

**ASSESSMENT OF MAJOR FACTORS AFFECTING TRIPLOID INDUCTION USING
HYDROSTATIC PRESSURE AND GROWTH ASSESSMENT OF TRIPLOID EASTERN
OYSTERS (*CRASSOSTREA VIRGINICA*) WITHIN THE BRAS D'OR LAKE**

by

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*Dedicated to my parents, **Alverna M. Sewell and Ian G. Sewell (Snr.)***

I can never thank you enough, my rock and fortress.

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ABSTRACT

Triploid oysters can be created through chemical or pressure treatments that induce a diploid embryo to become a triploid by blocking the expulsion of a polar body during development. Triploid oysters are desirable because they are sterile, faster growing, and have more energy to fight pathogens. Little is known about triploid induction in the Eastern oyster using hydrostatic pressure. The focus of these experiments were to evaluate different variables to elucidate their impact on triploid induction and assess the growth of triploid Eastern oysters grown in Canadian waters. The first objective of this study was to determine the most efficient pressure intensity and duration treatments that would yield the highest triploidy induction rates in the Eastern oyster. The second objective was to compare the growth of triploid vs diploid oysters in field conditions in the Bras d'Or lake, Nova Scotia.

LIST OF ABBREVIATIONS USED

Abbreviation	Definition
ABC	Aquaculture Genetics and Breeding Technology Center
ACOA	Atlantic Canada Opportunities Agency
ANOVA	Analysis of Variance
C	Control
CAD	Canadian dollars
CAIA	Canadian Aquaculture Industry Alliance
CB	Cytochalasin B
DAPI	Diamidino-2-phenylindole
DPF	Days Post Fertilization
FAO	Food and Agriculture Organization
M(I)	Meiosis I
M(II)	Meiosis II
MMC	Multiple Means Comparison
MIN	Minute
MSX	Multinucleate Sphere X
PB	Polar Body
NB	New Brunswick
NS	Nova Scotia
OIE	World Organisation for Animal Health
PB(I)	Polar Body I
PB(II)	Polar Body II
PD	Pressure Duration
PEI	Prince Edward Island
PI	Pressure Intensity
PSI	Pounds per Square Inch
SSP	Shellfish Sanitation Program
SW	Seawater

Trt	Treatment
US	United States
VIMS	Virginia Institute of Marine Sciences
3-N	3 Nuclei
6-DMAP	6- Dimethylaminopurine

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CHAPTER 1: INTRODUCTION

1.1 Project Overview

Aquaculture is one of the fastest growing sustainable food industries in the world. The FAO has highlighted aquaculture as a major source of the protein requirements for the growing human population, as agriculture and marine fisheries are unable to sustainably produce food quantities to supply this population growth (Subasinghe et al. 2009; Godfray et al. 2010). Due to its abundant coastal regions and access to natural resources, there is potential for the aquaculture industry in Canada to grow exponentially (CAIA 2018). To do so, various limiting factors such as climate change (Shackell & Loder 2012), pollution (Cauwenberghe and Janssen 2014; Cole and Galloway 2015; Sussarellu et al. 2016) and disease outbreaks (Ford and Tripp 1996; McGladdery & Stephenson 2005; Bower 2014) will have to be addressed so that they do not limit the growth of this food sector.

These limiting factors in industry expansion are mainly inhibiting growth of aquaculture in the Atlantic Provinces rather than on the Pacific coast of Canada. The aquaculture industry in Canada produced 39,927 tonnes of shellfish valuing 83 million Canadian dollars in 2014 (FAO 2016). The oyster industry contributed 29 million dollars of that total production value (FAO 2016). The Eastern oyster (*Crassostrea virginica*) accounts for 40% of the overall oyster production in Canada (CAIA, 2016). Recent statistics show that oyster production in Atlantic Canada had a value of \$36,547,000 CAD in 2015 with PEI leading in production (\$12,828,000), with NB second (\$7,423,000) and NS producing the least (\$1,186,000) (FAO 2016).

The NS oyster industry has faced a disease-related challenge which has impacted its potential for growth in comparison to PEI and NB. Since 2002, the Bras d'Or lake, once an economic catalyst for oyster production in Nova Scotia, has remained closed due to a disease

known as MSX caused by the protozoan parasite *Haplosporidium nelsoni* (McGladdery and Stephenson 2005). This disease continues to reside within the Bras d'Or lake and causes annual mortalities in oysters (personal communication with Rod Beresford 2018).

MSX disease prevalence has been well known due to the mass mortality events it has caused in Eastern oyster populations in North America (Ford and Haskin 1982). Delaware Bay and Chesapeake Bay are areas where the pathogen is fully established (Ford and Haskin 1982). In 1957, Delaware Bay was hit with the first outbreak, which killed up to 80% of the wild oyster population (Ford and Haskin 1982). Prior to this, the MSX disease agent was never identified (Ford and Haskin 1982). Along with the decimation of the wild populations, in both areas, 90 – 95% of aquaculture oysters were killed within two years (Ford and Haskin 1982). By 1960, oyster production within Delaware Bay was reduced by over 75% due to compound mortalities (Sindermann and Rosenfield 1967).

Endemic to the United States (Ford and Haskin 1982), MSX now has virtually no effect on current US oyster production. This is due to an investment in science that developed superior resistant oysters as a result of targeted breeding programs, genetic selection and implementation of triploid technology (ABC 2010; Dégremont et al. 2012). With advantageous traits such as faster growth, infertility, better taste, higher survivability and disease resistance (Ogle 1979; Stanley et al., 1984; Allen and Downing, 1986; Barber and Mann, 1991; Anderson 2000; Guo et al. 2009), the creation of triploid oysters is an advantageous technique that when applied, can potentially combat the effects of MSX on Eastern oysters in Canada.

By inhibiting first (I) or second (II) Polar body (PB) expulsion during egg development, triploid oysters can be created. Triploid induction is conducted either artificially with the use of physical or chemical means or through natural diploid- artificial tetraploid crosses (Longwell and

Stiles 1996; Guo et al. 1994; Eudeline et al. 2000a, b). Even though physical induction has proven to be less efficient in inducing triploids, its worth is shown through being virtually harmless to human beings in comparison to the use of chemicals. In addition, Canadian industry requests have been narrowed to physical induction means to prevent unwanted public criticism of chemical use in genetic alteration of the oysters. The focus of this project is to evaluate two major factors of treatment to elucidate their impact on triploid induction and assess the potential of triploid Eastern oyster growth in the MSX-prone waters of the Bras d'Or lake. In doing this, during the first phase of the project, a 3x3 multifactorial statistical design focusing on two major factors, pressure intensity and pressure treatment duration influencing triploidy induction rate will be assessed. Pressure intensity at 6000, 7000 & 7500 psi and pressure duration at 3, 5 & 10 minutes along with PB timing remained fixed at 60% PB(I) extrusion were the targeted variables of interest. The second phase of this project will involve the assessment of triploid Eastern oyster growth performance in comparison to their diploid counterparts after one year of open water bottom culture within the Bras d'Or lake. This marks the first time triploid oysters will be grown in bottom culture in the Atlantic provinces.

