The impact of ozonation of Atlantic cod, Atlantic salmon and rainbow trout eggs on hatching success, larval growth and survival

by

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Abstract

The direct exposure of eggs to ozonated water has generated interest as a means of ensuring pathogen-free eggs without the use of harsh chemicals. However, there are numerous knowledge gaps including safe contact times, exposure levels in both fresh water and sea water, and as potential long-term effects on aquaculture species such as Atlantic cod, Atlantic salmon, and rainbow trout. The effect of different ozone doses (0.5-1.0, 1.5- 2.0 and 2.5- 3.0 mg O_3 L^{-1} for 1.5 min) was tested on recently fertilized Atlantic cod and eyed salmonid eggs vis-à-vis the commercial disinfectant Perosan™ (0.004 mg L^{-1}) and Ovadine (100 mg L^{-1}) as well as an untreated control (similar handling and husbandry). The impact of ozone application was evaluated based on larval nucleic acid concentration, hatching success, larval growth, and survival. Overall, the study showed no negative effect of ozonation at < 3.0 mg O_3 L^{-1} for 1.5 min on cod eggs, cod larvae (up to 30 dph), and Atlantic salmon and rainbow trout eggs monitored until 85% yolk sac depletion.

The ability of ozone to eradicate *Saprolegnia diclina* infection on salmonid eggs as well the impact of ozone disinfection on *S. diclina* in the absence of salmonid eggs were also investigated. Results suggest that ozonation of *S. diclina* hyphal mats can reduce the growth of the fungus, with the level of impact being dependent on volume of *S. diclina* initially present.

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List of Abbreviations

ANCOVA - Analysis of Covariance

ANOVA - Analysis of Variance

BFNNV - Barfin Flounder Nervous Necrosis Virus

C - Concentration

cDNA - Complimentary Deoxyribonucleic Acid

CT - Contact Time

dd - Degree Days

DFO - Fisheries and Oceans Canada

DNA - Deoxyribonucleic Acid

dph - Days Post Hatch

EB - Ethidium Bromide

EDTA - Ethylenediaminetetraacetic acid

EF1α - Elongation Factor One alpha

GLM - General Linear Model

JBARB - Joe Brown Aquatic Research Building

K - Condition Factor

min - Minutes

NL - Newfoundland and Labrador

MT - million tonnes

PCR - Polymerase Chain Reaction

PSI - Pounds per Square Inch

QPCR - Quantitative Polymerase Chain Reaction

rRNA - Ribosomal Ribonucleic Acid

SGR - Specific Growth Rate

st. dev. - Standard Deviation

SIW - Sterilized Incubator Water

SL - Standard Length

STEB - Sarcosil Tris-EDTA

STW - Sterilized Tap Water

RNA - Ribonucleic Acid

T - Time

TMS - Tricaine Methanesulfonate

UV - Ultraviolet

VER - Vacuolating Encephalopathy and Retinopathy

VNN - Viral Nervous Necrosis

YSV - Yolk Sac Volume

1. Introduction

1.1 Atlantic cod

A global decline in exploitable wild Atlantic cod (*Gadus morhua*) stocks resulted in increased demand for sustainably produced, cultured Atlantic cod in the marketplace (Jobling & Pedersen 1995). Cod was historically considered an alternative species in aquaculture but the increased demand and high market value in the wake of declining fishing quotas resulted in renewed interest in large-scale production. Cod is well positioned to meet the growing demand due in part to the similarities in infrastructure and technology between its culture and that of other commercial established species as well as a wealth of historical research into both wild and farmed stocks (Brown 2003, Fahay *et al.* 1999).

The subsequent recovery of wild cod stocks effectively decreased the interest in cod aquaculture research. However the understanding gained from decades of research in husbandry and early juvenile rearing made Atlantic cod an ideal candidate for a research species on which to investigate the safety and efficacy of ozone. Atlantic cod therefore was selected as a model species in this study to examine the potential effect of ozone on marine finfish eggs and larvae reared in seawater.

1.2 Atlantic Salmon and Rainbow Trout

Salmonid culture is a relatively new component of aquaculture, however, in only a few decades it has dominated the market. Two genera in particular, *Salmo* and *Oncorhynchus,* capitalize a large portion of the global market (Stickney 2000) and within

these genera two species, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), account for approximately 14% of global aquatic animal production (FAO 2013).

Interest in the farming of salmonid species began in the 19th century, focusing first on the propagation of trout species, primarily for the purpose of stocking ponds and rivers in France (Greenberg 1960), Denmark and Norway as well as for their introduction into new regions of the world (Monahan 1993). Although France would be the first country to open a trout hatchery in 1851 (Greenberg 1960), it is in Denmark that the industry experienced the greatest success leading into the 20th century (Laird 1996) and to a lesser extent in southern Norway where cold water temperatures limited success of trout propagation (Monahan 1993). In part due to this temperature limitation of trout, as well as in response to declining numbers of wild fish stocks, Atlantic salmon replaced a large portion of the industry during the 20th century, in the process absorbing and adapting much of the existing infrastructure and technology developed for trout in the process (Monahan 1993). Salmon were favored by the industry for their high market value, amenability to growth in artificial conditions and wider tolerance to environmental conditions than trout.

It would not be until the 1980s, however, that salmonid aquaculture rapidly expanded into the market. In response to declining wild stocks (Laird 1996), high market demand and a greater appreciation of the nutritional value of fish by North American consumers, substantial aquaculture technology began to develop, particularly in Norway (Monahan 1993), and a rapid expansion of salmonid products was seen globally. In just forty short years, salmonid aquaculture has expanded into a multi-billion dollar business,

grown in over 68 countries and producing over 2.5 million tonnes of product in 2011 (FAO 2013).

Atlantic salmon have a widespread distribution, and can be found on either side of the Atlantic Ocean in both North American and European waters. Atlantic salmon are spawned and hatched in fresh water and remain there during the larval and fry stages, eventually travelling to the ocean where they remain for the rest of their natural lifespan (average 4 years), returning only to their freshwater origins between October to January to propagate (FAO 2004b).

In both wild and hatchery environments, fertilized Atlantic salmon eggs will 'eye' at about 250 degree days (dd: used to estimate the various stages of development, it is the sum of the mean temperature per day during incubation) and hatch at 500 dd. The larvae have a large yolk sac, an endogenous food reserve that sustains them for approximately 300 dd before exogenous feeding starts.

Rainbow trout have a relatively narrow distribution being found naturally only on the Pacific side of North America from Alaska to Mexico. However, through artificial introduction, rainbow trout can now be found on all continents except Antarctica (FAO 2005). Rainbow trout are capable of living in either fresh or salt water, or in both during certain periods, as strains and environmental conditions dictate. Spawning of rainbow trout occurs between January to May (FAO 2005).

Rainbow trout eggs incubate over a period of 370 dd, followed by hatching over 2-3 days. In hatchery conditions, a yield of 95% hatch is often achieved for a disease-free population. Like their salmon counterparts, rainbow trout hatch with a yolk sac food

reserve, which sustains them post-hatch until the commencement of exogenous feeding (FAO 2005).

1.3 Aquatic Disease in the Hatchery

Disease is currently the largest single cause of economic loss in aquaculture, resulting in billions of dollars in lost revenue in cultured aquatic species (OIE 2011). The importance of producing clean, disease-free fish and maintaining this status throughout all stages of development cannot be understated. Aquatic diseases are not a phenomenon resulting from aquaculture; however, the intensive rearing of aquatic animals in high densities and close proximity allows for disease outbreaks to amplify rapidly as can be seen in a hatchery setting. Pathogens can be introduced into a hatchery population by a number of means, including anthropogenic activities, transmission from another infected individual (horizontal transmission) or by the passage of the pathogen from broodstock to the egg through infected reproductive fluids (vertical transmission). The latter method can be particularly challenging to halt if disease-free broodstock are not available and can create a disease reservoir in fish hatcheries. The importance of interrupting transmission at this stage has led to the implementation of egg disinfection in aquatic facilities as a necessary biosecurity step, and is one of the most important methods of controlling transmissible diseases in an aquaculture facility. Current disinfection methods focus heavily on the application of strong chemicals to eliminate surface-borne pathogens, with approved chemicals varying with country, fish species, and timing of application.

1.3.1 Nodavirus

Found globally, except in South America, the RNA-virus family *Nodaviridae* is the causative agent of viral nervous necrosis (VNN), alternately vacuolating encephalopathy and retinopathy (VER) and fish encephalopathy in both warm and cold-water cultured teleost fish (Johnson *et al*. 2002, Munday *et al*. 2002, Nakai *et al*. 2009). VNN consists of an icosahedral-shaped, non-enveloped particle measuring between 25-34nm comprised of two single-stranded, positive sense RNA molecules (RNA1 and RNA2) (Mori *et al*. 1992). There are four distinct species of nodavirus in fish belonging to the genus *Betanodavirus*. Along with other cold water species such as Atlantic halibut (*Hippoglossus hippoglossus*), Atlantic cod are affected by the virus species designated barfin flounder nervous necrosis virus (BFNNV) (Johnson *et al*. 2002, Nakai *et al*. 2009). The first case of VER in Atlantic cod was diagnosed in 1999 in Nova Scotia, Canada followed shortly by an outbreak in Scotland in 2001 and then Norway in 2006 (Starkey *et al*. 2001; Patel *et al*. 2007). Vertical transmission has not been established in Atlantic cod, but there is evidence of this type of disease transfer in Atlantic halibut (Comps *et al*. 1994) as well as in European sea bass (*Dicentrarchus labrax*) and Asian (*Lates calcarifer*) sea bass, striped jack (*Pseudocaranx dentex*), barfin flounder (*Verasper moseri)* and sevenband grouper (*Epinephelus septemfasciatus*) (Nakai *et al.* 2009).

Nodavirus infection is characterized in cultured juveniles and older fish by anorexia, darkening of the skin, alternate periods of lethargy at the bottom or the tank, erratic swimming (including whirling, looping and spiralling) and/or loss of equilibrium including floating belly-up (Johnson *et al*. 2002, Munday *et al*. 2002). VNN produces no

gross clinical signs on the body or gills of fish nor does it produce unusual swimming patterns in the larval stage of development (Nakai *et al*. 2009). Internally VNN results in degeneration of the brain and retinal tissues as a result of vacuolative encephalopathy and histiocytic retinitis (Johnson *et al*. 2002).

1.3.2 Saprolegnia

Oomycetes are a class of mycotic organisms collectively known as water moulds. Though the class is comprised of eight genera, only three (*Achlya*, *Aphanomyces* and *Saprolegnia*) are significant in aquaculture (Bruno *et al.* 2011), of which *Saprolegnia* is the most common and economically devastating. Endemic populations of *Saprolegnia* sp. can be found worldwide in fresh water. The genus' life cycle, as with most water moulds, is obligate on a host (nectrotrophic), relying on an initial source of dead or decaying animal matter to complete its reproductive phases (Bruno *et al*. 2011). *Saprolegnia* sp. are capable of both sexual and asexual reproduction (Noga 1993), an adaptation which aids in their reproductive success and in establishing and maintaining strong populations. This ability to propagate and persist in variable conditions is in part what gives *Saprolegnia* sp. the power of affliction on a host population.

Saprolegnia sp. presents a particular obstacle to aquaculture for species with a life stage in fresh water. Infections can occur throughout the life stages, from egg to adult fish (Bruno *et al.* 2011), become epizootic within a population and remain and persist within a facility in water supply lines, causing chronic events leading to substantial economic losses. In a hatchery setting, fungal growths on eggs can cause significant mortality as moribund and/or dead eggs provide an ideal substrate for *Saprolegnia* spores and the

resultant growth can rapidly spread to adjacent healthy eggs (Meyer 1991). Saprolegnia infections are characterized by a white, wool-like or cotton-like growth on eggs and freshwater stages of a fish. This growth, which is visible to the naked eye, represent in fact large mats of mycelium. If left untreated, mats can fully envelop living eggs, killing them by either extensive damage to the cells (Van West 2006), suffocation (Meyer 1991), or a combination of both. In fish, Saprolegnia infections are not tissue-specific, although they are more frequently observed initially in the epidermal tissues around the head from which they radiate to the tail in a series circular growths (Van West 2006). Separate circular growths may expand until they contact as a series of large clusters, eventually enveloping the entire animal and resulting in cellular necrosis and death from osmoregulatory failure (Pickering & Willoughby 1982, Hatai & Hoshiai 1994).

1.4 Ozone

Ozone (O_3) is a molecule consisting of three atoms of oxygen. It is allotropic to oxygen and is capable of existing in a solid, liquid and gas phase. Ozone is highly oxidative, and acts on organic material by non-selectively destroying double bonds (Evans 1972); it is capable of oxidizing amino acids, fatty acids, proteins containing sulfhydryl groups and pyrimidine nucleotides, all biochemical compounds present in living organisms (Carmichael *et al*. 1982). It is this characteristic which enables ozone to be applied as a potent disinfectant.

Interest in ozone's application in aquatic systems began in the 1930's, when it was first introduced as a means of improving the quality of supply water for stocked fish. Initial studies determined ozone ameliorated water quality by oxidizing organic matter in

aquatic systems but also emphasized on its considerable toxicity to fish and aquatic organisms when supplied continuously (chronically) in supply water (Hubb 1930). Ozone continued to generate interest in water treatment in the decades that followed, focusing on water quality optimization and disease control in both flow-through and recirculating systems. It has been used successfully to remove nitrite as well as non-biodegradable organic material (Summerfelt & Hochheimer 1997) and to sterilize water supplies and discharge. It has been implemented as a means of improving water quality by removing particles and protein, reduce microflora (Summerfelt & Hochheimer 1997) and eliminate fish pathogens (Liltved *et al.* 2006) including infectious pancreatic necrosis virus (Liltved *et al.* 2006) and striped jack nervous necrosis virus (Arimoto *et al.* 1996).

The direct application of ozonated water to fish species as a means of disinfection against pathogens generated only minimal interest in the aquaculture industry in ensuing decades. Attempts at applying ozone successfully focused on chronic-ozone freshwater applications at the egg, fry and fingerling stages (Benoit & Matlin 1966; Wedemeyer *et al*. 1979, Grischkowsky *et al*. 1983) and yielded variable results. Two initial findings were that the non-selective oxidation of organisms by ozone generated a crucial surface area-volume relationship. The authors propose that this accounts for ozone's potential ability to control pathogens such as bacteria and fungi without exhibiting toxicity on fish eggs (Benoit & Matlin 1966). However, the application of ozone to rainbow trout (10- 13 cm) produced such extensive destruction of gill lamellar epithelium that it resulted in the death of the fish (Wedemeyer *et al*. 1979). Further research yielded favourable results when ozonated water was applied at the egg stage of development. It has been suggested that the membrane of the egg could provide a protective barrier for the developing

embryo against the oxidative action of ozone (Asbury & Coler 1980), allowing for the effective application of ozone as surface disinfectant to fish eggs.

