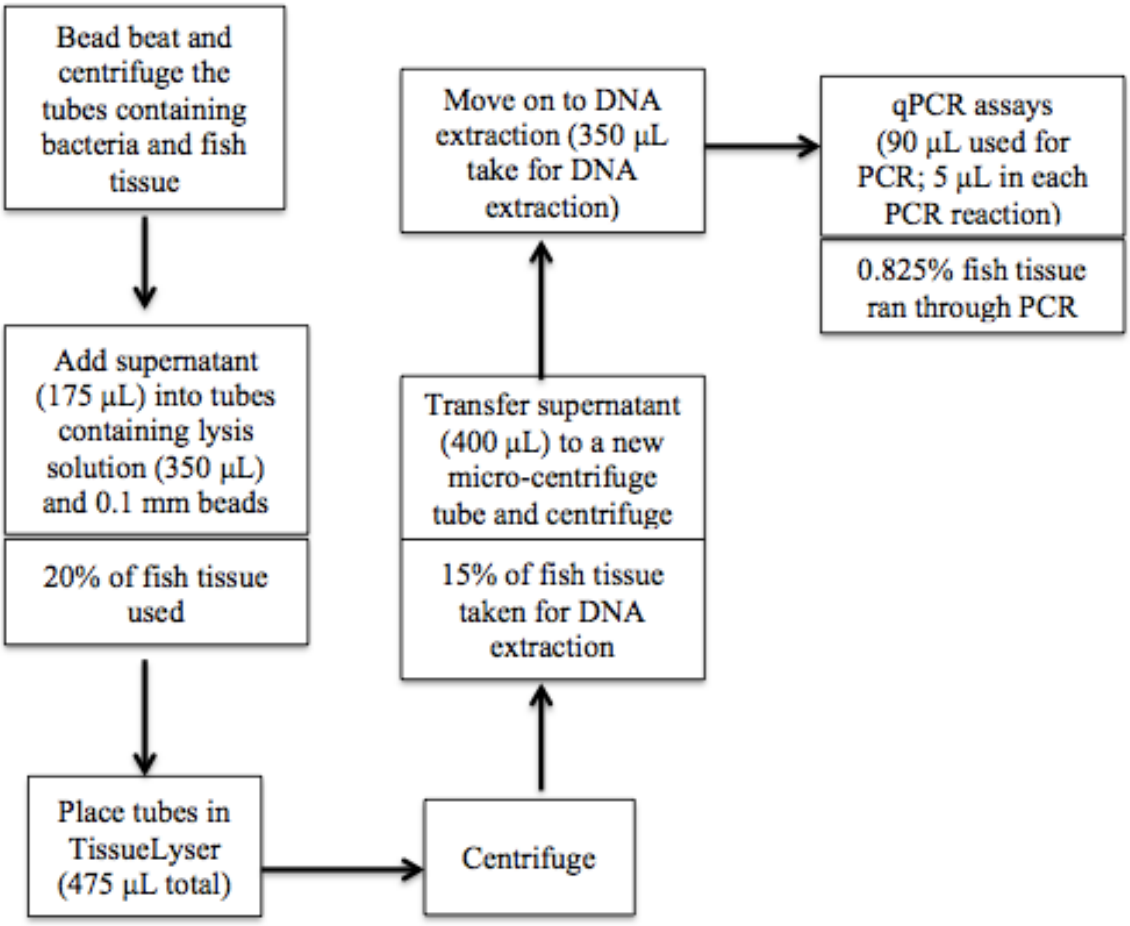

Figure 3.1 Flow chart displaying the simplified steps for preparing samples for DNA extraction, used by the Oregon State University Diagnostics Laboratory. The percentage of fish tissue used at each step is also noted.

Table 3.1 List of forward and reverse primers and probes for heat-shock protein 65 gene that were used for the qPCR assays. More details about the primers and probes can be found in Meritet *et al.*[9].

M. marinum Forward	5′ CAA CCC GCT CGG TCT GAA 3′
M. marinum Reverse	5′ CGA CCT CTT TGG CCG ACT T 3′
M. marinum Probe	5′ TCA CCG AGA CCT TGC 3′
M. chelonae Forward	5′ AAG GAA GTT GCC AAG AAG ACT GA 3′
M. chelonae Reverse	5′ CAG AGC CTG GGC AAG CA 3′
M. chelonae Probe	5′ ACG GCA CTA CTA CCG C 3′
M. haemophilum Forward	5′ GTT AAG GTG GCG TTG GAA GCT 3′
M. haemophilum Reverse	5′ TCC AGC CCG GAG TTG AAG 3′
M. haemophilum Probe	5′ CGC TGA AGC AGA TCG 3′

Table 3.2: *Mycobacterium* qPCR results for *M. chelonae* (H1E2) and *M. marinum* (OSU 214). Shaded areas indicate "positive" detection of bacteria. Interpretation of the Ct-values are as follows**: Ct = 0**, indicates target nucleic acid was not detected by the assay; **Ct ≤ 29**, indicates abundant amount of target nucleic acid present and is considered a "strong positive" result; **Ct = 30-38**, indicates a moderate amount of target nucleic acid present and is considered "positive" to "weak positive" result; **Ct > 38**, indicates a minimal amount of target nucleic acid present and is considered "weak positive" or "suspect." Each PCR reaction equals 5 µL.