1.2 The Eastern oyster

1.2.1 History & natural distribution

The last ice age event determined the natural distribution of the Eastern oyster. Eastern oyster distribution was driven by the changes in thermal and salinity gradient during this period (Hargis 1999; Steimle 2005). Proto-estuaries along the Atlantic and Gulf of Mexico continent shelf were areas in which this species resided. However, due to the volatility of thermal and salinity gradients during this period in time, only designated areas within these proto-estuaries were habitable (Hargis 1999; Steimle 2005). The end of the last ice age led to a significant increase in sea levels which created new estuaries through the flooding of valleys and coastal areas. This

coastal flooding moved oysters inshore and to new coasts. Documentations of this displacement of Eastern oysters to new areas have been recorded in various locations including New York, Massachusetts, Georges Bank, Maine and Nova Scotia (Merrill et. al 1965). Later, residual shell radio-carbonating showed evidence of oysters potentially entering both the Chesapeake Estuary and Hudson-Raritan systems (Hargis 1999). However, during the 1800s, there were many drastic changes in estuarine temperatures, mini-ice ages and even natural catastrophic events that challenge our knowledge of pre-colonial distribution (Merrill et. al. 1965). Distribution among the many Atlantic-Gulf estuaries is highly variable in terms of time scales, with oyster shell age ranging from decades to centuries hence reducing the creditability of post-colonial recordings (Merrill et. al. 1965).

Due to the high adaptability of the Eastern oyster to a wide ranges of environmental parameters, natural populations have been noted within habitats along the Atlantic coast through to the Caribbean Sea including areas such as the Atlantic Provinces of Canada, Gulf of Mexico, Panama and various islands within the Caribbean (Carlton and Mann 1996; Abbott 1974; MacKenzie 1997a; Jenkins et al., 1997; FAO 1978; Buroker 1983). Wallace (2001) noted additional natural populations in Venezuela, Argentina and Brazil. Apart from naturally residing populations, artificial seeding of this species has been occurring since the 1800s (Rueink et al. 2005). Successful seeded areas include Western Canada (British Columbia), Western US states, Hawaii, Fiji, Tonga, Japan, and the Mauritius-Indian Ocean (Rueink et al. 2005). Most of these importation efforts were conducted in an attempt to start commercial cultivation of Eastern oyster in these locations (Ruesink et al. 2005).

1.2.2 Habitat & Environmental tolerance

Oysters are highly tolerant to thermal and salinity fluctuations and can survive a wide range of aquatic conditions due to their natural range in the intertidal zone (Shumway 1996). However, despite their capacity to survive in a diverse range of environments, they do have a preference to specific ranges of conditions where growth and reproduction are optimized (Shumway 1996). Temperature and salinity are very important factors on the Eastern oyster's biological, physiological and behavioral functions (Shumway 1996). These two factors can impact survivability of the oyster and its reproductive capacity, which in turn, affects species recruitment (Shumway 1996). Apart from salinity and temperature, factors such as turbidity, dissolved oxygen, food availability, and space, the presence of metals, light, and pH are contributory to survival and productivity in any given environment (Shumway 1996; Rosesijadi 1996; Newell and Langdon, 1996). Environmental tolerance in the Eastern oyster varies with maturity, as the oyster becomes more mature it naturally becomes more robust to changing environments (Shumway 1996).

The adult Eastern oyster can survive within a thermal range of -2 to 36 °C (Galtsoff 1964). However, its optimal thermal range is from 20 to 30°C (Stanley and Sellers 1986). In terms of growth, temperature adaptations vary based on geography. Oysters have the ability to survive at very low temperatures even if frozen. This mechanism allows them to survive extremely harsh winters (Loosanoff 1965). However, during winter conditions the mantle is not actively making new shell so if physically disturbed during periods of extremely low temperatures, unrepairable shell damage may be fatal (Shumway 1996). The thermal tolerance of oyster larvae is inferior to adults as they are more fragile and prone to death (Calabrese and Davis 1970). In all life stages,

thermal ranges and rate of thermal change are two very critical factors in oyster health as fast fluctuations and lengthened exposure at low temperatures can induce stress and lead to death (Shumway 1996).

The Eastern oyster is also euryhaline, meaning it is physiologically adapted to tolerate a wide range of salinities (Shumway 1996). Consequently, its capacity to adapt has caused isolated populations in many geographic locations over time. Distinct zones of salinity tolerances in these populations are developed, thus causing confusion in documented publications about salinity tolerances. In adults, the optimal salinity tolerance ranges between 14 to 28 ppt (Galtsoff 1964) and in larvae, 10 – 27.5 ppt (Calabrese and Davis 1970).

In the wild, oysters reside in all three littoral zones depending on the presence of suitable natural and artificial substrate (Eastern Oyster Biological Review Team 2007). Population density however, is dependent on food availability, competition, predation, and available substrate. These are important ecological factors that influence reproduction, recruitment and species prolificacy (Galtsoff 1964). Oysters thrive on firm substrates such as rocks, hard or semi-hard mud, residual shells and even artificial objects or displaced infrastructure (Jenkins et al. 1997). Substratum is very critical for proper shell growth and development and so oyster larvae have the innate ability to choose suitable substrate (Galtsoff 1964). Oysters are gregarious settlers; an evolutionary adaptation possessed by most benthic bivalves with pelagic larval life stages (Verwey 1949). Gregarious settlement in oysters is an innate ability to settle on calcareous materials such as other oyster shells. Due to the gregarious nature of the oysters, when left undisturbed, populations form natural oyster reefs which are ideal environments for recruitment and survival (MacKenzie 1997a).

Water movement, climate and food availability affect the productivity of oyster reefs (Galtsoff 1964). The availability of food is directly related to both climatic conditions (Loosanoff

and Nomejko 1949) and tidal flow (Lund 1957a, b). Several studies have supported the ideology that there is a positive relationship between tidal flow and bivalve somatic growth (Kellogg 1903; Belding 1912; Kerswill 1949; Haskin 1952). However, growth has been more directly related to temperature and food availability (Kennedy 1996). Oysters are selective filter feeders (Galtsoff 1964). The main diet of adult oysters consists of phytoplankton, detritus and occasionally inorganic matter (Newell and Langdon 1996). Ingestion and food utilization are achieved by several organs' (labial palps, mouth, esophagus, stomach, crystalline style, digestive diverticula and midgut) coordinative work to ensure efficient use of food (Galtsoff 1964). The anus excretes fecal matter post-digestion and absorption (Galtsoff 1964).