A rising need for alternative means of disinfection to eradicate pathogenic organisms resistant to current disinfection methods, as well as a need to replace previous disinfection methods no longer considered safe or environmentally sustainable have created an ever-growing platform of research into safe dosing in both freshwater and saltwater species. In saltwater species, ozone has been used successfully applied to Atlantic halibut (*Hippoglossus hippoglossus*), turbot, haddock (*Melanogrammus aeglefinus*) and Atlantic cod eggs on a non-industrial sized scale (Grotmol *et al*. 2003, Buchan *et al.* 2006). In freshwater salmonid culture, ozone has been the focus of considerable research into treatment of fresh water for rearing fish (Øye & Rimstad 2001), although very little work exists on its application as a disinfectant to freshwater eggs. The impact of the disinfection process on eggs is affected by both the concentration of ozone applied (C) and the contact time (T). The strength of the disinfection is then scored as a combined value of concentration * time (CT). In order to be applied effectively, a safe dose of ozone incorporating both C and T should be determined for the species of interest (Grotmol *et al.* 2003, Liltved *et al.* 2006).

1.5 Overall Objectives

The intent of this research was to examine the safety and efficacy of the application of ozone to fish eggs in a hatchery at a semi-commercial scale through the use of ozonated water in either a saltwater or freshwater environment as the natural biology of the fish species dictates. To achieve this, three species, one saltwater species (Atlantic

cod) and two anadromous freshwater species (Atlantic salmon and rainbow trout) were exposed to 0.5- 3.0 mg L^{-1} O_3 to determine if there was a measurable effect on egg quality and larval performance following ozone application. By conducting the research on eggs potentially exposed to two pathogens, a virus (Nodavirus- Atlantic cod) and a common fungus (*S. diclina*- Atlantic salmon and rainbow trout), the study also sought to determine the efficacy of the application against transmissible aquatic pathogens.

2. The Effect of Ozonated Seawater Application on Atlantic Cod Egg Development and Larval Success

2.1 Introduction

Increased interest in the late-80s- early-90s in cod aquaculture resulted in a substantial research effort dedicated to improving egg quality, nutrition and environmental conditions, including water temperature, light regimes and water quality for cod (Roselund & Halldórsson 2007). The productions of fertile eggs of high quality, and subsequently the production of larvae with high survival and growth rates, are among the most important aspects of aquaculture (Abi-ayad *et al.* 1997). Stocking hatcheries with disease-free eggs is key to the production of healthy juveniles. Broodstock may be from either wild stocks or commercial stocks and carry with them the potential for pathogen transfer to their progeny (vertical disease transmission). Hatcheries must rely on broodstock screening, good husbandry and biosecurity to prevent the introduction and spread of pathogens. Health screening of potential broodstock is an important step in disease control; however current detection methods for some pathogens necessitate lethal sampling, a requirement that is not feasible from a production standpoint. As a result, effective egg disinfection prior to introduction into the hatchery is a key control measure to halt any vertical transmission from the broodstock to the progeny.

Chemical treatment is the primary method of disinfection in commercial hatcheries. Current chemical disinfection methods evaluated on Atlantic cod eggs include iodophores, chloramines, hypochlorites, glutaraldehydes, hydrogen peroxide and common antibiotics (streptomycin, penicillin) (Grotmol *et al*. 2003; Peck *et al*. 2004).

However, recent studies have shown that viral pathogens are more resistant to current disinfection methods than bacterial pathogens (Grotmol *et al*. 2003), leading to vertical transmission, disease outbreaks and/or latent and sub-clinical symptoms resulting in horizontal transmission to others in the population (Arimoto *et al*. 1996). One such pathogen is the RNA virus nodavirus, the causative agent of Viral Nervous Necrosis. Nodavirus is a major constraint on marine fish culture (Munday et al. 2002) and results in high mortality levels in larval and juvenile fish (Johnson *et al*. 2002). It poses a significant risk to larval culture and has been shown to transmit vertically in larval striped jack (*Pseudocaranx dentex*) (Arimoto *et al*. 1992). It is also highly resistant to disinfection methods and is capable of surviving temperature extremes, pH ranging from 2 to 9 (Frerichs *et al*. 1996), UV and concentrated chemical disinfection, including levels well beyond the tolerance or toxicity range of fish species themselves. However, previous work using ozone to eradicate the virus has been met with some success where levels were reached that could eliminated the virus without affecting the hatching rate of some gadoids (Grotmol *et al*. 2003). Ozone has been effectively applied to the eggs of several marine species, including Atlantic halibut, turbot, haddock and Atlantic cod eggs on a non-industrial sized scale (Grotmol *et al*. 2003; Buchan *et al*. 2006). However, ozone toxicity appears to be an issue, resulting in delayed or asynchronous hatch (Grotmol & Totland 2000) and reducing the hatch rates of several marine fish species (Mimura *et al*. 1999, Grotmol & Totland 2000; Battaglene *et al*. 2006). It has been proposed that oxidants formed during seawater ozonation may negatively impact the functionality of the chorion of the eggs thereby reducing hatch rates (Grotmol & Totland 2000, Grotmol *et al*. 2003). Prior work on the effect of ozone on Atlantic cod eggs led to the conclusion

that an optimum level for ensuring the destruction of fish pathogens might be attained that would not affect hatchability if exposure was limited to ≤ 2 minutes at a concentration of 2 mg O_3 L^{-1} (CT of ≤ 4: Grotmol et al. 2003). Operationally, ozonated seawater at 1.5- 2 mg O_3 L^{-1} for 1.5 minutes (CT of 2.25-3) has been used on Atlantic cod eggs kept at the Ocean Sciences Centre (NL, Canada) since 2004 in response to the use of nodavirus positive broodstock in initial production cycles. However, this procedure has never been validated scientifically and knowledge gaps are numerous in terms of exposure levels, duration, and toxicity to the gametes.

The intent of this research was to evaluate the effectiveness of three concentrations (0.5-1.0, 1.5-2.0, and 2.5-3.0 mg O_3 L^{-1}) of ozonated sea water for 1.5 minutes on a semi-commercial scale to disinfect recently fertilized Atlantic cod eggs in order to potentially control the vertical transmission of nodavirus as well as establish a toxicity threshold and determine any long term effects on larvae. The success of the ozonation procedure was measured using parameters of larval quality and ozone efficacy: larval hatch rates (% hatch), larval survival (%) at 30 days post hatch (dph), RNA:DNA of larvae at 0, 10, 20 and 30 dph and nodavirus detection (by qPCR) on eggs and larvae. The ozonation procedure was also compared to the usage of Perosan™ (5.1 % peroxyacetic acid, 5-15% acetic acid, 21.7% hydrogen peroxide- ZEP Manufacturing, Atlanta, GA, U.S.A.) a commonly employed disinfectant in marine finfish hatcheries. The ratio of RNA to DNA (RNA:DNA) is an index of short-term changes in the growth rate of larvae, which reflects changes in protein synthesis rates, and hence potential larval growth, over periods as short as 1-4 days (Caldarone 2005). The physiological basis of this indicator is due to the constant amount of deoxyribonucleic acid (DNA) in somatic

cell versus the varying concentration of ribonucleic acid (RNA) due to protein synthesis (Buckley 1984, Rooker & Holt 1996, Caldarone *et al*. 2003). Hence, organisms in good condition will tend to have higher RNA:DNA than those in poor condition (Chícharo & Chícharo 2008).

2.2 Material and Methods

2.2.1 Atlantic Cod Eggs

Prior to the commencement of the study, a review of the handling of animals and research protocol were met with full approval from the Institutional Animal Care Committee of Memorial University of Newfoundland (Protocol # 10-09-DH).

Communally spawned Atlantic cod eggs were collected at the Joe Brown Aquatic Research Building (JBARB) of the Ocean Sciences Centre, Logy Bay, NL from January 27 to February 28, 2011. The broodstock were captive wild fish, initially transferred to the facility in 2005. This stock was photo-advanced by 6 months and had previously been found to be nodavirus positive (Provincial veterinary services, DFA-NL). Eggs to be used for these trials were collected once daily at 8 a.m. from an external egg collector, which accumulates eggs from within the tank into a Nitex® bag suspended in sea water. In order to obtain a batch of eggs of a similar age for treatment, the collector was emptied at 6 p.m. the night prior to collection and reset with a clean, empty Nitex® bag. Only fertilized eggs spawned within the previous 14 hours in the broodstock tanks were employed for each trial.

2.2.2 Ozone System

Ozone for egg disinfection was generated using a corona-discharge ozone generator (Pacific Ozone O-series model 01- Pacific Ozone Technologies, CA, USA) previously installed in the hatchery of the JBARB. Ozone was produced and directed from the ozone generator cells into a venturi injector, where ozone was injected into sea water by means of a vacuum created when pumped sea water is forced through a conical device, creating a differential pressure between the inlet and outlet ports (Lenntech 2009; Ozone Solutions n.d.). Ozonated sea water was in turn pumped into the egg contact chamber located inside a fume hood. The contact chamber consisted of a small 25L fibreglass tank with a conical bottom leading to a valve. When the valve was opened, ozonated sea water filled the chamber from the base up and was pumped in at a constant rate throughout treatment. As the water reached the top of the chamber, excess water escaped via an overflow line on the top of the tank. Within the chamber, a round basket consisting of Nitex® mesh was held in place by a polyvinyl chloride base, holding the egg basket in place and allowing sea water to be pumped through the base, coming in direct contact with the eggs before any ozone dissipated (Figure 1).

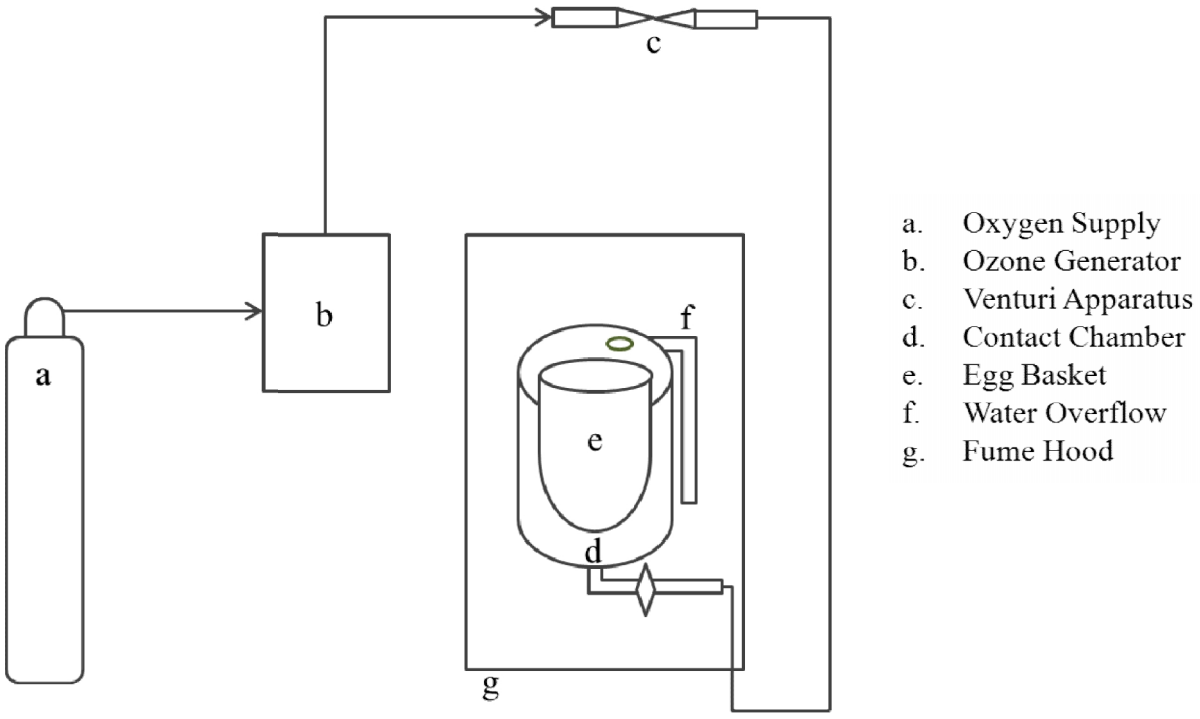

Figure 1: Egg ozonation system at the Ocean Sciences Centre.

2.2.3 Egg Quality

Viable Atlantic cod eggs are buoyant and only egg batches with an excess of 1800 mL floating eggs (a sufficient volume to proceed with all treatments within a single batch) were considered for this study. Egg quality was assessed in 100 eggs per batch by examining the fertilization success (# of eggs showing cell division out of 100), the cell stage (# of cells), and six parameters of blastomere normality as revised for Atlantic cod by Penney *et al*. (2006) from Shields *et al*. (1997) as follows: (1) uniformity of cell size and shape (all cells are of equal size visually), (2) adhesion (adjacent cells are contacting), (3) margins (cell margins are well resolved), (4) clarity of cell cytoplasm (cell cytoplasm is not obscured by darkening of the cell membrane or presence of vacuoles of unknown composition), (5) symmetry (bilateral symmetry of blastomeres about the axes are symmetrical) and (6) cell number (even number indicating normal cell division patterns). Any eggs showing variations outside of these criterions were counted and normality was expressed as:

$$[(\#\text{ of eggs} - \#\text{ of eggs with variation})\ (\#\text{ of eggs})^{-1}] * 100.$$

Finally, the diameters (mm) of 10 eggs per batch were measured and recorded.

Egg quality was reassessed in all of the treatment groups as described at 5 hours post-treatment. This period allowed for cell division to occur while ensuring that the number of cells would still be able to be counted visually under the microscope.

2.2.4 Treatment

Floating eggs from a single batch were divided into volumes of 300 mL. As ozone concentration is affected by organic load, it was important that treatment replicates were of the same volume across all batches. Eggs were briefly removed from water, measured

into five volumes of 300 mL, and each of these volumes was placed in a beaker containing 1L of 6°C sea water (filtered (5 μm) and UV-treated). The beakers were held at 4°C in a cooler to maintain the temperature of the eggs for up to one hour while awaiting disinfection treatments. Beakers were removed separately from the cooler immediately prior to disinfection.

Each beaker was randomly assigned to one of five treatments as follows: untreated Control, 0.004 mg L^{-1} Perosan™, or either 0.5-1.0, 1.5-2.0 or 2.5-3.0 mg O_3 L^{-1} disinfection treatment for 1.5 minutes. A total of 10 replicate batches (1500 mL of eggs per batch, 300 mL per treatment) were tested with 4 treatment groups and 1 control group.

Ozone concentrations were determined by spectrophotometry using an Ozone Vacu-vials® kit (CHEMetrics, Calverton, VA, U.S.A.) as per the manufacturer's instructions. When the sea water within the contact chamber was at the desired concentration of ozone, eggs to be treated with ozonated sea water were carefully poured directly into the ozone contact chamber basket. They remained in the system for 1.5 minutes. The contact period was terminated by the removal of the basket from the system. The eggs were then transported within the basket to the hatchery and rinsed into the incubators with clean hatchery water. Both the ozonated sea water and the hatchery water were maintained at 6°C throughout the experiments so that eggs were not temperature shocked.