***Mycobacterium chelonae* (H1E2)**												
	Ct-values									**Lowest concentration Detected**	**CFUs/mL in each sample**	**CFUs per PCR reaction (5 µL)**
Bacterial Concentrations	***M. chelonae***			***M. haemophilum***			***M. marinum***					
10^8	21.64	21.35	22.17	0	0	0	0	0	0	10^5	61,000	437
10^6	33.14	33.3	30.99	0	0	0	0	0	0			
10^5	32.24	33.53	33.16	0	0	0	0	0	0			
10^4	38.5	37.71	0	0	0	0	0	0	0			
10^3	0	0	0	0	0	0	0	0	0			
10^2	0	0	0	0	0	0	0	41.15	0			
10^1	39.37	38.52	40.52	0	0	0	0	0	0			
Tissue only	0	0	0	0	0	0	0	0	0			
***Mycobacterium marinum* (OSU 214)**												
	Ct-values									**Lowest concentration Detected**	**CFUs/mL in each sample**	**CFUs per PCR reaction (5 µL)**
Bacterial Concentrations	***M. chelonae***			***M. haemophilum***			***M. marinum***					
10^8	0	0	0	0	0	0	20.66	20.47	19.95	10^4	3,700	27
10^6	0	33.43	0	0	0	0	28.66	28.1	29.94			
10^5	0	0	0	0	0	0	31.66	32.23	31.64			
10^4	42.47	0	0	0	0	0	35.1	36.75	35.98			
10^3	0	0	0	0	0	0	39.91	41.13	37.56			
10^2	0	0	0	0	0	0	40.6	0	0			
10^1	0	0	0	0	0	0	0	0	0			
Tissue only	0	0	0	0	0	0	36.62	0	0			

Chapter 4: Conclusion

The overall aim of this study was to provide information to assist with the control of important pathogens in zebrafish facilities. The two specific aims of this thesis were 1) to examine the effects of cryopreservation and general subzero conditions on zebrafish pathogen survival and 2) evaluate the clinical sensitivity of *Mycobacterium* real-time qPCR assays. As the use of zebrafish animal models continues to increase, any information that can be learned about the survival and possible transmission of current and potential zebrafish pathogens is essential. Having a way to identify such pathogens, to the species level, in actual fish tissue samples is also a key resource that can aid in the continued survival of zebrafish colonies in research facilities.

Sperm cryopreservation has allowed zebrafish resource facilities to maintain a wide range of mutant and transgenic lines, but the possibility of vertical transmission of pathogens in sperm is a concern. *Mycobacterium chelonae*, *Mycobacterium marinum* and *Pseudoloma neurophilia* spores, pathogens known to cause two of the most common diseases in zebrafish, mycobacteriosis and microsporidiosis, were able to survive subzero temperatures both in the presence (the ZIRC method) and absence of a cryoprotectant. *Edwardsiella ictaluri*, a devastating yet rather uncommon pathogen of zebrafish, showed minimal reduction in viability following freezing and thawing in cryopreservant, while eggs of the nematode *Pseudocapillaria tomentosa* showed no survival. Previous studies have demonstrated the ability of microsporidia and mycobacteria to survive subzero

temperatures, not to mention cryopreservation is a common method used to store such pathogens. The impermeable, thick, waxy cellular wall of mycobacteria is likely what allows them to survive such low temperatures, as it may give the cells enough time to dehydrate and avoid internal ice crystal formation which can be detrimental to the cells.

The cryoprotectant used in the ZIRC method most likely contributed to the survival of *E. ictaluri* as there was a significant 1-2 log drop in bacterial survival when they were frozen without cryopreservant. In contrast, for *Pseudocapillaria tomentosa* eggs, it is likely that their thick shell prevented the cryoprotectant from reaching the internal regions of the eggs.

At ZIRC and other zebrafish resource facilities fish showing signs of infection or that are found dead in the tanks, are examined using various methods such as histology, staining, and PCR, in order to determine the cause of these conditions. This same process is done on fish from which sperm samples are extracted, so it is important to have a PCR assay that is able to detect pathogens in actual tissue samples as opposed to just water or saline samples The clinical sensitivity of the mycobacteria real-time qPCR assays, used and developed by the Oregon Veterinary Diagnostics Laboratory (OVDL) at Oregon State University, was calculated by using zebrafish tissue spiked with known *Mycobacterium* concentrations. The lowest concentration detected for *M. chelonae* was the 10^5 sample, which contained 61,000 CFUs/mL in each tube and about 437 CFUs per 5 μL PCR reaction. The lowest detected concentration detected for *M. marinum* was the 10^4 sample, which had 3,700 CFUs/mL in each tube and 27 CFUs per PCR reaction.

In conclusion, based on these data four out of the five zebrafish pathogens we tested can survive sperm cryopreservation and have the potential to be vertically transmitted. Much of the focus for avoiding vertical transmission in *in vitro* fertilization has been on sterilizing the donor eggs and using pathogen free females with the assumption that most pathogens cannot survive cryopreservation or at least that the concentrations will be significantly reduced. However, our results show that some pathogens are able to withstand such drastic conditions. We suggest that zebrafish facilities as well as other aquaculture and agriculture facilities that implement sperm cryopreservation should consider testing sperm samples prior to freezing them or using them for *in vitro* fertilization. Because the current protocol entails testing the fish that produced the sperm or are the products of the cryopreserved sperm, another suggestion might be to send such fish samples to diagnostics laboratories that have had the clinical sensitivity of their PCR assays evaluated and not just the analytical sensitivity. This would allow for more certainty that the PCR assays used can truly detect pathogens in actual tissue samples.

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