Food consumption, sorting and selection in both larvae and adult are highly regulated in the Eastern oyster (Newell and Langdon 1996). Consumption rate is dependent on particle size, type of food and nutritional constituent of the food item (Baldwin 1995; Baldwin and Newell 1995a, b; Kennedy 1996). Other direct factors affecting consumption is food abundance, excessive siltation, and water turbidity (Lund 1957; Lund 1957a, b). Prior to ingestion, sorting occurs via the labial palps and gills where 2 to 15 cells are processed at any given time (Newell and Langdon 1996). Some particles are rejected as pseudofaeces and others ingested and digested (Beninger et al., 1999). In contrary to mammalian anatomy, peristalsis is absent in bivalves, food is transported throughout the body via ciliary motion (Galtsoff 1964). Food preference in the Eastern oyster is for particles ranging from 1 – 30 μm in size (Mackie 1969; Quast et al. 1988; Newell and Langdon 1996). Despite all the factors involved in food utilization, maximum growth is mainly dependent on food availability, environmental and genetic influences (Shumway 1996).

1.2.3 General anatomy

The Eastern oyster, of the phylum Mollusca and family Ostreidae is an inequilaterally-structured bivalve shellfish species (Seed 1983). Oysters belonging to the Ostreidae family are similar in their physiological behaviours and anatomical construct (Galtsoff 1964). The Eastern oyster has an elongated shell structure consisting of two calcareous valves; with the left having a cupped shape and the right, flattened like a lid (Yonge 1980). The external shell colour varies among individuals and includes random patterns of green, brown, gray and white (Kay 1979). The inner shell surfaces are smooth and white around the adductor muscle attachment point, which has a similar colour to the muscle itself (Stewart 1989). Both valves are attached by a robust hinge, the left valve's cup-like depression reduces towards the attachment point. (Galtsoff 1964). The composition, shape and durability of the shell of the Eastern oyster is determined by its environment (Wendell and Malone 1994). When grown on hard substrate, the shell is thick and cup-shaped. On soft substrate, the shell tends to be thin with less depression in its left valve (Stanely and Sellers 1986). Other factors such as overcrowding, light, air exposure and mechanical disturbances affect shell quality (Galtsoff et al. 1947).

The major organs of the Eastern oyster are the shell, mantle, adductor muscle, gills, stomach, and heart. These all work together in coherence to ensure proper growth and development of the oyster throughout its life. The shell is the oysters' primary protective mechanism (Galtsoff 1964). The adductor muscle allows the opening and closing of the shell (Galtsoff 1964; Eble and Scro 1996). The mantle works along with the gill to create negative water current which guides food particles towards the oyster. It is then sorted and transported to the labial palps prior to ingestion (Eble and Scro 1996). Although the mantle aids in many other supporting biological processes, its main function is shell formation (Galtsoff 1964). The stomach, located in the central

region of the visceral mass, contains various secondary organs such as the caecum, crystalline sac, and epithelial layer which contributes to its overall main function. Sorting and storage of undigested food particles and conduct digestive, absorptive, and excretory activities are jobs conducted by these secondary organs (Galtsoff 1964).

The Eastern oysters' open circulatory system consists of the heart, arteries, veins, and sinuses which all aid in the distribution and transportation of haemocytes in the haemolymph (Galtsoff 1964). The circulation of hemolymph is controlled by the pulsating action of the heart. Haemolymph is pumped to the arteries and access organs in open sinuses, ultimately being distributed throughout the entire body via muscular contractions (Galtsoff 1964).

1.2.4 Reproduction

The Eastern oyster is a protandrous hermaphrodite (Fretter and Graham 1964). These are animals that have the ability to change sex from male to female at some point in their life. At first maturity, 70% of the new spawners are males. Sex change tends to occur between spawning season after all gametes are resorbed where a portion of the population are converted to females (Needler 1932a; Coe 1934; Thompson et al. 1996). Oysters may alternate between sexes due to physiological influences such as dietary needs, environmental conditions, and internal and external stressors (Tranter 1958; Bahr and Hillman 1967; Davis and Hillman 1971; Ford et al. 1990). Hermaphroditism does sporadically occur in the Eastern oyster at low rates (Thompson et al. 1996). Age and environment are major factors influencing this physiological process (Galtsoff 1964). In large older populations, sex ratios tend to favor females resulting in female dominant populations (Burkenroad 1931). Eastern oysters usually spawn during the warmest months of the year; this may vary based on geographic location.

Shell growth can be used as an indicator for oysters that have reached sexual maturity. Singh (1978) showed that spawning occurring in individuals at 25 mm in size. In female oysters, the ovary, gills, mantle and adductor muscle all play major roles in spawning (Galtsoff 1964). These organs collectively coordinate to facilitate the transport of eggs from within the oyster to the water column where fertilization occurs (Galtsoff 1964). Prior to the release of eggs from the ovary, the mantle prepares a pathway for movement of the eggs towards the gills. During ovulation, the eggs move from the ovary into the mantle, the mantle then creates a pulsating motion periodically exposing an entrance to the gills (Galtsoff 1964). The unfertilized eggs are released through a small opening created by the gills and expelled into the water column. This release of gametes is aided by the adductor muscle that continuously opens and closes the shells in a rapid manner (Galtsoff 1964). Expulsion of the eggs is distinguished by its widely dispersed fluffy white appearance and may last up to an hour depending on a number of mature eggs available for release (Galtsoff 1964). A well conditioned female oyster can produce up to 114 million eggs in a single reproductive cycle (Buroker 1983)

In the male oyster, the sperm travels from the spermary where it is developed. It is then transferred through the genital duct and into the water column via respiratory outflow (Galtsoff 1964). During ejaculation, the pallium maintains an open position (Galtsoff 1964). Sperm ejaculation in oysters is distinguished by a flow of a stream-like milky substance. Spawning in male oysters may persist for several hours (Galtsoff 1964).

Natural spawning is influenced by water temperature, salinity and chemical composition (Hayes and Menzel 1981). Spawning is a seasonal phenomenon which usually occurs during summer where water temperatures are above 20°C and salinity above 10 ppt (Eastern Oyster Biological Review Team, 2007). In the Maritimes, oysters spawn between June and August;

however, spawning can be extended if suitable environmental temperatures persist (Kennedy and Battle 1964). At the end of the spawning season, gonads are resorbed into the tissues of the oyster and are used as a source of energy and nutrients. Spawning occurs on an annual basis and gonadal development in both sexes recommences as water temperature begins to increase (Thompson et al. 1996).