The JBARB protocol for Perosan™ treatment consisted of a static bath of 40 mL of Perosan™ in 10L of 6°C sea water for 1 minute, followed by 4 minutes of rinsing with sea water by repeatedly filling the basin to remove any remaining solution. The control

group underwent similar handling to the other treatments including transfer to an egg basket, followed by seawater rinsing (5 minutes) and subsequent transfer to an incubator.

2.2.5 Egg Incubation and Sampling

Eggs were incubated in 50 L conical flow-through incubators with a flow of 25 L $hour^{-1}$ (one turnover per 2 hours) with gentle aeration. Temperature in the incubators was monitored daily (average: 5.9 ± 0.2 °C) and used to calculate individual batch degree days. Any dead eggs were removed daily from the incubators by stopping water and aeration to the tank and allowing viable eggs to float to the surface and dead eggs to sink during a period of 5- 10 minutes. Dead eggs were then collected through the bottom valve of the incubator and the total volume measured before disposal to assess the volume remaining in the incubator. Once larval hatch was observed in a tank, removal of dead eggs was terminated as larvae could inadvertently be removed during the process.

All sampling equipment was treated with RNaseZap® (Ambion, Austin TX, U.S.A.) prior to sample collection. Atlantic cod eggs were placed on a 0.150 mm Nitex® screen to remove excess water prior to collecting triplicate samples of 1.5 – 2.0 mL of eggs from each treatment in RNase/DNase free microcentrifuge tubes and frozen in dry ice prior to storage at -70°C for future analysis for nodavirus presence. Egg samples were collected immediately prior to disinfection treatment and at 1 hour post-treatment for nodavirus detection.

2.2.6 Larval Rearing and Sampling

After all eggs within a treatment hatched, the larvae were removed from the incubator and placed in a bucket containing sea water. Larval counts were completed as

per the JBARB standard method by measuring the total volume of water within each bucket, and gently agitating the water to create an even distribution of larvae throughout the water column. Three 50 mL sample were then removed and the number of larvae hatched was determined by counting the amount in each sample and extrapolating to the total number of larvae in the incubator using the mean of three counts. All larval counts were completed in the same manner.

Due to a limited availability of larval rearing tanks, the 0.5- 1.0 mg O_3 L^{-1} group was terminated at this stage. This treatment group was selected as it represented the lowest dose of ozone and therefore the least likely treatment to exhibit ozone toxicity. From the remaining 4 treatments (Control, Perosan™, 1.5-2.0 mg O_3 L^{-1} and 2.5-3.0 mg O_3 L^{-1}) 30,000 larvae were counted in the same manner as previously described for the larval hatch and placed into new buckets filled to ¾ with incubator water and transferred to 500 L tanks. In the event that fewer than 30,000 larvae hatched within a treatment, the total number of larvae present was recorded as mentioned above and all larvae were moved. Larvae were acclimated to the tanks by slowly adding small volumes of tank water to each bucket over the course of an hour. Once placed in the tanks, larvae were maintained under 24 hour light at 10 ± 1°C in flow-through sea water which was monitored daily to maintain a dissolved oxygen content of greater than 95% saturation. Larvae were fed a diet of rotifers (*Branchionus plicatilis*- cultured with Ori-culture and enriched with Ori-green (Skretting- Bayside, N.B., Canada)) three times daily at 1 a.m., 9 a.m. and 5 p.m.

Larvae were sampled at 0, 10, 20 and 30 dph, from each treatment group for nucleic acid analysis (n=10) and standard length/myotome height (body height at the

anus) measurements (n=10). Any equipment used for sampling was treated with RNaseZap (Ambion) prior to larval collection. Larvae were placed on a 0.150 mm Nitex® screen to remove excess water. Single larvae were then sampled, placed in RNase/DNase free microcentrifuge tubes and frozen in crushed dry ice. Tubes were stored at -70°C until further analysis of RNA and DNA content.

At 30 dph, all the larvae were siphoned from the tank and final numbers of larvae were determined in the same manner as at hatch. Following sampling and counting, all larvae were terminated with 100 mg L^{-1} tricaine methanesulfonate (TMS- Syndel Laboratories, Qualicum Beach, BC, Canada).

2.2.7 Larval Morphometrics

Larval condition factor (K) was calculated from standard length (mm) and myotome height (mm) as per Puvanendran *et al*. (1999) as described in Koslow *et al*. (1985) where:

$$(K) = \text{myotome height} * \text{standard length}^{-1}$$

Length-specific growth rate (SGR) was calculated according to Jobling (1994):

$$SGR = 100[\ln (SL_{t2}) - \ln (SL_{t1})] * (t2-t1)^{-1}$$

where $SL_{t(x)}$ represent standard length measurements in mm and t2-t1 represents the time period in days.

2.2.8 Nucleic Acid Analysis

RNA and DNA concentrations were determined using a 1-dye (ethidium bromide)/1-enzyme (RNase) assay using a fluorescent microplate reader as per Caldarone *et al*. (2001). In this process, a single larva is submerged in 1% N-laurosarcosine which

dissociates proteins from the nucleic acids. Centrifugation (14,000g, 15 minutes at room temperature) isolates the nucleic acid of the larvae into a supernatant portion, which is then removed and loaded into a 96-well microplate along with the fluorescent dye ethidium bromide (EB: 3,8-diamino-6-phenyl-5-ethylphenanthridinium- Sigma-Aldrich, St. Louis, MO, USA). EB increases the fluorescence of the nucleic acids 20-30x and the resulting fluorescence is measured by the microplate reader and can be quantified relative to a standard curve created using molecular grade 18S- and 28S-rRNA (bioWorld, Dublin, OH, USA) purified from calf liver. Following enzymatic digestion of the RNA using RNase (Sigma-Aldrich), the process is repeated to measure the DNA of the same sample, this time relative to a DNA standard curve created using genomic ultrapure calf thymus DNA (Sigma-Aldrich).

RNA and DNA standard solutions concentrations were determined using a NanoDrop 2000 (Thermo-Scientific, Mississauga, ON, CA) spectrophotometer. Standard solutions of RNA and DNA were aliquotted into RNase-DNase-free microcentrifuge tubes in volumes of 160µL and 400 µL, respectively, and stored at -70°C until used. As needed, tubes were removed from storage and held on ice until fully thawed. Once thawed, solutions were either used or disposed of in the same day. All nucleic acid stock and calibration curve solutions were held on ice at all times.

All reagents and standard solutions were prepared as per Caldarone *et al*. (2001).

Using RNA and DNA stock solutions, calibration curves were created daily as shown in Table 1.

Table 1: Nucleic acid stock solution calibration curve concentrations.

Nucleic Acid	Tube ID	µL of stock solution	µL of 0.1% STEB	Concentration (ug mL^{-1})
RNA	R-1	1.25	240	0.148
	R-2	2.5	240	0.296
	R-3	5.0	240	0.592
	R-4	10.1	240	1.183
	R-5	20.0	240	2.367
	R-6	40.0	240	4.733
	R-7	80.0	240	9.467
DNA	D-1	1.0	550	0.042
	D-2	2.0	550	0.084
	D-3	4.0	550	0.168
	D-4	8.0	550	0.336
	D-5	16.0	550	0.672
	D-6	32.0	550	1.344
	D-7	64.0	550	2.688

A BIO-TEK KC4 Synergy HT microplate reader capable of measuring fluorescence was used to analyze the samples. Black 96-well microplates (Costar 3915, Corning Incorporated Tewksbury, MA, USA) were used in the microplate reader and the optic position of the reader was set to top reading. Excitation and emission levels were set at 530 nm and 590 nm, respectively.

Aliquots from each dilution factor were loaded in triplicate into the plate beginning with a blank of 0.1% sarcosil Tris-EDTA buffer and proceeding from lowest to highest concentrations.

The plate was then transferred to the microplate reader generating a standard calibration curve for each of the nucleic acids. Only standard curves with a R^2 value of greater than 0.98 were used.

In order to reduce reading error, larvae from all treatments within a batch at a single age were analyzed on each 96-well plate. For every batch (n=10), 4 samples per treatment and time (0, 10, 20 and 30 dph) were processed. Samples were loaded in triplicate and the final nucleic acid concentration reflects a mean of three fluorescence readings.

As larvae increased in age (and size), their nucleic acid content increased and exceeded the range levels of the standard curve. As a result, the nucleic acid supernatant of the larvae was diluted prior to being loaded in the well plate. By modifying the dilution guidelines in Caldarone *et al*. (2001), an optimized dilution system was created as shown in Table 2.

Table 2: Dilution factors used for larval cod samples.

Fish Size (mm)	Approximate days post hatch	Dilution Factor	µL of supernatant	µL of 0.1% STEB
< 4.7	0	0	500	-
4.7-6.0	10	1.33	750	250
6.0-7.2	20	2	500	500
7.2-8.4	30	4	250	750

Following the DNA-attributed fluorescence reading, DNase was added to all wells on the plate, and a third and final reading was taken. The intent was to ensure the purity of the reagents and the standard curve solutions and was only completed once per set of reagents and/or standard curve nucleic acid stock solutions. If fluorescence levels were found to be negligible on the first plate they were assumed to be negligible on all subsequent plates and the step was therefore omitted (Caldarone *et al.* 2003).

2.2.9 Nodavirus Detection

Total RNA was extracted from 70 ± 2 mg of Atlantic cod eggs in triplicate using aRNeasy® Lipid Tissue Mini Kit (Qiagen Inc., Missisauga, ON, CA) as per the manufacturer's instructions for each of the 10 batches. The concentrations of RNA (μg μL^{-1}) was determined using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo-Scientific) and integrity of the RNA was assessed through electrophoresis using 1 μg of total RNA on a 1% agarose gel (1g of agarose dissolved in 90 mL of purified water and 10 mL of 10X Tris-borate electrophoresis buffer) containing 1.3 μL EB (10 mg mL^{-1}) resulting in discrete bands corresponding to the 18S and 28S rRNA under UV light.

First-strand cDNA was synthesized from 1 μg RNA and oligodt primers using the QuantiTect® Reverse Transcription kit (Qiagen Inc.) which first eliminates genomic DNA prior to reverse transcription of the RNA to cDNA. All samples were processed as per the manufacturer's instructions.

Nodavirus positive RNA samples were obtained from Fisheries and Oceans Canada, Moncton, N.B, for use as a positive control. The concentrations of total RNA from the samples were determined using a NanoDrop 2000 UV-Vis spectrophotometer

(Thermo-Scientific) and cDNA was synthesized using a QuantiTect® Reverse Transcription kit (Qiagen Inc.) as previously described.

Quantitative PCR primer sequences for nodavirus detection were provided by Dr. Nellie Gagné (Department of Fisheries and Oceans, Moncton) and were internally sourced detection sequences for in-house nodavirus detection (Table 3). However, before use in qPCR assays, sequences were compared for similarities with other nucleotide sequences using the Basic Local Alignment Search Tool (BLAST; nucleotide suite) as described in Alstchul *et al*. (1990). Significant hits were obtained against published piscine nodavirus sequences (E value = 0.0006) such as *Lates calcarifer* nervous necrosis virus, *Trachinotus ovatus* nervous necrosis virus, *Umbrina cirrosa* nodavirus, Barfin flounder nervous necrosis virus and others. Elongation factor 1 alpha (EF1α) was used as the normalizer gene and the sequences of the primers used are shown in Table 3. Amplification efficiencies were calculated for one random sample from the negative control and positive control groups using a five point 1:9 dilution series starting with 50 ng of cDNA, with the reported value (Table 1) being the average of the two.

qPCR amplification of 2µL of 25ng/µL cDNA was completed in a 25µL/well reaction consisting of Power SYBR Green I dye chemistry (Applied Biosystems, Foster City, CA, USA) and 20nM of both the forward and reverse primers. The qPCR was completed in 7500 Fast Real-time PCR system (Applied Biosystems) using the 7500 Software (version 2.0.5) in a 10 minute amplification cycle of 95°C followed by forty cycles of: 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Fluorescence was set at the 72°C stage.

Table 3: Gene name and primer sequence for qPCR used in nodavirus detection.

Gene Name	Primer ID	Sequence (5' → 3')	Gene Bank Acc. No.	Amplication Efficiency (%)
Elongation Factor 1-α	EF1α-f	GAT GCA CCA CGA GTC TCT GA	DQ40237.1	97.5
	EF1α-r	GGG TGG TTC AGG ATG ATG AC		
	NNV-F	AAT TCA GCC AAT GTG CCC CGC AAA		91.5
	NNV-R	GTT CTG CTT TCC CAC CAT TTG GC		

2.2.10 Statistical Analysis

The age of the eggs within a batch, based on cell number, were evaluated using 1-way ANOVAs. The effect of treatment on egg survival, larval hatch rates and larval survival were calculated by a randomized block general linear model with one fixed ('treatment') and one random ('batch') term. Effect of treatment on length-specific growth rate was calculated using the same model; however the effect was measured at each time point (10, 20 and 30 dph). Effect of treatment on larval condition factor, RNA, DNA and RNA:DNA was calculated using a series of one-way analyses of variance (ANOVA) with Tukey's honestly significant difference for pairwise comparisons of differences in treatments. If the data set failed the equal variance assumption ($P \leq 0.05$), a non- parametric Kruskal-Wallis ANOVA on ranks was performed. In all analyses, statistical significance was set at $P = 0.05$. Statistical analyses were completed in STATISTICA™ Version 10 (StatSoft Inc., Tulsa, OK, USA) and SigmaPlot 12.0 (Systat Software Inc., Chicago, IL, USA).

2.2.11 Results

Egg quality results are based on the 2-16 cell eggs from 10 separate batches with a fertilization rate of 49% or higher (Table 4). The ages of the eggs within each of the batches were found to be statistically similar ($P= 0.418$- 0.996). There was no difference in egg survival ($P= 0.421$) during the incubation stage (Figure 2). All egg batches hatched between 92.4- 108.8 dd; hatch was synchronous in all batches except batch 1, where the Controls hatched 11.9 dd prior to the other four treatments (Table 5). The results showed no statistical difference in hatch rates ($P= 0.280$) of Atlantic cod eggs between treatments

despite a downward trend with increasing ozone concentration and a lower hatch rate in the Perosan^{TM} treatment (Figure 3).

There was also no statistically significant difference in larval length-specific growth rate between treatments at 10, 20 or 30 dph (Figure 4). The ANOVA results of K showed an increasing variation between treatments with time within select batches (Table 6). Statistical differences from treatment were more frequent at 30 dph than at 0, 10 or 20 dph but with no specific trend in the manner the treatments differed.

Significant differences in RNA concentration of Atlantic cod larvae as a result of treatment were only seen in batch 10 at 0 dph (P= 0.003) and in batch 4 at 20 dph (P= 0.007); however in batch 4 this difference was no longer observed at 30dph (P= 0.072) (Table 7). Overall RNA concentrations ranged from 3.182 ± 1.02 to 23.12 ± 10.23 μg larva^{-1} between 0 and 30 dph. Differences in DNA concentration of larva as a result of treatment were twice as frequent, with detectable differences seen in batches 1 and 4 at 0 dph (P= 0.002, P= 0.014 respectively), in batches 3 and 4 at 20 dph (P= 0.043, P= 0.004) and batch 2 at 30 dph (P= 0.016). As with RNA, the differences observed in batch 4 were no longer detectable at 30 dph (P= 0.109). DNA concentrations ranged from 1.07 ± 0.362 to 8.50 ± 3.70 μg larva^{-1}. Finally, the RNA:DNA showed statistically significant effects of treatment at 0, 10, 20 and 30 dph and within several batches (1, 2, 3 and 10) with each age showing a unique effect of treatment (Table 7). In most instances where differences were found Control values (RNA, DNA, RNA:DNA) were higher than the corresponding batch treatment values. RNA:DNA values were between 1.3 to 3.73 throughout the experiment.