1.2.5 Life cycle

The Eastern oyster can live up to 20 years. Within two years, they can reach up to 115 mm in size. Sexual maturity can be reached by as early as 4 months (Buroker 1983). Oyster larval stages can last up to 3 weeks; however, with the presence of favorable environments, time to metamorphosis can be significantly reduced (Castagna et al 1996). During spawning, fertilization occurs within the water column which leads to the development of the first larval stage known as the trochophore. Trochophore larvae remain pelagic and non-feeding; relying on endogenous nutrition (Galtsoff 1964; Kennedy 1996). The trochophore stage lasts from 24 – 48 hours depending on water temperature (Easter Oyster Biological Review Team 2007). The D-stage larvae is the next stage of development where the oyster begins to feed on a diet consisting of bacteria, phytoplankton, and detritus (Kennedy 1996). Food quality and quantity is very critical during the larval stages and is highly correlated with survival (Underwood and Fairweather, 1989).

Similar to other bivalves, excessive mortality is not uncommon as larvae are very susceptible to predation, unfavorable environments and disease exposure (Underwood and Fairweather, 1989). In a hatchery operation, artificial environmental conditions, proper husbandry, and feed are important factors for survival in larvae (Wallace 2008). Throughout the larval stages, the oyster persistently develops and undergoes various morphological changes (Galtsoff 1964). During the latter stages of larval development, the eyed larval stage is achieved where its sensory

capacity is enhanced and the larvae begins to move away from light. At this stage, the larvae develop an organ known as a “foot” and reach the life stage known as a pediveliger larvae. This foot allows the larvae to explore benthic environments and find a hard substrate for settlement (Kennedy 1996). Typical pediveliger behavior includes swimming with its foot retracted between its valves. When on substrate the larva reveals its foot and begins to crawl. This behaviour is repeated until the larvae finds desirable substrate. If not settled during this stage, the larvae die (Galtsoff 1964). The larvae undergo metamorphosis after settlement. The foot is completely resorbed and an adhesive fluid is secreted from its byssal glands which aid in the adherence of its left valve to suitable substrate. At this stage the larvae is sessile and known as a “spat” in the industry (Galtsoff 1964). Sessile larvae will continue growing and mature into adults in approximately one-year post-metamorphosis (Galtsoff 1964).

1.3 Oyster industry

1.3.1 History of the oyster industry in North America

Oysters, as a biological resource, have been fished in North America by First Nations communities since before the early 1600’s. However, since the settlement of European immigrants in the 1600’s harvesting of wild oysters was apparent (Stevenson 1894). Simple man-made harvesting tools, such as rakes and tongs, were used for harvesting (de Charlevoix 1744). As European populations increased, exploitation of wild oyster populations became increasingly abundant and harvest practices were then changed from simple tools to dredging sea-beds (Stevenson 1894). Quantitative production data showed yields of 710,000 bushels in 1839 (Kennedy and Breisch 1981). The discovery of many large reefs in Tangier Sound of Chesapeake Bay led to the quick expansion of the oyster fisheries (Steven 1894). Lime, a key constituent in oyster shells were mainly used for creating road beds, agricultural lime, mortar, poultry feed, and

concrete-like material used for construction called “tabby” up until the 1800’s (Hargis and Haven 1999). Overfishing of New England’s oyster beds through dredging in the 1800’s led to their departure to find other areas such as New Jersey and Virginia with larger populations of wild oysters (Stevenson 1894). The first large-scale commercial fishery launched by New England oyster fishermen began dredging oyster reefs along Chesapeake Bay (Kirby 2004). Several laws were passed for the prohibition of Oyster commercial fishing by 1820 due to extensive dredge-related damages on subtidal reefs along Chesapeake Bay (Winslow 1881; Lenihan and Peterson 2004).

Proceeding to the early 1800’s, oyster meat - once a delicacy only accessible by the privileged, quickly became available to all due to the substantial increase in demand and price depreciation. A new approach to oyster production was emerging and performing exceedingly well. In 1830, a legislation was passed allowing 1-acre of bottom lease to individuals for oyster culture (Power 1970). Due to the growing industry, another legislation was enacted in 1865 and 1867 allowing an increase to 5-acre bottom leases for oyster culture (Power 1970). Oyster culture peaked during the 1880’s through to the early 1900’s. Highest outputs amounted to 27 million bushels annually thus surpassing all other countries combined production (Ruge 1898). Production in US between 1880 and 1910 reached 160 million pounds of oyster meat annually (Lyles 1969). This level of market demand was short lived as numerous economic and social issues led to its decline. The earliest signs of overexploitation of oyster populations were evident around the mid 1800’s (Winslow 1881, 1882; Lenihan and Peterson 1928, 2004). Oyster consumption grew substantially as the 19th century approached, leaving a bottleneck scenario where there was increasing demand but limited oyster sources. Oyster farming was the one of the only potentially sustainable ways to supply these demands.

The Eastern oyster is one the four fundamental oyster species upon which the North American oyster industry was built. This species is naturally distributed widely along the Atlantic coast (Buroker 1983) and is found in many major geographic areas such as Canada, the Gulf of St. Lawrence, the Caribbean, Mexico and Brazil (Buroker 1983). Three other fundamentally important species were the Olympia oyster (*Ostreola conchaphila*), European flat oyster (*Ostrea edulis*) and the Kumamoto oyster (*Crassostrea sikamea*). The Olympia oyster, naturally residing from Alaska to California while the other two oyster species imported from Europe and Asia. Finally, the Pacific oyster (*Crassostrea gigas*) native to the Pacific coast of Japan (Quayle 1988) was also imported to various areas North America to aid in the development of the oyster industry.

The availability oyster meat within the US was mainly dependent on the oysters' physiological tolerance to climatic and seasonal changes (Mackenzie 1996). The majority of oyster sales occurred between November and February. During this time of year, oysters became fattened and were more desirable thus increasing demand (Mackenzie 1996). A proportional increase in oyster meat was evident from the beginning of fall through to winter. In contrast, throughout the summer season, oyster sales ceased as a result of spawning (Mackenzie 1996). During spawning, physiological fat reserves and energy is converted to gonads which creates an undesirable taste and texture in the oyster (Allen and Downing 1986).

A history of disease occurrences and bad publicity has been documented throughout the North American oyster industry. The Pure Food and Drug Act was instated in 1907 by the US government which highly regulated the cultivation, packaging and distribution of live oysters (Killian 1918). It was thought that this would positively influence the industry; however, due to bad publicity, oyster consumption became less desirable (Killian 1918). Throughout this period, labelled as a "Dark Age", oyster farms suffered a consistent decreased in annual income (Killian

1918). Disease related incidences within the industry aided in this demise. The largest typhoid outbreak in American history, occurring in 1924 due untreated sewage that was passed on to humans via oyster consumption (Rippey 1994). This outbreak claimed over one hundred lives and led to many illnesses that were tracked back to a specific group of oysters that were harvested from the Raritan Bay south of New York and New Jersey (Kochiss, 1974). Growing skepticism surrounding oyster consumption led to further decreased interest in oysters thus causing further reducing the production value of the species during this time. (Kochiss, 1974).

Adding to these unfavorable events, the third “Dark age” approached. Again, attacked by another major disease (this one killing oysters instead of people); the MSX outbreak in the late 1950’s (Ford and Haskin 1982). As soon as the oyster industry started to gain popularity and became widely accepted, an infectious protozoan parasite, *Haplosporidium nelsoni*, wiped out 90-95% of the existing oyster population in Delaware during the epizootic event (Ford and Haskin 1982). *H. nelsoni* wasn’t the only pathogen causing mortalities; another, *Perkinsus marinus* led to compounding mortalities across the US (Andrews 1996). This pathogen was responsible for the disease known as ‘Dermo’. These compound mortalities persisted in US in areas such as Delaware Bay, Chesapeake Bay, Florida, and Massachusetts among many others (Krantz et al. 1972; Fegley et al. 2003; McGladdery and Stephenson 2005). Both diseases caused mass mortalities during the summer months and infected oysters that survived through the summer would either die during overwintering or the following summer after being severely weakened. Efforts turned to a selective breeding program to develop strains of native oysters that possessed resistant capabilities to both MSX and Dermo. These programs were conducted at Rutgers University and Virginia Institute of Marine Science. Through intensive research and testing, they were successful in significantly reducing mortality events from these pathogens (ABC 2010). Triploidy was also developed to aid

in creation of genetically stronger oysters with more tolerance to diseases exposure and also faster growth (ABC 2010).

Another MSX mitigation strategy include supplying smaller oysters known as cocktail oysters than take approximately 2-4 years to reach market size rather than growing traditionally sized oysters which have a longer growing period of 3-6 years (Government of Canada, 2015). This shift will reduce the exposure time of the oysters to MSX thus reducing its impact on oyster stocks. (Canzonier 2004; Ross et. al. 2012). The *H. nelsoni* pathogen that causes MSX is known to be more virulent in adult oysters when compared to juveniles, a shift to cocktail oyster production will aid in MSX alleviation as younger oysters are less prone to the effects of MSX (Haskin and Andrews 1988). These strategies to mitigate impacts of disease were key in producing a self-sustainable, growing and profitable US oyster industry.

1.3.2 History of the oyster industry in Canada

Historical evidence of oyster culture in Canada exists that dates back to medieval times after the Roman Empire. The first documentation of oysters being consumed in North America was recorded in 1608 by Samuel de Champlain, who noted its edible appeal (Lavoie 1978). However, oysters were fished and farmed for many years by First Nations communities prior to Champlain. This discovery sparked a business agreement for oyster production in the Gulf of St-Lawrence. Oyster producers located in Isle-Saint Jean, now known as Prince Edward Island, agreed to supply the famous Malpeque oysters to Canadian French consumers which led to the creation of the oyster industry today (Lavoie 1978). North American colonization by various groups, including the French introduced oyster culture throughout the US and Canada (Lavoie 1978).

Throughout Canadian history, oysters have held cultural significance and have remained essential in the livelihoods of indigenous residents (Vercaemer et al. 2010). This fundamental species was adopted to large scale production post-European settlement. The first commercial oyster operation in Canada started on the Atlantic coast in 1865 in both provinces of Prince Edward Island (PEI) and New Brunswick (NB). The Eastern oyster, a species native to the Atlantic coast of Canada, is highly adapted to thrive in warm estuarine waters (Carriker and Gaffney 1996). This species, naturally residing in oyster reefs, were transplanted to bottom leases and grown to market size (Lavoie 1995; Mathieson 1912). Canadian provinces such as New Brunswick, Prince Edward Island and Nova Scotia provide ideal areas for culturing this species due its large coastal areas.

In 1876, the influence of the industrialization era aided in the expansion of potential market for Canadian oysters allowing substantial growth within the Canadian oyster industry (Morse 1971). This expansion was partly due to the Canadian Intercontinental Railway that started operating in 1876 (PEI Department of Agriculture, Fisheries and Aquaculture 2005). Oyster cultivation on the west coast started in the 1920's and involved the non-native Pacific oyster (*Crassostrea gigas*) (FAO 2015). Prior to this, documentation of marketing oysters native to British Columbia, the Olympia oyster (*Ostrea lurida*), was seen as early as 1884 (Quayle 1988). Furthermore, *O. lurida* was the only oyster used for production prior to the later introduction of the Eastern oyster in 1903 (Quayle 1969). Both the Eastern oyster and Olympia oyster performed poorly on the west coast (Quayle 1969). However, the Eastern oyster thrived in its native range throughout the Atlantic provinces of Canada up to this very day.

An overall increase in demand for oysters during the 1950s, led to the focused implications of 'spat collectors' on commercial leases to aid stock replenishment on the Atlantic coast (FAO 2015). A different method of oyster seed accumulation was adopted in British Columbia to produce

hatchery-raised seed (Quayle 1988). The improvement of knowledge on oyster aquaculture, coupled with monetary resources and implementation of necessary infrastructure, aided in its transformation from a fisheries-based industry to an aquaculture-based industry (Quayle 1988). Due to the high interest in Canadian oysters from both local and international markets, in the 1960s, Fisheries and Oceans Canada narrowed their focus to conducting shellfish-based aquaculture research to further understand the implications of farming oysters and their impact on their pre-disposed environments (FAO 2015). During the later 1950's, oyster seed importation ceased due to the excessive populations that were present in British Columbia. By 1990, Canadian oysters made up 0.6% of the overall world supply; most of which were Pacific oysters (FAO 1993).

Bottom culture, a primary method of oyster farming in Canada since the early 1800's, has now been mostly converted to off-bottom culture (Doiron 2008). In bottom culture, oysters are grown in bags on seabed leases from juvenile stages until they reach market size as adults. In contrast, for the newly developed method of off-bottom culture, also known as suspended culture, oysters are grown on rope or in beds suspended by buoys until they become marketable. The first oyster lease on the Atlantic coast, in Prince Edward Island, was granted authorization for production using bottom culture method in the 1850s (CAIA 2016). Bottom culture possessed a more simplified approach to oyster farming (Comeau 2009); however, suspended culture was overall more advantageous (Bataller et al. 1999). The main advantage of increased growth was now possible based on increased food exposure through tidal influx and elevated temperature (Comeau et al. 2010). Growing in popularity, suspended culture provided a wider range of advantages in oyster culture; facilitating easier harvesting practises, benthic predator alleviation and optimal waste removal (Comeau 2013). Using suspended culture, oyster growth periods could be reduced by 2-4 years.