No statistically significant difference ($P = 0.480$) was detected in larval survival at 30 dph among the treatment groups (Figure 5).

Results of the qPCR did not detect the presence of nodavirus in any pre-treatment samples from any batch. Amplification of the nodavirus positive controls using the in-house nodavirus sequences from DFO-Moncton were successful at all dilutions tested (mean C_T: 12.725 ± 0.140 at 1.5 x 10^2 ng to C_T: 26.855 ± 0.303 at 1.5 x 10^{-3}).

Table 4: Fertilization rate (%) and cell stage (%) of eggs.

Batch	Fertilization (%)	Cell Stage (%)	
		2-16	16-64
1	57	100	0
2	82	100	0
3	49	63	37
4	94	100	0
5	77	74	26
6	100	88	12
7	98	99	1
8	99	94	6
9	99	94	6
10	99	100	0

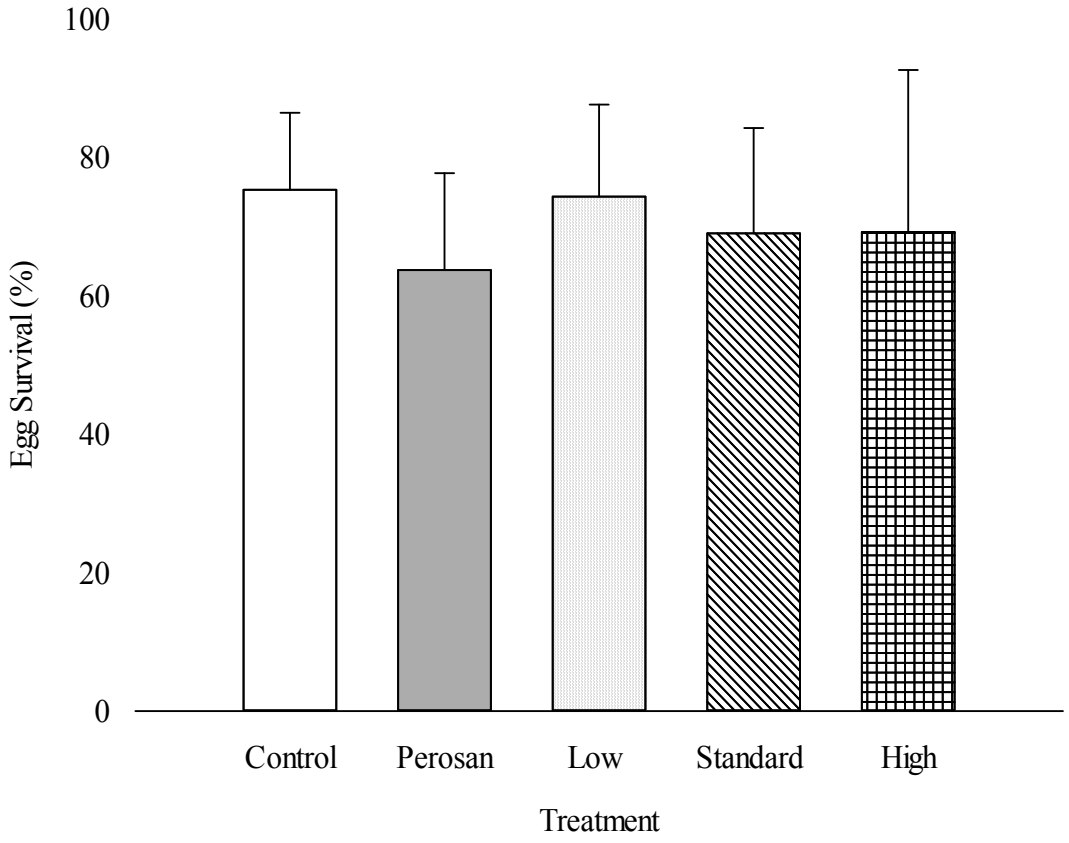

Figure 2: Mean egg survival ± st. dev. at -1 dph of Control, Perosan, 0.5- 1.0 mg O_3 L^{-1} (Low), 1.5- 2.0 mg O_3 L^{-1} (Standard), and 2.5- 3.0 mg O_3 L^{-1} (High) Atlantic cod egg batches 1-10.

Table 5: Age (dd) at 100% hatch for each of the Atlantic cod egg batches tested (C: control, P: Perosan, L: Low 0.5- 1.0 mg O_3 L^{-1}, S: Standard 1.5-2.0 mg O_3 L^{-1}, H: High 2.5- 3.0 mg O_3 L^{-1}).

Batch	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Hatch (dd)	C: 92.4 P,L,S,H: 104.3	108.8	100.1	97.2	101.2	96.0	94.6	100.0	101.5	95.8

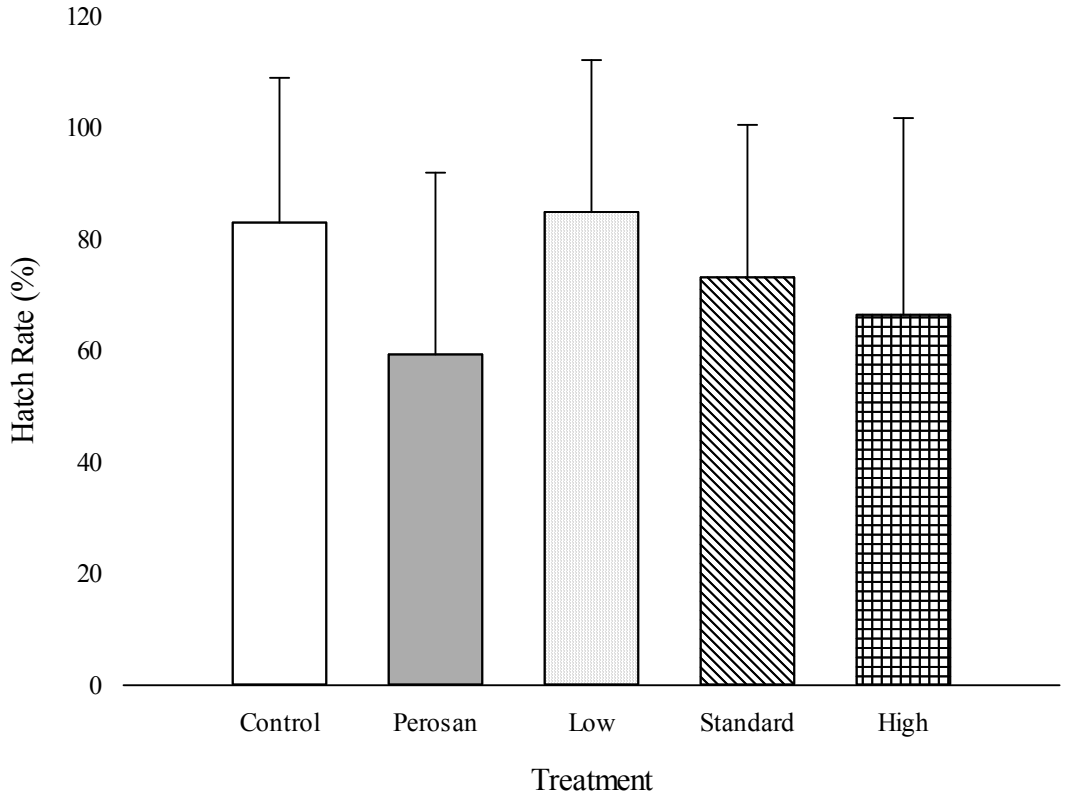

Figure 3: Mean hatch rate (%) ± st. dev. at 0dph of Control, Perosan, 0.5- 1.0 mg O_3 L^{-1} (Low), 1.5- 2.0 mg O_3 L^{-1} (Standard), and 2.5- 3.0 mg O_3 L^{-1} (High) Atlantic cod egg batches 1- 10.

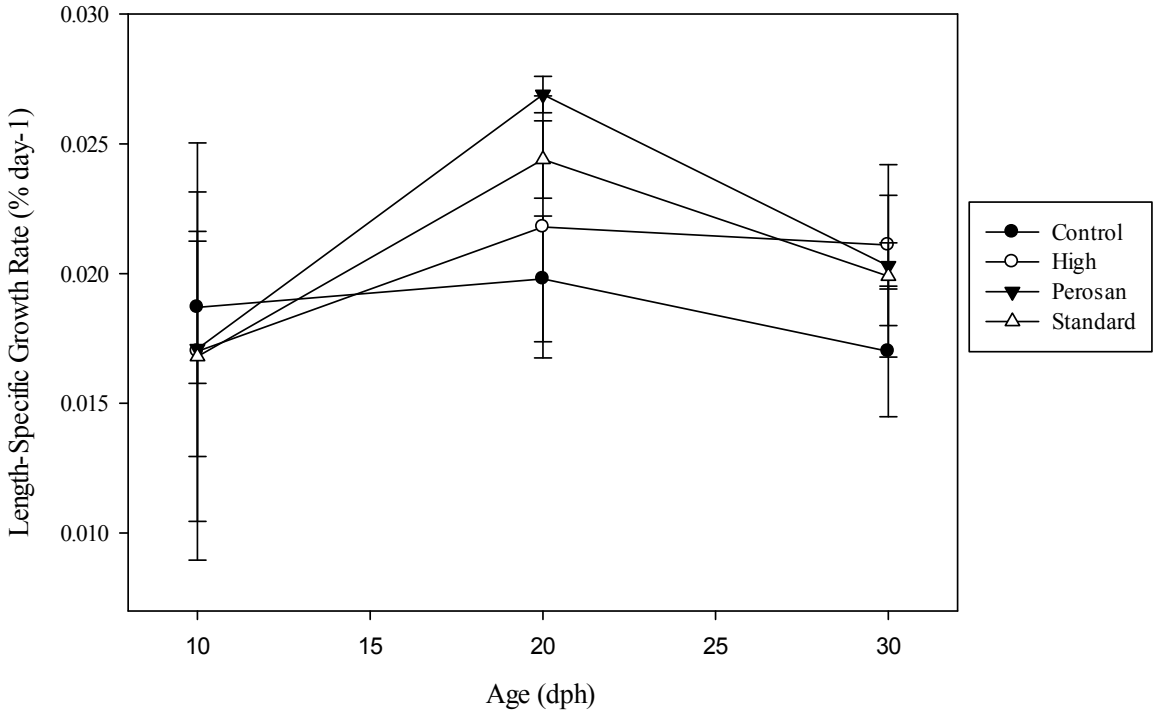

Figure 4: Mean length-specific growth rate of treatments ± st. dev. (Control, Perosan, 1.5-2.0 mg O_3 L^{-1} (Standard), and 2.5- 3.0 mg O_3 L^{-1} (High)) up to 30 dph for batches 1-4.

Table 6: Mean larval condition factor (K) (± st. dev.) and statistical results of 5 treatments (Control, Perosan, 0.5- 1.0 mg O_3 L^{-1} (Low), 1.5-2.0 mg O_3 L^{-1} (Standard), and 2.5- 3.0 mg O_3 L^{-1} (High)) at 0, 10, 20 and 30 dph for batches 1-10.

Age	Batch	Treatment					df	F	P	Differences
		Control	Perosan	Low	Standard	High				
0	1	0.0566 ± 0.0029	0.0563 ± 0.0018	0.0602 ± 0.0058	0.0598 ± 0.0062	0.0595 ± 0.0059	4	2.185	0.080	
	2	0.0571 ± 0.0038	0.0575 ± 0.0030	0.0557 ± 0.0014	0.0569 ± 0.0018	0.0593 ± 0.0051	4	2.271	0.070	
	3	0.0565 ± 0.0020	0.0608 ± 0.0064	0.0578 ± 0.0041	0.0594 ± 0.0053	0.0585 ± 0.0050	4	1.751	0.149	
	4	0.0576 ± 0.0049	0.0580 ± 0.0054	0.0560 ± 0.0042	0.0575 ± 0.0052	0.0573 ± 0.0037	4	0.394	0.812	
	5	0.0610 ± 0.0056	0.0580 ± 0.0016	0.0575 ± 0.0015	0.0580 ± 0.0031	0.0601 ± 0.0047	4	2.697	**0.038**	
	6	0.0561 ± 0.0016	0.0563 ± 0.0021	0.0573 ± 0.0018	0.0561 ± 0.0021	0.0559 ± 0.0016	4	1.448	0.227	
	7	0.0572 ± 0.0039	0.0580 ± 0.0053	0.0568 ± 0.0034	0.0565 ± 0.0034	0.0577 ± 0.0048	4	0.300	0.877	
	8	0.0568 ± 0.0053	0.0564 ± 0.0055	0.0563 ± 0.0057	0.0554 ± 0.0035	0.0558 ± 0.0037	4	0.176	0.950	
	9	0.0539 ± 0.0025	0.0564 ± 0.0048	0.0559 ± 0.0045	0.0573 ± 0.0051	0.0560 ± 0.0055	4	1.096	0.365	
	10	0.0548 ± 0.0044	0.0592 ± 0.0052	0.0571 ± 0.0030	0.0570 ± 0.0046	0.0552 ± 0.0040	4	2.542	**0.047**	P > C
10	1	0.0616 ± 0.0037	0.0618 ± 0.0053		0.0637 ± 0.0076	0.0668 ± 0.0057	3	2.704	0.054	
	2	0.0609 ± 0.0061	0.0599 ± 0.0051		0.0609 ± 0.0079	0.0621 ± 0.0066	3	0.286	0.084	
	3	0.0602 ± 0.0059	0.0631 ± 0.0039		0.0604 ± 0.0049	0.0626 ± 0.0057	3	1.328	0.273	
	4	0.0650 ± 0.0062	0.0627 ± 0.0059		0.0654 ± 0.0061	0.0635 ± 0.0053	3	0.701	0.555	
20	1	0.0627 ± 0.0096	0.0645 ± 0.0050		n.d.	0.0681 ± 0.0031	2		*0.051*	
	2	0.0659 ± 0.0051	n.d.		0.0679 ± 0.0069	0.0669 ± 0.0047	2	0.484	0.620	
	3	0.0732 ± 0.0050	0.0785 ± 0.0074		0.0692 ± 0.0066	0.0716 ± 0.0044	3	6.432	< **0.001**	P > S, H
	4	0.0748 ± 0.0064	0.0734 ± 0.0053		0.0724 ± 0.0038	0.0757 ± 0.0075	3	0.906	0.444	
30	1	*0.0727 ± 0.0061*	*0.0800 ± 0.0074*		n.d.	*0.0797 ± 0.0075*	2	5.199	**0.010**	H, P > C
	2	0.0738 ± 0.0075	n.d.		0.0806 ± 0.0079	0.0766 ± 0.0094	2	2.507	0.094	
	3	0.0826 ± 0.0130	0.0899 ± 0.0056		0.0801 ± 0.0075	0.0855 ± 0.0055	3	3.696	**0.017**	P > S
	4	0.0899 ± 0.0068	0.0906 ± 0.0096		0.0891 ± 0.0065	0.0710 ± 0.0076	3	22.393	< **0.001**	P, C ,S > H

Table 7: Individual batch results of RNA, DNA and RNA:DNA statistical analysis at 0, 10, 20 and 30dph for batches 1-10.