During 2002, there were unexpected elevations in oyster mortalities in Cape Breton on the Atlantic coast of Canada (McGladdery and Stephenson 2005). Assessment of oyster samples taken from the affected area (St. Patrick's Channel) led to the discovery of a new pathogen that had never been before in Canada (McGladdery and Stephenson 2005). Following more intensive assessment of samples, the disease was found to be MSX, which previously became endemic to the United States. Its presence was confirmed by an OIE reference laboratory and emergency procedures to contain the disease were immediately enforced which included the discontinuation of aquaculture practices within the Bras d'Or lakes (McGladdery and Stephenson 2005). Another precautionary measure was to temporarily cease all transfers of shellfish species during 2003 to analyze the potential spread of MSX to other bivalves. This temporary restriction was abandoned after evidence showed that other shellfish species were not reservoirs for MSX (McGladdery and Stephenson 2005). The introduction of this parasite to Canada was suspected to be caused by ballast water displacement from affected areas within the United States (McGladdery and Stephenson 2005).

In 2013, further development in suspended culture was seen, when UV-resistant Vexar™ bags were fitted to floats to allow partial sun and air exposure (Mallet et al. 2009). These bags remain partially exposed at the water surface and can periodically turn 180° which significantly reduces tunicate biofouling (Mallet et al. 2009). These oyster bags are now popularly known as the 'Oystergro™'. Conveniently, during winters, these bags could be detached and placed on bottom while floats were filled with water and sunk to the seafloor until the coming spring (Mallet et al. 2009). All Atlantic Provinces have now adopted suspended culture thus causing a significant shift from bottom culture (McDonald 2010). Despite PEI currently being the highest producer of

oysters on the Atlantic coast, NB is currently more technologically advanced in modern suspended culture practises and equipment (McDonald 2010).

1.3.3 Open water culture

Oyster culture is heavily influenced by ocean properties such as water temperatures, salinity gradients, ocean movement, ecosystems, benthic environments and planktonic organisms (Galtsoff 1964; Shumway 1996). Oyster growth varies year to year due to fluctuations in climate and oceanic parameters. There has always been a co-dependent relationship between the ocean and atmosphere in respect to oyster production (Loosanoff and Nomejko 1949; Lund 1957 a, b). Weather development and climate change are very important in oyster cultivation (Loosanoff and Nomejko 1949; Lund 1957 a, b; Galtsoff 1964). When culturing oysters, oysters are subjected to various oceanic and atmospheric influences. Luckily, the Eastern oyster is widely adapted to various ranges of salinities, temperatures, substrates and suspended solid concentrations (Galtsoff 1964; Andrews 1979a). In a natural scenario, bivalves are gregarious in nature, hence they are usually found in concentrated populations (Galtsoff 1964). The natural benefit to this would be promoting 'survival of the fittest' where strong members within the population would have more access to the limited resources. However, in an aquaculture setting, this is seen as a limitation as it encourages intraspecific competition among peers (Hadley and Manzi 1984; Jarayabhand and Newkirk 1989; Fréchette and Lefaiivre 1990; Parsons and Dadswell 1992; Fréchette 1998). In bivalve culture, a non-linear inverse relationship is noted between stocking densities and growth (Rheault and Rice, 1995). Furthermore, in some cases, this same type of relationship is seen between stocking density and survival (Fréchette and Lefaiivre 1990). In addition to this, it is important to note that oyster shell structure, may become compromised due to high stocking

densities (Taylor et al. 1997b). Oyster farming is based on the availability to control stocking densities, growth rates, survival and proper shell composition (Lavoie 1996).

Oyster aquaculture is divided into two types: ‘suspended culture’ otherwise known as ‘off-bottom culture’ and ‘bottom culture’ (FAO 2004). Bottom culture is the simplest method of culturing oysters; this involves growing of naturally-produced or hatchery-based oyster spat on the ‘seafloor’ (FAO 2004). Recent developments within the industry created hatcheries that can provide nursery environments of oyster spat. This aids in the overall proliferation and survival of the next generation of oysters. (FAO 2004). Upon reaching 25 – 30 mm in size, oyster spat are transplanted to the seafloor to grow until they become market size (FAO 2004). Bottom culture is relatively inexpensive in respect to harvesting; however, a stable benthic environment is a necessity (FAO 2004). It is important to maintain stable substrate as substrate type and crowding can impact oyster growth and quality (Galtsoff 1964). One of the main issues with bottom culture is predation (Galtsoff 1964). This can be reduced by implementing various new forms of bottom culture, a few of which are: bag-and-longline culture, rack-and-bag culture, Standway tube culture and Lantern nets (FAO 2004). Each form possesses their own advantages and disadvantages; however, the principle remains the same (FAO 2004).

Off-bottom culture is the newly modernized way of farming oysters (Lavoie 1996). In this method of culture, oysters are grown in various types of mesh bags that are floated with buoys off the seafloor. Spat gained through either hatchery-rearing or natural populations are placed in these containers to prevent predation (Lavoie 1996; Comeau 2013). There are many modified forms suspended culture, with each having their own advantages and disadvantages (Lavoie 1996). This method of culture is more desirable as it provides many benefits to its use, few of which are: increased survival, biofouling control, high quality shell structure formation and product efficiency

and consistency (Galtsoff 1964; Quayle & Newkirk 1989; Lavoie 1996; Bastien-Daigle et al. 2007).

Despite the chosen method of culture, oysters harvested are marketed in various ways. On the Atlantic coast of Canada, oysters are sold to high end restaurant and distribution companies as whole oysters. In BC, oysters are both processed and sold as value-added product in addition to being sold as whole oysters. (FAO 2004).

1.3.4 Hatchery based culture

Having a consistent supply of oyster is critical to the long-term stability of the industry (Supan 1995). As a result of diminishing wild stocks attributed to overexploitation, environmental degradation and disease proliferation, efforts were directed to oyster enhancements through the development of proper hatchery techniques in the 1950s (FAO 2018)

Broodstock conditioning is a preparatory procedure promoting gamete production out of season in brood prior to spawning. Gamete development can take up to 8 weeks (Castagna et al. 1996). To ensure synchronicity during conditioning, all oysters should be at the same developmental stage prior to its initiation (Helm et al. 2004). Broodstock were traditional chosen based on desirable traits such as fast growth rate, disease resistance, and environmental tolerance among many other traits (Loosanoff and Davis 1963). Hatchery-rearing systems provide ideal environments that can be carefully manipulated and controlled to induce gonad development and gametogenesis out of season (Helm et. al 2004). The key factors that impact hatchery performance are regulating artificial housing temperature, maintaining dietary needs and proper rationing of algae (Helm et. al 2004). The first step in broodstock conditioning is the removal fouling organisms and inorganic matter from the oysters' shell. Subsequently, the brood are placed in tanks containing clean filtered seawater (Castagna et al. 1996). The water temperature is gradually elevated along with food concentrations to encourage the onset of gonadal ripening (Castagna et

al. 1996). To maximize conditioning performance, broods should be exposed to artificial environments mimicking their initially pre-disposed environments (Helm et. al 2004; FAO 2004). Natural factors such as seasonal changes, thermal fluctuations, and algal food source are highly influential on conditioning success (Castagna et al. 1996).