Age	Batch	RNA				DNA				RNA:DNA			
		df	F	P	Differences	df	F	P	Differences	df	F	P	Differences
0	1	4	1.621	0.224		4	7.454	**0.002**	L > H, P	4	1.067	0.409	
	2	4	0.851	0.515		4		*0.539*		4	0.840	0.521	
	3	4		*0.588*		4		*0.255*		4		***0.030***	*C > L, S*
	4	4	2.631	0.083		4	4.747	**0.014**	C > H	4		*0.258*	
	5	4	1.410	0.281		4	0.753	0.573		4		*0.913*	
	6	4	0.507	0.731		4	2.311	0.105		4	0.585	0.679	
	7	3	3.123	0.081		3	3.609	0.059		3		*0.625*	
	8	4		*0.855*		4	2.683	0.072		4	2.675	0.073	
	9	4	0.277	0.888		4	0.233	0.915		4	0.205	0.931	
	10	4	6.380	**0.003**	C > S	4		*0.073*		4	16.988	**<0.001**	C, P >S > L
10	3	3	2.666	0.100		3	1.780	0.209		3	5.480	**0.015**	H > C
	4	3	2.671	0.095		3	3.270	0.059		3	1.262	0.331	
20	1	2	0.001	0.999		2	0.007	0.993		2		*0.941*	
	2	3	0.630	0.609		3	0.343	0.794		3		***0.023***	C > P
	3	2	2.702	0.127		2	4.768	**0.043**		2	0.525	0.611	
	4	3	6.581	**0.007**	C > P, H	3	7.925	**0.004**	C > P, S, H	3	2.739	0.090	
30	1	2	0.402	0.681		2		*0.397*		2	4.463	**0.050**	P > C
	2	2	4.230	0.051		2	6.836	**0.016**	C> H	2	3.039	0.098	
	3	3	2.325	0.127		3	2.664	0.095		3	1.571	0.248	
	4	3		*0.072*		3		*0.109*		3		0.060	

Values in bold are significantly different at P = 0.05. Italicized values were analyzed by a non-parametric Kruskal-Wallis one-way analysis of variance on ranks, which subsequently lacks a F-statistic.

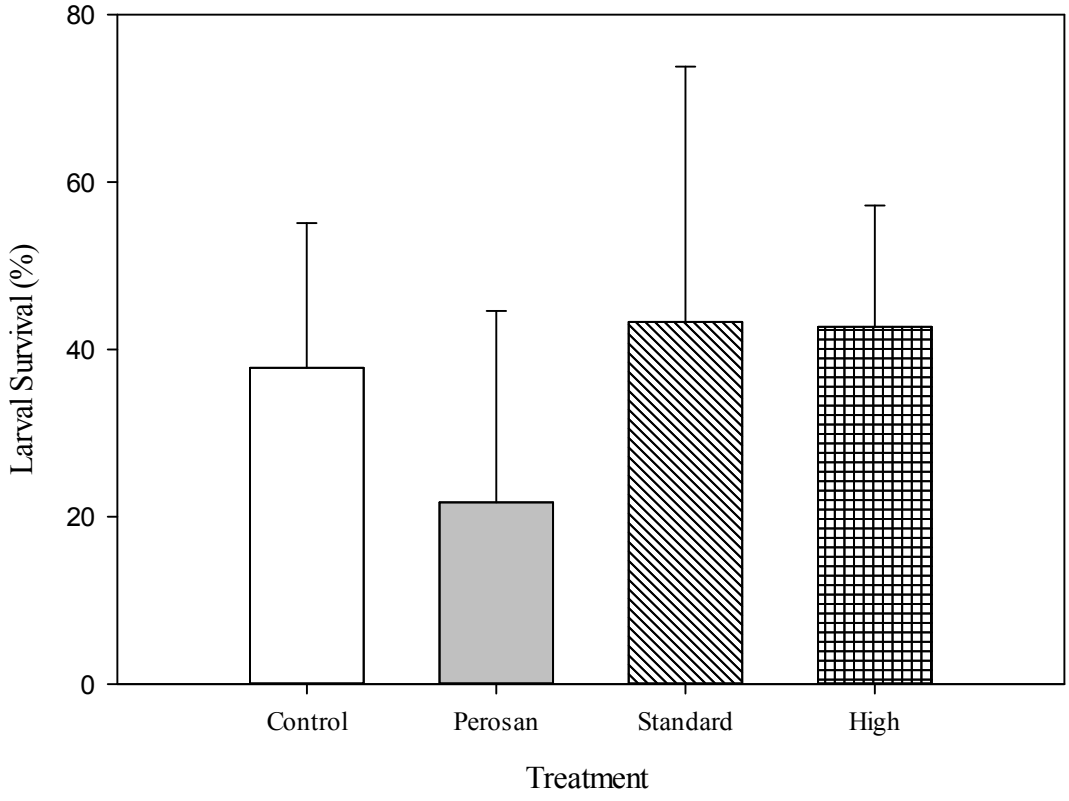

Figure 5: Mean larval survival ± st. dev. at 30 dph for the Control, Perosan, 1.5- 2.0 mg O_3 L^{-1} (Standard), and 2.5- 3.0 mg O_3 L^{-1} (High) treatments.

2.3 Discussion

As ozone oxidizes non-selectively, ascertaining a safe dosage for treating Atlantic cod eggs without reaching toxicity to the embryo is very important. High ozone exposure has been shown to reduce hatching rates in fish larvae and it has been hypothesized that this occurs due to a reaction between the oxidants formed during ozonation with the chorion protein polymer, leading to a resistance by the latter to the hatching enzyme (Grotmol and Totland 2000). An ozone disinfection process needs to be developed so that it destroys the pathogen without harming the larvae. This study attempted to evaluate the effectiveness of salt water ozone as a safe and efficient disinfectant on Atlantic cod eggs against the vertically transmitted virus nodavirus. To this end, we assessed several indices of larval condition both at the morphological and cellular level in order to determine if the disinfection was impacting normal growth, which is ultimately a major factor in survival of larval fish (Buckley 1984).

Despite the collection of eggs from potentially nodavirus-positive broodstock, none of the batches contained nodavirus-positive eggs pre-treatment. qPCR testing successfully amplified nodavirus-positive control samples, however all ten egg batches examined tested negative for the virus; consequently, we were unable to evaluate the potential and/or efficiency of using ozone to control nodavirus. Therefore the results presented are only representative of the toxicity of ozone disinfection in a healthy cod population, and may not reflect the response of virus-carrying fish.

2.3.1 Egg Quality and Hatching Success

Ozone disinfection has been examined as a viable disinfectant in numerous marine species. At the embryonic stage of development, three common side effects of

ozone usage have been reported: reduced hatching (Buchan *et al.* 2003; Grotmol *et al.* 2003, Battaglene & Moorehead 2006, Ballagh *et al.* 2011), asynchronous hatch (Grotmol & Totland 2000) and delayed hatch. Delayed hatch or reduced hatching success have been reported as results of salt water ozone disinfection in Atlantic halibut, striped trumpeter (*Latris lineata*) and Japanese flounder (*Paralichthys olivaceus*) eggs (Mimura *et al*. 1999, Grotmol & Totland 2000, Battaglene *et al.* 2006). This study found that embryos treated with ozone within 18 hours of fertilization (2- 32 cell stage) had equivalent egg survival and hatched synchronously when compared with either the untreated control or Perosan™- treated eggs at levels up to 2.5- 3.0 mg O_3 L^{-1} for 1.5 min (CT of 3.75- 4.5). There was a single outlying batch (#1) in which the treatments hatched asynchronously; all ozone-treatments and the Perosan™- treated eggs hatched 2 days (11.9 dd) later than the control group for batch #1 only. These results support those previously reported by Grotmol *et al*. (2003) who found that when ozone was applied to Atlantic cod eggs two days prior to expected hatch at levels of $\leq$ 2.2 mg O_3 L^{-1} for 0.5 min (CT of $\leq$ 1.1) most ozone treated groups hatched in the same period as the control (Grotmol *et al.* 2003). The consistency in results between the two studies despite the differences in timing of ozone disinfection suggests that Atlantic cod eggs are tolerant to ozonation throughout their embryonic development. This observation suggests a potential flexibility to the application of the ozone-disinfection process within a facility, particularly at a commercial scale. In this study, the choice to ozonate eggs within hours of fertilization coincided with the transfer of the eggs from the broodstock tanks to hatchery, with the aim of halting transmission of disease within the hatchery.

Ozone disinfection at levels up 2.5- 3.0 mg O_3 L^{-1} for 1.5 min (CT of 3.75- 4.5) applied at the 2- 32 cell stage of embryonic development did not affect hatch rate or timing of hatch. Hatch rate was not significantly different between the control and any of the disinfection treatments and hatching occurred within the normal range (88 dd) for this species (Brown *et al.* 2003). Nonetheless, across the range of ozone concentrations used in this study a downward slope can be observed in the number of larvae hatching as the ozone concentration increased to the range of 2.5- 3.0 mg O_3 L^{-1}. This trend suggests that if higher ranges of ozone were to be used, hatch rate may decline, although this would require further testing to confirm. This observation is consistent with what is known of the ozone tolerance of Atlantic cod eggs, and that of other marine fish species. For Atlantic cod, Grotmol *et al*. (2003) found that when ozone levels below 2 mg O_3 L^{-1} for 2 minutes (CT of 4) were used, the result was a normal larval hatch. In Atlantic halibut, a safe level of ozone treatment to produce normal hatch was found to be 0.3 to 1 mg O_3 L^{-1} or 4 mg O_3 L^{-1} for up to 0.5 minutes (CT of 0.15-0.5, 2- Grotmol & Totland 2000), while haddock eggs will hatch normally when exposed to 3.0 mg O_3 L^{-1} for up to 6.7 minutes (CT of 20.1) and in striped trumpeter up to 1.0 mg O_3 L^{-1} for as much as 5 minutes (CT of 5) is considered safe (Battaglene & Moorehead 2006). Likewise, in each of the species cited above higher levels or longer exposure times beyond these ranges produced a decrease in overall hatching success.

2.3.2 Condition Indices

As the long-term effect of ozone treatment applied during the egg stage had not been previously examined for Atlantic cod, three separate indices of larval condition were

measured in order to accurately determine if treatment was impacting growth and development of the larvae up to 30 dph.

The RNA:DNA is an index of growth rates in larval fish, and is one of the most commonly used indices of nucleic acid content (Chícharo & Chícharo 2008). RNA:DNA has been used extensively in studies to measure the impact of temperature and nutrition/starvation on growth in cod; however, to my knowledge, this is the first instance of its use to measure the impact of ozone disinfection on larval growth. The results of this study found that disinfection treatment did not impact larval growth and statistically significant differences were found in only 25% of the batch/age periods considered. There was considerable variability in RNA:DNA between egg batches, particularly at 0 dph when RNA:DNA ranged predominantly from 1.3- 3.73, but reached as high as 6.54 in one batch. Larval RNA:DNA variability has been observed to be high during the yolk sac stage, which in Atlantic cod extends up to 5 dph when exogenous feeding commences (Kjorsvik *et al*. 1991), and caution in extrapolating from this time period for RNA:DNA growth models has been suggested (Westerman *et al*. 1994; Folkvord *et al*. 1996; Caldarone *et al*. 2003). Individual larval growth is known to vary greatly, even within a single treatment, leading to difficulties in establishing the mean growth patterns (Caldarone *et al*. 2003). This was evident in this study. The RNA:DNA between treatments within a single batch was less variable than when comparing across batches. Each batch was therefore examined as a single unit, examining the effect of disinfection treatment relative to the Control. No true pattern was observed at any age however disinfection was overall negative on the RNA:DNA as compared to the untreated controls up to 20 dph. By 30 dph there was no significant difference in any of the ozone-treated

groups and in batch 1, the Perosan™- treated group had actually grown faster than the Controls. Mean values of RNA:DNA for each of the four ages ranged from 2.19± 0.4 to 3.14± 1.6, with an overall mean of 2.53 ± 0.75 for all larvae in all treatments. Nutritional studies conducted on cold-water marine fish species have shown similar levels including 2.32 and 2.61- 2.65 at 0 dph for Walleye pollock (*Theragra chalcogramma*- Canino, 1994) and Atlantic cod respectively (Clemmesen *et al*. 2003), 2-3 (14 dph- fed Atlantic cod– Buckley *et al*. 1999) and 2.75- 3.0 (20 dph fed Atlantic cod– Caldarone *et al*. 2003). Thus, despite the inter-individual and inter-batch variability in RNA:DNA and the statistical differences observed in certain batches, there is no evidence to suggest that any of the disinfection methods negatively impacted growth up to 30 dph.

This conclusion is also supported by the absence of effect of disinfection on the other condition indices: condition factor and length-specific growth rate. The condition factor measures the myotome height as compared to the total length of the animal. As with the RNA:DNA, statistically significant differences between treatments were infrequent, occurring in less than 25% of the comparisons and with no clear trend in differences. Moreover, the condition factors of all the larval groups were very similar to those of Atlantic cod larvae reared under optimum prey concentrations in a growth and survival study (Puvanendran & Brown 1999).

Length-specific growth rates were not statistically different at any larval age. Initial growth rates were low and did not increase over the sampling period. Under these rearing conditions, expected SGRs should have commenced closer to 2.2% day^{-1} by 10 dph and increased in a linear manner to > 3.0 day^{-1}.

Finally, larval survival levels in all the treatments ranged from 21.7% to 43.3%, with the highest survival in the two ozone-treated larval groups. Larval survival is highly variable but Puvanendran and Brown (1999) achieved a survival rate of approximately 24% for Atlantic cod reared under similar conditions but varying prey densities (4000-16000 prey L^{-1}) at 14 dph and no statistical difference between survival at 14 and 42 dph. In a later study, Puvanendran and Brown (2002) observed levels of 40% at 42 dph when examining optimal photoperiod and light intensity were incorporated.

Egg quality and age were found to be consistent between all batches, therefore, the results seem to indicate that the variation in larval condition in response to treatment may be a function of the genetic differences of larvae among and within batches. Batch effect as the strongest effect on larval survival rather than disinfection, has been shown in spotted halibut eggs (*Verasper variegatus*) treated with iodophor but only in the instance that initial egg quality in the whole batch was variable (Hirazawa *et al*. 1999). In the current study, initial egg quality and cell stage were checked in every batch to ensure consistent quality between batches and that any effect was the result of treatment and not poor eggs. Hirazawa *et al*. (1999) have reported an increased sensitivity to disinfection in eggs based on the spawning period, with eggs collected early in the spawning cycle of a fish being less sensitive to treatment. The eggs for this study were taken from communally spawning fish; therefore one batch may contain the genetic derivatives of multiple parents, both female and male. This method of egg collection is the least invasive to the animals and allows for more natural spawning of the fish, yet it renders it impossible to identify the genetic pairing within the tank. Evidence from a single commercial spawning event in Atlantic cod has shown that only a small portion of a

given population actually contributes to a single egg batch event (Herlin *et al.* 2008) and that from within that spawning, not all parents contribute to the same degree (Hansen *et al*. 2004). Taking these two factors together, an increased sensitivity to disinfection from one parent could greatly increase the effect of treatment within a single spawning event (batch) yet not impact the overall production.

This study was run on a semi-commercial scale over the course of one month of spawning to accurately assess the impact of ozone on eggs and the feasibility of the system itself. While there were select instances of statistical differences (25% of eggs tested) in the RNA:DNA and condition factor (< 25% of eggs tested) within batch treatments suggesting that disinfection was impacting the eggs, there was no specific trend to indicate one treatment was more damaging than another. Furthermore, length-specific growth rate was found to remain consistent between all treatments and final survivals at 30 dph were comparable between all groups including the control. These results support the conclusion that ozone application at ≤ 3.0 mg L^{-1} for 1.5 minutes does not negatively impact fertilized 2-16 cell Atlantic cod eggs and suggest ozone may be a good candidate for the disinfection of salt water fish eggs.

3. Safe Dosing of Ozone for Disinfection of Atlantic Salmon and Rainbow Trout Following Exposure to *Saprolegnia diclina*

3.1 Introduction

Saprolegnia are a genus of ubiquitous freshwater moulds, capable of infecting both fish and eggs, with devastating consequences. They present a major constraint to the culturing of fish worldwide and result in extensive economic losses in aquaculture (Meyer 1991). From the mid-1930s and for the next five decades, *Saprolegnia* infections in both eggs and fish were effectively controlled within fish rearing facilities with applications of malachite green (N-methylated diaminotriphenylmethane) (Foster and Woodbury 1936, Culp & Beland 1996). Malachite green was highly efficacious against fungi (Alderman 1985), however, it has been banned in most countries including Canada, the US, Japan, China and within the European Union since the 1980s due to findings that is carcinogenic, mutagenic, and teratogenic. It poses a serious health risk to the human operators, to fish-consumers and even to the fish to which it was being applied. The loss of malachite green as fungicide prompted new interest in investigating alternate disinfection methods.

The criteria for success of an effective fungicide to treat *Saprolegnia* would be to identify a method of disinfection that would be equivalent in efficacy to malachite green, but which is safe for the applicant, the consumer, the fish and the environment as a whole. There is a limited list of candidate methods capable of eradicating *Saprolegnia*, and of the methods identified, none have yielded the same efficacy as malachite green (Bruno *et al*. 2011). Currently bronopol, hydrogen peroxide and formaldehyde have

generated the most promising results based on the aforementioned criteria, although each presents limitations. Bronopol (2-bromo-2-nitropropane-1,3-diol), is marketed under the trade name Pyceze (Novartis Animal Vaccines, Braintree, UK) and is an approved fungistatic. It is considered to be the most effective preventative treatment for mycotic infections (Pottinger & Day 1999) but in many countries it must be administered under veterinary direction. Hydrogen peroxide is an effective fungicide although it requires a precise dosing for safe application (with respect to the animal), which is temperature dependent and does not have full regulatory approval for use on food fish at any life stage in Canada (Burka *et al*. 1997). Formaldehyde (or formalin in the 37% aqueous state) is the most frequently applied fungicide at the hatchery level and is approved for use in Canada and the United States (Burka *et al*. 1997). However, it prompts concerns for health of the operator, as it is a recognized carcinogen (Marking *et al*. 1994, Forneris *et al*. 2003).

Ozone is environmentally friendly, producing few harmful by-products, breaking down into oxygen as end product and in a relatively short period of time (Summerfelt 1997). Unventilated, it presents a respiratory risk to the operator, as ozone gas is toxic to humans, as it is a strong oxidizer. Ozonated water will release ozone gas during the application process, however when administered in a properly ventilated area with the appropriate air quality monitoring including a detector designed to measure ozone, the risk to the operator is minimal.

Ozone has a long history in freshwater hatchery applications. It has been employed for decades for its biocidal properties in water treatment to improve water quality and to eliminate waterborne pathogens in inflow and effluent; ozone has been

shown to effectively reduce aquatic viruses and bacteria by up to 99.99% (Liltved *et al*. 1995). Experiments focused on the application of this technology more specifically to improving egg quality and fish health through chronic low level concentrations in system water have been met with varied success. Chronic exposure to ozonated water resulted in the destruction of gill lamellar epithelium and hydromineral inbalance and ultimately death in rainbow trout (Wedemeyer *et al*. 1979). In contrast, a recirculating aquaculture system incorporating ozone improved Atlantic salmon growth and survival (Sutterlin *et al*. 1984) and chronic ozone application to river water supplied to summer steelhead trout (*Oncorhynchus mykiss*) resulted in significantly reduced mortality as well as larger fish as compared to those grown in untreated river water (Tipping 1988). At the egg and larval stage, acute exposure to low levels of ozone to sockeye salmon (*Oncorhynchus nerka*) throughout the incubation period extending to larval hatch resulted in high mortality up to the eyed-stage, although the authors acknowledge that ozone levels were inconsistent and significantly higher than originally intended (Grischkowsky *et al*. 1983). As a disinfectant, ozone efficacy for the eradication of *Ceratomyxa shasta* in cutthroat trout (*Oncorhyncus clarki*) was found to be successful at a CT of 0.84 (Tipping 1988). When used in repeated applications of 0.01, 0.03 or 0.2 mg O_3 L^{-1}, at two-day intervals for 46-60 days ozonated water effectively controlled saprolegniasis in brown trout (*Salmo trutta*) eggs incubating in California trays (Forneris *et al*. 2003).

The purpose of this study was two-fold: to determine if there were any short-term effects on larval performance following egg ozonation and to determine ozone's efficiency in eradicating *S. diclina*. The project included the testing of ozone application at two concentrations (0.5- 1.0 mg L^{-1} and 2.5- 3.0 mg L^{-1}) on eyed Atlantic salmon and

rainbow trout eggs using a prototype ozonation system. The efficacy of ozone was examined as well as its performance vis-à-vis the commercial disinfectant Ovadine (10-12 % povidone iodine- Syndel Laboratories Ltd, Qualicum Beach, BC, Canada).

Treatment success was evaluated using measures of egg and larval success (% survival), growth (larval weight and length) and growth rate (yolk sac conversion efficiency).

3.2 Materials and Methods

Prior to the commencement of the study, a review of the handling of animals and research protocol were met with full approval from the Institutional Animal Care Committee of Memorial University of Newfoundland (Protocol #12-04-CC).

3.2.1 Saprolegnia diclina Source

*Saprolegnia diclina (*ATCC® 42061™ F11 strain isolated from Atlantic salmon) was obtained (Cedarlane, Burlington, ON, CA) as a pure, live culture propagating on cornmeal agar. Fresh colonies were maintained by aseptically transferring an agar plug of *S. diclina* with the mycelium facing downward onto a new plate of cornmeal agar (17.0 g Cornmeal Agar; US Biological, Swampscott, MA, USA) with 1-3 drops of sterile tap water (STW) on the surface of the new plate as per the manufacturer's instructions (American Type Culture Collection, Manassas, VA, USA). These plates were incubated at 20°C under aerobic conditions, and monitored for growth and moisture content. When sufficient growth was achieved (on average every 48 hours), new plugs from fresh growing ends of the colonies were excised and plated onto fresh cornmeal agar plates as described above (Stueland *et al*. 2005).

3.2.1.1 *S. diclina* Hyphal Cluster and Cyst Preparation

Saprolegnia hyphal clusters and cysts were prepared as per Stueland *et al*. (2005). Briefly, agar plugs with hyphae-growth were excised and moved to glucose yeast broth containing 10 g glucose (Sigma-Aldrich, St. Louis, MO, USA) and 2.5 g yeast extract (Amresco, Solon, OH, USA) in 1 L H_2O and allowed to incubate for 2 days at 20°C, producing bundles of hyphae. These bundles were then harvested for ozonation or removed from the broth and washed with STW before further incubation at 20°C for 24 hours in STW. This final incubation step allowed for zoospore production to occur. The zoospore suspension was then filtered (0.5 mm pore) into a clean glass bottle. Cysts were counted by volume using a haemocytometer and adjusted to create a 1.0 x 10^4 spores L^{-1} suspension no more than 3 hours prior to use (Thoen *et al*. 2011). Viability of the spore suspension was checked by placing one drop into glucose yeast broth and incubating at 20°C until germination was visible.

3.2.1.2 Pre-infected Dead Egg Preparation

Pre-infected dead eggs (eggs to be infected with the pathogen and introduced into a clean population) were used as the source of *S. diclina* infection. As per Stueland *et al*. (2005) live eggs were immersed in 60°C water for a period of 1 minute to kill them. Thereafter groups of eggs were placed in 1 L glass bottles containing *S. diclina* spores at a concentration of 1.0 x 10^4 spores L^{-1} and incubated at 20 ± 1°C overnight. Following this incubation period, eggs were removed and rinsed with STW then transferred to sterilized incubator water (SIW) and incubated for a further 48 hours at 11 ± 1°C. The eggs were inspected under a stereomicroscope both at the initial rinsing and just prior to

introduction into the clean egg batches to ensure hyphal growth on the pre-infected dead eggs was both present and even (Thoen *et al*. 2011).

Presence of *S. diclina* on eggs was evaluated as per Fregeneda-Grandes *et al*. (2007). Eggs were initially placed in glass vials, with one egg per vial also containing SIW and transported from the Fisheries and Marine Institute (St. John's NL, CA) to the laboratory (Northwest Atlantic Fisheries Center, St. John's NL, CA). Prior to disinfection, eight eggs were removed from every tray housing eggs regardless of whether they were exposed to *S. diclina* or not. Then, immediately following treatment (prior to placement in the incubation trays), 10- 15 eggs per treatment (10 for unexposed eggs, and 15 for exposed consisting of 10 eggs and 5 pre-infected dead eggs) were removed and placed in 250 mL of SIW. At the laboratory, eggs were handled within a biosafety cabinet to avoid contamination. Eggs were individually rinsed three times with SIW and then placed in a Petri dish containing 20 mL of SIW water. After 24 hours of incubation at 20°C, eggs were examined under a stereomicroscope for fungal growth. If fungal growth was observed, mycelia were removed and placed onto glucose-peptone agar (Willoughby 1994) containing 200 μg mL^{-1} chloramphenicol. If no growth was observed, the eggs were placed in a new Petri dish containing SIW water and the process was repeated at 24-hour intervals for 72 hours. When present, mycelia growth on agar would undergo successive passes on the media at 48-hour intervals to ensure growth of *S. diclina* was pure. Following this final step, samples were disposed of.

3.2.1.3 Atlantic Salmon Eggs

Thirty-thousand disease-free hatchery stock eyed Atlantic salmon eggs (age 381.7 dd, diploid) were transferred from Northern Harvest Sea Farms (Stephenville, NL, CA) to

the Fisheries and Marine Institute, Memorial University of Newfoundland (St. John's, NL, CA) by ground transportation in 7 ± 1 °C water. Eggs were held pre-experiment in vertical incubation units supplied with 8°C UV-treated fresh water at a density of 5,000 eggs $tray^{-1}$.

3.2.1.4 Rainbow Trout Eggs

Forty-thousand disease-free eyed rainbow trout eggs (245 dd, diploid) were transported by air from AquaSearch ova Aps (Jelling, Denmark) to St. John's, NL and housed at the Fisheries and Marine Institute. Upon arrival, eggs were acclimated as per the supplier's instructions, which indicated that eggs should be submerged in a supply of hatchery water adjusted to match the temperature within the shipping container using chlorine-free crushed ice. Thereafter the temperature was raised by 1°C $hour^{-1}$ by gradually adding hatchery water to the container. Eggs were held pre-experimentally in vertical incubation units supplied with 9.5°C UV-treated fresh water at a density of 5,000 eggs $tray^{-1}$.

3.2.1.5 Salmonid Trials Infrastructure

Eggs were incubated throughout the study in 8-tray vertical incubation units. Four units were used in this study and each was modified to create an individual water supply to each tray, with two effluent lines, which discarded directly into the effluent sump. In this way, water from one tray did not mix with water from another (Figure 6). This modification controlled for the movement of *S. diclina* from one replicate tray to another, as *S. diclina* is a waterborne contaminant (Johnson *et al*. 2002). As a result, the study was

better able to replicate a semi-commercial-scale salmonid hatchery while also avoiding cross-contamination of the replicates.

Water source for the incubation system was pathogen-free ground water, which was degassed within the facility. All water for the incubation system within the quarantine unit housing the vertical incubation stacks was UV-treated prior to entering the system.

Atlantic salmon fry were raised in a single raceway containing dividers to separate the different fish groups (Figure 6). A single water supply line provided freshwater to the entire unit.

3.2.1.6 Ozone System

The freshwater ozonation system was a prototype system designed for plug-and-play use. Two major differences existed in this model as compared to the saltwater system used in the Atlantic cod experiment (Section 2.2.2). The first is the addition of a unique fume hood for the contact chamber. This fume hood rests on top of the contact chamber and dissipates gases out of the room and away from the user. The second addition is a user-interface panel. This panel provides a touch-screen control of the various mechanical components of the system including the pumps, fans and ozone generator. The software also featured a pre-programmable option, allowing the user to set the ozone concentration within the chamber and the contact time prior to disinfection. The latter option was not used for the purposes of this experiment. Ozone concentrations were instead determined by spectrophotometry using an Ozone Vacu-vials® kit (CHEMetrics, Calverton, VA, U.S.A.) as per the manufacturer's instructions.

As with the saltwater system used for Atlantic cod disinfection (Chapter 2), ozone for egg disinfection was generated using a corona-discharge ozone generator (Pacific Ozone O-series model G11- Pacific Ozone Technologies, CA, USA). Water flow dynamics remained the same, with fresh ozonated water flowing through the base of the tank and overflowing out a line at the top of the contact chamber.

3.2.1.7 Effect of Ozone on *S. diclina* Hyphae

To measure the effect of ozone on *S. diclina* itself, in the absence of salmonid eggs, bundles of hyphae were harvested of pure *S. diclina*. Ten fresh plates of *S. diclina* from source material were commenced and incubated as described previous, for 48 hours and then excised as agar plug and placed into ten Petri dishes containing glucose-yeast broth and further incubated for 48 hours. The resultant hyphal bundles were cut away from the agar plugs using a sterile scalpel blade. These bundles were then weighed on an analytical balance and separated into three portions using a sterile scalpel before being transferred aseptically to individual histology cassettes and sealed into sterile whirl pack bags. From each original excised agar plug, three hyphal mats in histology cassettes were created, and these were then assigned to a treatment set: control (no treatment but similar handling), Low O_3 (0.5 -1.0 mg L^{-1}) or High O_3 (2.5 - 3.0 mg L^{-1}). In order to avoid desiccation of the hyphae, all treatments were used within three hours of harvesting.