Algae species needs to be consistently produced and supplied to brood as a food source to promote ideal gonadal development in oysters. These algae species, varying in nutritional content, size, and digestibility, are cultured separately in tall, transparent fiberglass tubes known as Kalwalls (FAO 2004). During culture, these single-cellular organisms are supplied with an artificial source of carbon dioxide and light to promote population growth. New algae cultures are created through the inoculation of small quantities of algal cells from a pre-existing established culture. Continuous exponential growth is promoted by increasing the size of holding tubes along with the volume growth media and maintaining the necessary environment needed to aid growth (Helm et al. 2004). *Tetraselmis* (*T. chuii*, *T. tetrahele* and *T. suecica*), *Isochrysis galbana* (*T-ISO*), *Pavlova lutherii* and *Chaetoceros muelleri* are some of the most commonly used marine algal species for oyster conditioning. Usually, a combination of all species at various percentages are used to fully supply broodstocks' nutritional needs during conditioning (Helm et. al 2004). Population density is very important when providing oysters with cultured algae; feeding too much algae can lead to sorting stress (Galtsoff 1964), while underfeeding can impede the growth of high quality gametes (Helm et. al 2004). Ideal feeding quantities can be calculated using dry body weights of a subset from the general population. To commence conditioning, a feeding ratio of 2 - 4% of the average dry body weight should be fed daily (Helm et. al 2004). This ration, without exceeding 6% body weight per day, will promote fast gamete development during conditioning (Helm et. al 2004). Algae production is a limiting factor in hatchery systems as it is very expensive

and time consuming to maintain cultures. Ideally, hatchery-raised oysters are placed in the natural environment as soon as they become spat to limit the reliance on laboratory-reared microalgae (FAO 2004).

Spawning is the process where eggs and sperm are expelled into the water column from the oysters' body cavities and fertilization takes place (Helm et al. 2004). This phenomenon is better known as 'broadcast spawning'. Subsequent to successful conditioning, spawning under hatchery conditions can be achieved through various means: thermal shock and stripping (Helm et al. 2004). Thermal shock spawning is initiated by instantly removing oysters from cold seawater and periodically placing them in warmer seawater up to 30°C (FAO 2004). This temperature fluctuation will trigger the release of gametes into the water column. The release of gametes is characterized by the gaping motion seen in each individual oyster along with white milky stream/cloud being expelled. At this point, the oyster should be removed and placed in its own container with warm seawater to allow full release of its gametes (FAO 2004).

Ripened adults can also be stripped as a method of harvesting gametes; however, this procedure results in sacrificing each oyster (Helm et al. 2004). Firstly, the oyster is shucked to remove the upper portion of the shell. The gonads are located just above the digestive organs upon first glance of the body cavity. Incisions are repeatedly made using a scalpel to allow the expulsion of gametes; warm seawater should be used to wash the gonads into individual containers (Helm et al. 2004). When stripping, puncturing digestive organs should be avoided as this may contaminate the sample thus leading to potentially lower fertilization success (Helm et al. 2004).

Sexing oysters and gonad quality assessment is usually conducted through microscopic observation under x40 to x100 magnification. Healthy eggs are distinguishable by their initial pear shaped appearance while healthy sperm by their motility (Helm et al. 2004). Female Eastern

oysters are a highly fecund species that can produce up to 115 million eggs per spawning (Galtsoff 1930). However, typical productive quantities range from 10 to 20 million eggs per female during spawning season (Galtsoff 1964). Prior to fertilization, excessive debris and tissue is washed away using various fine screen sizes where larger particles are trapped and only eggs similar to the screen size below (20-30 μ m) can be withheld. The procedures mentioned below for stripping female oysters are similarly conducted for males. A small portion of sperm is used to fertilize eggs. To encourage normal larval development, excess sperm should be removed from the fertilized batch of eggs and microscopic monitoring should be periodically conducted (FAO 2004).

Oysters are highly sensitive during the larval stages (Helm et. al 2004). Large aerated tanks are used to house fertilized eggs while they develop into trochophore larvae (FAO 2004). It is necessary to maintain a clean environment during these stages to prevent high mortalities (Helm et. al 2004). A constant supply of algae is needed for larval rearing after they have passed the trochophore (non-feeding) stage. Regular husbandry practices involve grading the larvae based on size to regulate feeding competition among batches and water changes to remove waste and maintain clean water (Helm et. al 2004). Over a few weeks, the larvae will develop until they become pediveligers (Galtsoff 1964; Helm et. al 2004). At this stage, they have the ability to settle onto micro-particles such as micro-cultch or granulated oyster shells (approximately 0.025 mm in size) (FAO 2004). To encourage settlement, pediveligers are placed in downwellers where they are exposed to a gentle 'down-welling' water force along with the provision of algae (Coon et. al 1986). This procedure, also known as 'remote setting' was first documented in Pacific oysters at Oregon State University in 1972 (Lund 1972). Subsequently, various replications of this procedure were successfully accomplished (Budge 1973; Henderson 1983; Gibbons 1988; Bohn 1989).

Following settlement, oyster larvae are moved to upweller rearing systems. Upwellers provide ideal environments for sessile larvae. Oxygenated water concentrated with algae is provided to the larvae in an upwelling motion. Sessile larvae grown in this system tends to grow faster than in downwellers (Loosanoff and Davis 1963). Finally, after 4 weeks, they are transferred to the wild where they are provided with a consistent source of natural foods and are either seeded to bottom leases or grown to market size through suspended culture (Anderson 2000).

1.4 Eastern oyster aquaculture production

1.4.1 Production in Atlantic Canada

Aquaculture production in the Atlantic Canada started out from humble beginnings. In the late 1900s, aquaculture production, comprising of both Fin-fish and shellfish culture, amounted to \$567,841,000 producing 114,204 tonnes of aquaculture product (FAO 2016). Of that total value, oyster production had a value of \$13,278, 000. Oyster production allocated solely to the Maritime Provinces (PEI, NB, and NS) amounted to \$7,678,000 of total said value.