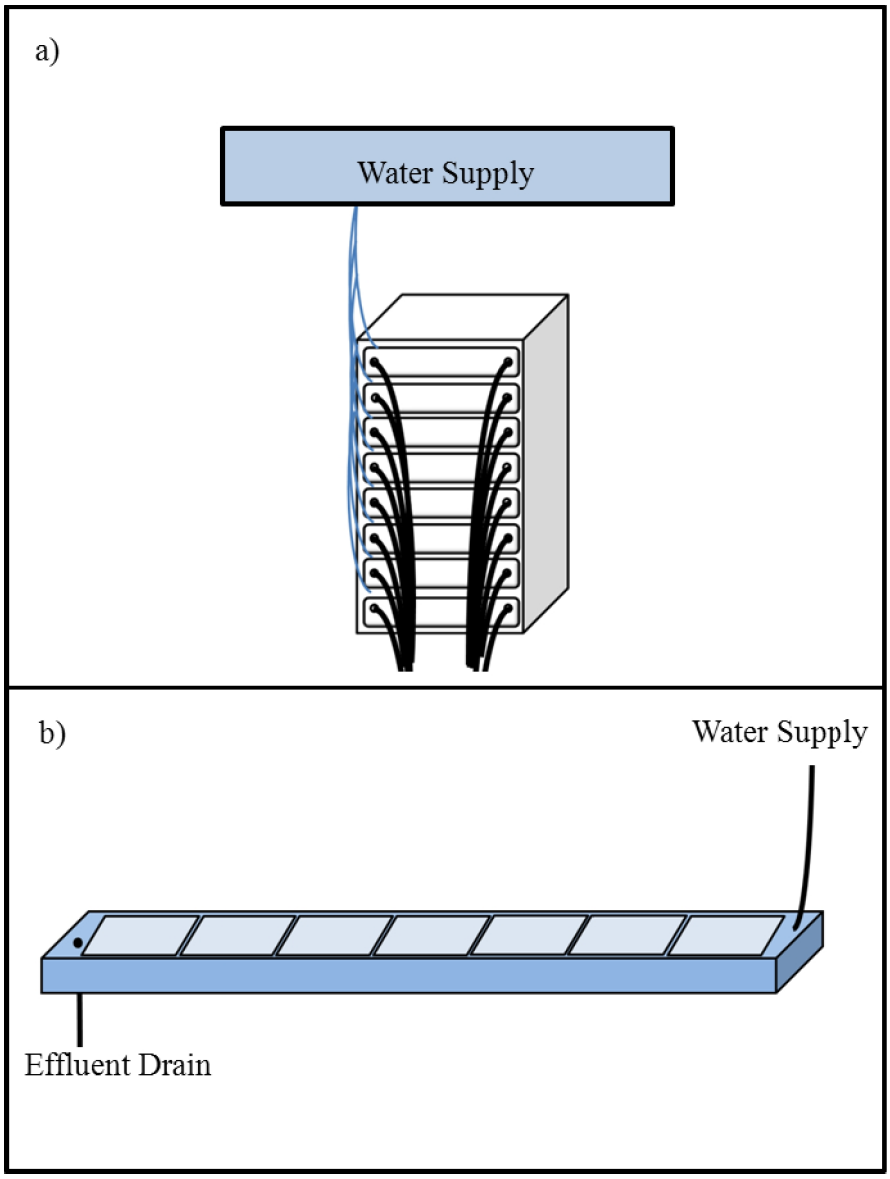

Figure 6: Salmonid trial infrastructure: a) modified vertical incubation unit and b) multi-tray raceway for salmon fry.

The histology cassettes containing *S. diclina* hyphae bundles were placed in a Nitex® mesh bag and submerged directly into the ozonation chamber for 1.5 minutes at the desired ozone concentration of either 0.5- 1.0 mg O_3 L^{-1} or 2.5- 3.0 mg O_3 L^{-1}. The same procedure was followed for the controls except that ozone concentration within the chamber was 0.0 mg L^{-1}. Immediately following treatment the histology cassettes containing *S. diclina* hyphae bundles were removed from the chamber and submerged in 500 mL of sterile tank water. Following this rinsing, the water was drained and the cassettes were placed in new whirl pack bags for transfer from the ozonation area to the clean biosafety cabinet.

Following transfer, all hyphal bundles were aseptically removed from the histology cassettes and placed into new Petri dishes containing 30 mL of glucose-yeast broth and incubated at 20°C for 72 hours. At 24 hours intervals, bundles were removed from the broth and any new growth was measured. The change in mass over 24 hours was measured as:

$$(Mass_{t2} - Mass_{t1}) * (t2\text{-}t1)^{-1}$$

where $t_{(x)}$ represents time in days.

3.2.1.8 Infection

One-half of the total quantity of eggs per species was exposed to *S. diclina* through the introduction of pre-infected eggs. Twenty-four hours prior to disinfection, pre-infected eggs were evenly dispersed across the surface of the eyed-eggs in each tray at a ratio of 1 infected egg: 25 clean eggs. Pre-infected eggs remained in the population of eggs throughout treatment and handling and were only removed twenty-four hours post-disinfection when egg mortalities were collected.

3.2.1.9 Treatment

In order to avoid any cross-contamination of the unexposed and *S. diclina*-exposed eggs, unexposed eggs were always handled before exposed eggs and gloves were changed between the groups. Each group was handled using separate equipment and were separated spatially to opposite ends of the workstation. Prior to treatment, all trays in the vertical incubation system containing unexposed eggs were removed and the eggs were combined into a single bucket containing an excess of fresh water. The same procedure was repeated for the *S. diclina*-exposed eggs. Immediately prior to treatment, the water was removed from the bucket and the eggs were transferred to a graduated beaker to determine the total volume of eggs. This volume of eggs was then subdivided into four treatment groups of equal volume. The four volumes were then randomly assigned to one of the treatments groups (Table 7):controls (similar handling but no chemical disinfection), Ovadine solution, Low O_3 (0.5- 1.0 mg O_3 L^{-1}) or High O_3 (2.5- 3.0 mg O_3 L^{-1}).

The Ovadine treatment was completed as per the manufacturer's instructions for the surface disinfection of salmonid eggs, which recommends mixing a 10 mL L^{-1} solution of Ovadine and submerging the eggs in this solution for 10 minutes at a minimum volume of four times that of the eggs. Eggs were then removed from the solution by means of a strainer, dipped in a bowl containing clean hatchery water to remove any remaining disinfectant and finally placed in a vertical incubation tray with a supply of UV-treated fresh water to flush away any remaining solution.

Table 5: Summary of Atlantic salmon and rainbow trout experimental groups and applied treatments.

Treatment #	Saprolegnia present	Ozone (mg L^{-1})	Ovadine (mg L^{-1})	Contact Time (min)
1	Yes	0.0	100.0	10.0
2	No	0.0		
3	Yes	0.5- 1.0	0.0	1.5
4	No	0.5- 1.0		
5	Yes	2.5- 3.0		
6	No	2.5- 3.0		
7 (Control)	Yes	0.0	0.0	0.0
8 (Control)	No	0.0		

Ozone concentrations were determined by spectrophotometry using an Ozone Vacu-vials® kit (CHEMetrics, Calverton, VA, U.S.A.) as per the manufacturer's instructions.

As with the salt water system, when the water within the contact chamber was at the desired concentration of ozone, eggs to be treated with ozonated fresh water were carefully submerged in the contact chamber using a free-floating Nitex® bag. The eggs remained in the system for a 1.5 minute contact, which was terminated by their removal from the system. The eggs were then transported within the Nitex® bag to the hatchery and rinsed into a bowl containing clean hatchery water. The ozonated water and the hatchery water were maintained at the same temperature throughout the experiments to eliminate any temperature shock to the eggs. Eggs were then transferred to the vertical incubation trays and maintained with a supply of UV-treated fresh water.

3.2.2 Husbandry and Sampling

3.2.2.1 Atlantic Salmon

Atlantic salmon egg and larval mortalities were counted and removed daily by manual picking. At hatch, total larval lengths (mm) and weights (g) were measured in each of the trays. Following hatch, egg shells were removed from the trays by passing a handheld sieve through the trays taking care to avoid the larvae. Once the larvae had reached 85% ± 5% yolk sac absorption, lengths (mm) and weights (g) were measured and then a subset of the population was moved to a raceway. Salmon fry feed (0.5 mm- Corey Nutrition, Fredericton, NB, CA) was introduced by hand from days 0-7 in the raceway, and thereafter to excess by automated feeders. Measurements for length and weight were repeated at 10, 20 and 30 days post transfer. Larvae were euthanized with 100 mg L^{-1}

TMS (Syndel Laboratories, Qualicum Beach, BC, Canada) following measurements at 30 dph.

3.2.3 Rainbow trout

Rainbow trout egg mortality was also counted and dead eggs removed daily from the trays. Larvae were measured for total length (mm), weight (g) and yolk sac volume (YSV) at hatch (0 dph), midway through the endogenous feeding period (8 dph) and at 85% ± 5% yolk sac re-absorption (16 dph). Yolk sac volume was calculated as per Koskinen *et al*. (2002) where YSV= $\pi/6$(yolk sac length * yolk sac height^2). Rainbow trout larvae were euthanized following the re-absorption of their yolk sac with 100 mg L^{-1} TMS.

3.2.4 Larval Morphometrics

Length measurements were taken at 0.7x magnification using a stereoscope to measure total length of the larvae in millimeters (± 0.1mm). Weight was measured using an analytical balance (± 0.0001g). Specific growth rates were derived for both length (mm) and weight (g) as per Jobling (1994):

$$SGR=100\ [\ \ln(X_{t2}) - \ln(X_{t1})](t2\text{-}t1)^{-1}$$

where $X_{t(y)}$ represents the morphometric measurements and t2-t1 represents the time period in days.

Yolk sac conversion efficiencies (YSC) were estimated as per Fraser *et al*. (2008):

$$YSC= (\text{size at yolk re-absorption - size at hatch}) (\text{yolk sac volume})^{-1}$$

where size is larval length in millimeters.

3.2.5 Statistical Analysis

In each of the analyses below, treatment and status (exposed vs. unexposed) were the categorical predictors for the Atlantic salmon, and only treatment was a categorical predictor for rainbow trout. The effect of treatment on weight and length of larvae and fry was measured with an analysis of covariance (ANCOVA). For both species, mean initial larval weight/length was the continuous variable. The yolk sac conversion (trout) and cumulative survival of both salmonid species was measured using a general linear model (GLM). Cumulative percent survival was square root transformed prior to analysis as the data set is based on proportions and all values were greater than 70%.

Change in S. diclina weight at 24, 48 and 72 hours was measured using a GLM with treatment as the only categorical variable. Data were square root transformed prior to analysis as the data set is based on proportions and all values were between 0- 30%.

Statistical significance was set at P= 0.05. In the event of a statistically significant difference, Tukey's HSD was used to determine the difference between treatments. Statistical analyses were completed in STATISTICA™ Version 10 (StatSoft Inc., Tulsa, OK, USA).

3.3 Results

The pre-infected dead eggs used to introduce *S. diclina* to the live Atlantic salmon eggs all exhibited growth of *S. diclina* at the time of introduction into the vertical incubation stacks. However, none of the eggs successfully infected adjacent naïve live eggs.

The pre-infected eggs used to introduce *S. diclina* to the rainbow trout eggs as well as the eggs collected from the trays pre- and post-treatment resulted in no growth in

all treatments and all replicates within each treatment at 24, 48 and 72 hours when incubated at 20°C. Therefore, only effect of treatment was considered for rainbow trout (n= 15- 16 trays per treatment).

Ozonation of *S. diclina* hyphal mats in the absence of any salmonid eggs resulted in a significant reduction in hyphal weight in the 0.5-1.0 mg O_3 L^{-1} and 2.5-3.0 mg O_3 L^{-1} treatment groups 24 hours post-treatment ($P< 0.01$)(Figure 7). At 48 hours post-treatment, this reduction in weight as compared to the control was still visible in the 2.5-3.0 mg O3 L^{-1} treatment ($P= 0.02$). However at 72 hours post-treatment the significant difference in fungal weight between treatments (0.5- 1.0 mg O_3 L^{-1} and 2.5- 3.0 mg O_3 L^{-1}) and the Control was no longer present. ($P= 0.100$).

There was no statistically significant difference ($P> 0.05$) among treatments in Atlantic salmon or rainbow trout at any life stage in terms of length (Atlantic salmon: P= 0.842; rainbow trout: P= 0.951) (Table 9, 11), weight (rainbow trout: P= 0.316) (Table 12), yolk sac absorption (rainbow trout: P= 0.783) (Table 13) or specific growth rate for weight (rainbow trout: P= 0.472, 0.981) or SGR for length (rainbow trout: P= 0.334, 0.996,) (Table 14). There was also no difference in percent survival in Atlantic salmon (P= 0.800) (Table 10). There was a statistically significant difference in survival in the rainbow trout ($P< 0.001$) between the control and all the other treatments (Ovadine, 0.5-1.0 mg O_3 L^{-1} and 2.5- 3.0 mg O_3 L^{-1}). The control had a significantly higher percent survival (81.8%) as compared to the Ovadine (74.1%), 0.5-1.0 mg O_3 L^{-1} (74.5%) and 2.5- 3.0 mg O_3 L^{-1} (73.6%). The survivals between the disinfection treatments were similar within one percent (Table 15).

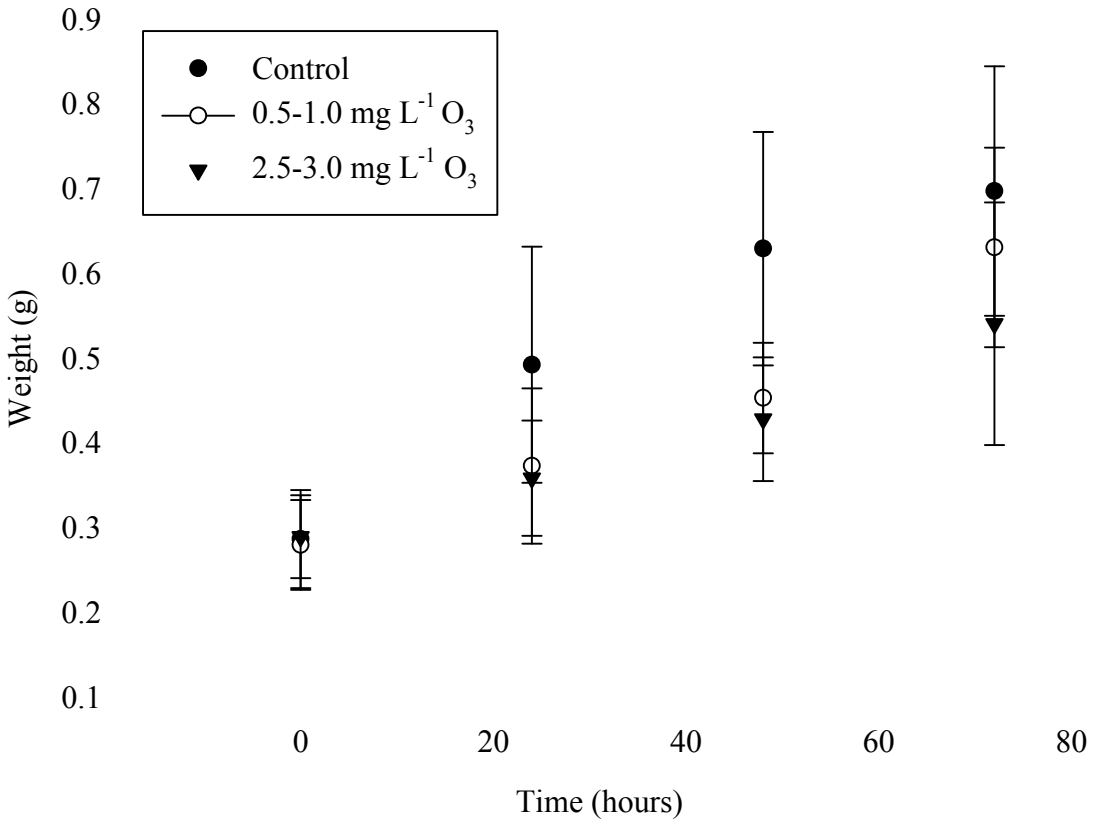

Figure 7: Change in hyphal weight (g) of *S. diclina* at 24, 48 and 72 hours following no treatment (Control), 0.5- 1.0 mg O_3 L^{-1} and 2.5- 3.0 mg O_3 L^{-1}.

Table 6: Summary of mean final length (cm) of Atlantic salmon larvae at yolk sac absorption.

Treatment	n (larvae)	Mean	st.dev	df	F	P
Control	40	2.335	0.144			
Ovadine	40	2.330	0.136			
Low	40	2.295	0.177			
High	40	2.355	0.016	3	0.28	0.842
X-Control	40	2.318	0.139			
X-Ovadine	40	2.315	0.133			
X-Low	40	2.343	0.148			
X-High	40	3.225	0.141			

* X: live eggs exposed to pre-infected dead eggs

Table 7: Summary of survival (%) of Atlantic salmon larvae at 85 ± 5% yolk sac absorption.

Treatment	n(tray)	Mean	st.dev	df	F	P
Control	8	84.589	4.839	3	0.33	0.800
Ovadine	8	84.741	4.089			
Low	8	82.679	5.192			
High	8	83.592	5.869			
X-Control	8	81.156	5.916			
X-Ovadine	8	82.565	3.681			
X-Low	8	82.340	3.212			
X-High	8	84.374	4.079			

* X: live eggs exposed to pre-infected dead eggs

Table 8: Summary of mean length (mm) of rainbow trout larvae at 85 ± 5% yolk sac absorption.

Treatment	n (tray)	Mean	st.dev.	df	F	P
Control	15	26.511	0.640	3	0.115	0.951
Ovadine	16	26.440	0.454			
Low	16	26.355	1.005			
High	16	26.486	0.719			

Table 9: Summary of mean weight (g) of rainbow trout larvae at 85 ± 5% yolk sac absorption.

Treatment	n (tray)	Mean	st.dev.	df	F	P
Control	15	0.130	0.006	3	1.208	0.316
Ovadine	16	0.127	0.008			
Low	16	0.127	0.007			
High	16	0.130	0.010			

Table 10: Summary of mean yolk sac conversion efficiency of rainbow trout larvae from hatch to 85 ± 5% absorption.

Treatment	n (tray)	Mean	st.dev.	df	F	P
Control	15	0.0406	0.0108	3	0.359	0.783
Ovadine	16	0.0403	0.0099			
Low	16	0.3914	0.0085			
High	16	0.0370	0.0131			

Table 11: Specific growth rate of larval rainbow trout at 8 and 16 dph

Specific Growth Rate	Age	Treatment	n (tray)	Mean	st.dev.	df	F	P
Length	8 dph	Control	15	4.400	0.690	3	1.155	0.334
		Ovadine	16	4.227	0.628			
		Low	16	3.945	0.860			
		High	16	4.242	0.571			
	16 dph	Control	15	2.567	0.440	3	0.019	0.996
		Ovadine	16	2.617	0.612			
		Low	16	2.594	0.781			
		High	16	2.286	0.506			
Weight	8 dph	Control	15	3.864	1.073	3	0.851	0.472
		Ovadine	16	3.714	1.649			
		Low	16	3.409	1.568			
		High	16	4.210	1.395			
	16 dph	Control	15	2.805	1.119	3	0.059	0.981
		Ovadine	16	2.689	1.414			
		Low	16	2.683	1.150			
		High	16	2.605	1.564			

Table 12: Summary of square root transformed trout survival.

Treatment	n (tray)	Mean	st.dev.	df	F	P	Comments
Control	15	9.0428	0.1437	3	6.95	<0.001	Control > Ovadine, Low, High
Ovadine	16	8.1217	0.3465				
Low	16	8.6266	0.2617				
High	15	8.5647	0.4785				

3.4 Discussion

The objective of this study was to determine the effects of freshwater ozone disinfection of eggs on early growth and survival of two salmonid species of commercial importance to aquaculture, as well as to determine ozone's suitability as a safe and efficacious treatment against the mycotic infection *S. diclina.* The impact of ozone application was examined vis-à-vis the predominant egg disinfection method, Ovadine (an iodophore derivative) as well as an untreated control group. The disinfection applications were tested at the eyed-stage of development, as this is a critical point in salmonid hatcheries for egg movement, transport and export. Eggs were monitored daily from eyed development up to larval yolk sac reabsorption for survival, hatching success, growth (length, weight) and yolk sac conversion efficiency (a growth index examining the rate at which yolk (nutrient) is converted to tissue).

The genus *Saprolegnia* has been historically considered to be a secondary pathogen (Neish 1977) but more recent research indicates that it may also act as primary pathogen including the *S. diclina* species, which is the predominant species identified on salmonid eggs (Kitancharoen *et al*. 1997, Fregenada- Grandes *et al.* 2007). Live Atlantic salmon eggs have been shown to be refractory to *Saprolegnia* sp. and exposure to zoospores is insufficient to induce an infection (Thoen *et al*. 2011). As such, the challenge model adopted in this study was the introduction of dead, *S. diclina* pre-infected eggs into a clean population of live eggs twenty-four hours prior to disinfection. Pre-killed healthy eggs were infected with *S. diclina* spores and following 72 hours of incubation, were visually inspected prior to use to ensure infection with *S. diclina*.

In the first experiment, all pre-infected Atlantic salmon eggs showed positive growth of *S. diclina* when inspected under a stereomicroscope. In the rainbow trout experiment, the inspection of pre-infected trout egg found no visible growth of *S. diclina* immediately prior to introduction. This absence of mycotic infection on the pre-exposed dead eggs was confirmed post-treatment by the follow-up sampling of egg batches 96 hours after the initial exposure infection as well as in laboratory incubation, which continued to show zero growth of *S. diclina* on the unused portion of pre-exposed dead eggs over a period of one week. Because of the increasing age of the rainbow trout eggs and therefore their proximity to hatch, it was not possible to produce a new batch of pre-infected dead eggs to attempt the *S. diclina* infection prior to hatch, making it necessary to proceed with the ozone experiment using the zero-growth dead eggs. For this reason, the rainbow trout eggs were considered to be pathogen-free throughout the experiment and data collected from this group was analyzed as only four treatment groups (no infected-state groups). Conclusions from that experiment were therefore only representative of the effect of the disinfectant applications on rainbow trout eggs, and not their ability to eradicate *S. diclina*.

In both Atlantic salmon and rainbow trout eggs, hatching success was comparable among all of the treatments. For Atlantic salmon, these results showed no effect of *S. diclina* exposure on hatching success. There was no significant effect at 0.5- 1.0 mg O_3 L^{-1}, 2.5- 3.0 mg O_3 L^{-1} or in the Ovadine application as compared to the untreated Control. Hatching success of both salmonids was also comparable with optimal hatching levels for the respective species in an industry-scale setting. This result indicates that at < 3.0 mg O_3 L^{-1} for up to 1.5 minutes, ozonated freshwater can be applied at the eyed-stage of

development and will not result in delayed or decreased hatch, a reported side-effect of ozone toxicity in some marine fish species (Mimura *et al*. 1999, Grotmol & Totland 2000, Battaglene *et al.* 2006). Safe application of ozone at this crucial hatchery stage could allow for further development of ozone as a disinfectant against pathogens as ozone is capable of destroying and bacteria and therefore aid in the reduction of disease transfer (Liltved *et al*. 1995).

The effect of ozone treatment on larval success was measured in terms of larval growth. Atlantic salmon larval growth was measured for total length up to 85 % yolk sac depletion. There was no impact of disinfection on total length of Atlantic salmon larvae. Rainbow trout larval growth was measured in three different ways: length, weight and yolk sac conversion efficiency. All three disinfection applications yielded results that were similar to the control group. As with the salmon, growth of larval rainbow trout was comparable to levels normally achieved for this species under similar hatchery conditions.

Atlantic salmon survival was above 81% in all treatments and there was no effect of treatment up to 85% yolk sac reabsorption. Survival in rainbow trout up to 85% yolk sac reabsorption resulted in the only measureable difference between the control and disinfection treatments in either experiment; there was a significant decrease in survival in all the disinfection treatments (73.5- 74.5%) as compared to the Control (81.8%). However larval survival between eggs treated with 0.5- 1.0 mg O_3 L^{-1}, 2.5- 3.0 mg O_3 L^{-1} or Ovadine (100 mg L^{-1}) varied by only one-percent, suggesting the impact of disinfection on the trout is equivalent. These results are supported by the limited data available on ozone disinfection in salmonid eggs: when treated with continuous, low

levels of ozone throughout the egg development stages, sockeye salmon eggs achieved a hatch rate of 78.6% (Grischkowsky *et al*. 1983). Similarly, freshwater application of ozone in sturgeon (*Acipenser persicus*) eggs at 0.15 mg O_3 L^{-1} yielded hatch rates of 76.4% (Ghomi *et al*. 2007). Overall, survival in both species was satisfactory during the endogenous feeding period. The reduction in hatching success due to disinfection does not outweigh the long-term benefits of halting disease transmission within a population. Though, further research is necessary to ensure ozonation efficacy in stopping specific pathogen (fungus, virus, bacteria) transmission in eggs.

Rainbow trout larvae were terminated once the treatment had reached >85% yolk sac reabsorption. However, Atlantic salmon larval growth and survival was carried through to the first 30 days of exogenous feeding, housed in individual trays within one raceway. This raceway had a single water source, with water passing from the first tank to the second and so on until it exited at the drain. The raceway was not located within the quarantine facility, but in an open aquatic facility which houses other salmonids at various life stages. The communal nature of the lab resulted in an outbreak of a white, cotton-like mold presumed to be a *Saprolegnia*. Despite intervening measures, it was not possible to eradicate the growth without compromising the study. This infection resulted in high rates of mortality within all of the treatment trays, but particularly impacting the trays furthest from the water source where water quality was lowest and build-up of organic material was highest. Therefore, while it was possible to maintain many of the Atlantic salmon fry for an additional 30 days of exogenous feeding, the resultant data were too severely compromised to allow for any conclusions to be drawn. This outbreak within the raceway, although detrimental to the study, only served to highlight the

extremely aggressive and devastating impact *Saprolegnia* sp. can have within an aquatic facility.

The effect of ozone treatment on *S. diclina* indicated that 0.5 -1.0 mg O_3 L^{-1} and 2.5 - 3.0 mg O_3 L^{-1} for 1.5 minutes both significantly impacted the growth of this species. The oxidation of the hyphal mats resulted in a reduction in mass and therefore a decreased rate of growth twenty-four hours post-treatment. In the lower concentration applied, 0.5 -1.0 mg O_3 L^{-1}, *S. diclina* recovered growth rates comparable with the control (0.0 mg O_3 L^{-1}) within 48 hours. The *S. diclina* exposed to 2.5 - 3.0 mg O_3 L^{-1} also recovered as compared to the control, but required 72 hours post-treatment to return to comparable levels of growth. Ozone treatment of 0.01, 0.03 or 0.2 mg L^{-1} to brown trout (*Salmo trutta fario*) infected with *Saprolegnia* resulted in a lack of visible hyphal growth for a period of approximately one week (Forneris *et al*. 2003). The difference in *Saprolegnia* recovery time between the studies may be due to differences in the extent of the *Saprolegnia* infection rather than efficiency of the treatment method. The hyphal mats tested within this study exceed the hyphal mass normally found on individual eggs especially if manual picking of dead or visibly-infected eggs occurs pre-treatment, which is the most effective method of fungal control in the hatchery (Ghomi *et al*. 2007). Therefore as there seems to be no discernible differences in toxicity towards Atlantic salmon or rainbow trout eggs at either of the ozone levels tested as a single dose, it is recommended that the higher dose of 2.5 - 3.0mg O_3 L^{-1} for 1.5 min be applied in the control of *S. diclina* infections in vertical incubation trays.

4. Summary

The purpose of ozone application to fish eggs is that the treatment be developed as an alternate method of disinfection against disease to those currently in use in aquaculture today. To be effective, ozone must eradicate the target pathogen while not rendering harm (either immediate or long term) to the eggs themselves. The intent of this research was to investigate these aspects of ozone efficacy and safety in three species of interest to aquaculture. The applications were focused at specific stages in egg development, which corresponded with hatchery handling or transport points during production for the respective species. Although neither portions of the research were successful in establishing any results in the context of ozone efficacy against nodavirus (Atlantic cod) or *S. diclina* (Atlantic salmon, rainbow trout), the aspect of applying ozone safely yielded largely positive results.

The treatment of recently fertilized Atlantic cod eggs with 0.5- 1.0, 1.5- 2.0 or 2.5- 3.0 mg O_3 L^{-1} vis-à-vis the commercial disinfectant Perosan™ (0.004 mg L^{-1}) and an untreated control revealed no trend or changes in either egg quality or larval condition, which would have suggested a negative impact of treatment on the eggs or the resultant larvae up to 30 dph.

Similarly, treatments of Atlantic salmon and rainbow trout eggs at the eyed-stage of development with ozonated freshwater suggest that at $\leq$ 3.0 mg O_3 L^{-1} for 1.5 minutes (CT $\leq$ 4.5) ozonation does not negatively impact the growth or survival of eggs as compared to the commercial disinfectant Ovadine (100 mg L^{-1}). Rainbow trout were

found to be more sensitive to both disinfection methods, showing equivalent levels of hatch in disinfected batches but lower but as compared to the untreated control.

These results support the conclusion that ozone can be applied safely to both Atlantic cod (a saltwater model species) as well as Atlantic salmon and rainbow trout (freshwater) when the correct concentration and contact time were evaluated. This study concluded that there is no negative effect of ozonation at < 3.0 mg O_3 L^{-1} for 1.5 min. The efficacy of the application in eradicating vertically transmitted pathogens will require more research.

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