Oyster production values in 2001 were led by PEI, which produced \$ 6.27 million, followed by NS producing \$1.3 million and the least production value of \$772,000 produced by NB (FAO 2016). Despite the increasing productivity by the industry, it was a known fact that production had the potential to increase by 400% without saturating the market (ACOA 2013). However, an overall reduction in the Atlantic oyster industry occurred in the early 2000's when NS was challenged with an OIE reportable disease that had never before been present in Canada (Stephenson et al. 2003). The MSX disease outbreak caused by the pathogen, *H. nelsoni* in the Bras d'Or lake led to severe depletion of residing oyster populations in 2002. Since MSX is an OIE reportable disease, the Bras d'Or lakes remain closed for oyster export (Stephenson et al. 2003). Nova Scotian oyster industry suffered a loss in production value by \$232,000 by the end of 2002 (FAO 2016). PEI production value depreciated to \$546,000 in 2002. Despite this fall, PEI

remained top producer in 2002. An increase in production output was since observed in the NB oyster industry to \$1,173,000 in the said year (FAO 2016).

By 2008, overall production value within the all the Atlantic Provinces continued to depreciate. What used to be a \$16 million industry in 2001 was now valued at \$13,476,000 (FAO 2016). An interesting increase in oyster production value following 2008, was seen in 2010. Despite this decrease, PEI continued to lead with a \$5,654,000 industry followed by NS and NB valuing \$652,000 and \$628,000 respectively (FAO 2016). Approaching 2010, the decreasing trend with industry made an increasing turn. The industry had now appreciated in value to \$18,876,000 during 2010. All individual provinces within the Atlantic region also appreciated in value with PEI still leading in production. New Brunswick had now out-performed NS with an industry valuing \$3,355,000 while NS amounting to \$720,000 (FAO 2016).

The aquaculture industry in Canada produced 39,927 tonnes of shellfish valuing 83 million Canadian dollars in 2014. Oyster aquaculture made up 29 million dollars of that total production value (FAO, 2016). The Eastern oyster accounts for 40% of the overall oyster production in Canada, with the latter 60% attributed Pacific oyster culture coming from in British Columbia (CAIA, 2016). Most recent statistics of oyster production value in Atlantic Canada showed a further increase to \$36,547,000 in 2015. Prince Edward Island continues to lead in production (\$12,828,000), with NB second (\$7,423,000) and NS producing the least (\$1,186,000) (FAO 2016).

1.4.2 Present status of oyster aquaculture in Atlantic Canada

Canadian oyster industry's main producers are BC, PEI, NS, and NB (FAO 2016). However, BC, the main producer of Pacific oysters, has been out-performing all the stated Atlantic Provinces. This is mainly due to slow progression in technological improvements and lack of both

labour and monetary investment in the industry. These short-comings seen within the Atlantic Provinces, when addressed appropriately, may lead to significant advancements in the near and long-term future. Furthermore, to understand the industry-related productive stratification seen within the Atlantic provinces, PEI and NB continue to advance in industry growth while NS shows very little advancements. Monetary earnings within each Atlantic province were \$12.8 million, \$7.4 million and \$1.8 million for PEI, NB, and NS respectively in 2015 (FAO 2016). The oyster industry in NS has faced many challenges which has impacted its potential for grow in respect to PEI and NB. In order to combat these issues, an overall rejuvenation and re-construction of the NS industry's approach and practises is necessary (Bagauov et al. 2016)

One of the most detrimental events in Nova Scotia's oyster industry's history was the outbreak of MSX (McGladdery and Stephenson 2005). This disease caused by a pathogenic protozoan parasite (*H. nelsoni*) decimated the Cape Breton (CB) oyster industry back in 2002. Wiping out almost all the oysters in production within the Bras d'Or lake watershed, this resulted in a stagnant oyster industry up to this very day (Personal communication with Rod Beresford 2018). The CB oyster industry, which was once seen as an industry growth catalyst within NS, complemented with production values among the highest within the province had been terminated due to the potential for disease transfer to other areas and lack of oyster stock (Collaborative Environmental Planning Initiative, 2016). Solutions for disease alleviation in Canada, in respect to the pathogen itself have yet to be discovered (Newell 1985; Burreson and Ford 2004; Savoie – Swan 2012; Ross et al. 2012)

A fearful industry has led to lack of investment from both stakeholders, government and oyster farmers (Ross et al. 2012). Coupled with this, stratified relationships among farmers has led to minimal unified growth within the NS oyster industry (Ross et al. 2013). Stratification in the

form of individualism is evident as farmers continue to compete against each other rather than together to meet market demands (Bagauov et al. 2016). MSX mitigation efforts can be presented in the form of three approaches. The Nova Scotia oyster industry supplies two sizes of oysters: traditional and cocktail (Government of Canada, 2015). A simple industry-shift from the production of traditionally-sized oysters (3-6-year-old) to cocktail oysters (2-4-year-old) could significantly alleviate the mortalities associated with MSX (Ross et. al., 2012). The conversion of supply to a cocktail oyster-based market would allow for the culturing of oysters that are less prone to MSX, as the pathogen only affect adult oysters during the latter part of production (Haskin and Andrews 1988).

Secondly, a more unified approach may drive significant growths within the industry. In the form of production, pooled oyster supply can fill a larger demand. Small farmers could contribute their amounts to this overall demand generating income to fuel their growth. First Nation communities' involvement in this overall effort would allow for more growth as they own many dormant oyster leases. Activating these leases could help in filling the market demands. This in-turn would spark economic enrichment and growth within rural areas (Bagauov et al. 2016). The advancement of technology is the final approach that could aid in the re-build of the industry. Presented in the form of off-bottom techniques (Lavoie 1996), polyploidy (Allen and Downing 1991; Allen et al. 1993; Matthiessen and Davis 1992) and selective breeding (Roch 1999) present a potential for growth within the industry could be exponential. Oysters grown in suspended culture grow significantly faster than oysters grown on bottom and have less biofouling and increased survival (Galtsoff 1964; Quayle & Newkirk 1989; Lavoie 1996; Bastien-Daigle *et al.* 2007); these are all beneficial opportunities for MSX alleviation and further industry growth. Polyploid technology has proven to be a key factor driving growth within the US oyster industry

and has been one of the main methods of MSX alleviation (ABC 2010). Application of this technology to the NS oyster industry would create more options to combat MSX. Breeding technologies in the United States have advanced to the point where MSX- resistance strains have been developed (ABC 2010). These practises could be implemented in NS oyster stocks to breed a strain of oysters that could thrive in MSX-prone waters such as the Bras d'Or lake.

1.5 Polyploidy in marine bivalves

1.5.1 Oyster triploidy in North America

Mass production of triploids in oysters on a commercial scale was made possible by the ground-breaking research conducted by partnering Virginia Institutions; Virginia Seafood Council and Aquaculture Genetics and Breeding Technologies Center, Virginia Institute of Marine Sciences (VIMS) in 2009 (ABC 2010). Prior to this, there have be numerous intensive studies conducted to understand the physiological potential of triploid oysters in North American waters (Stanley et al. 1981; Allen 1987, 1993; Allen and Downing 1990, 1991; Barber and Mann 1991; Mathiessen and Davis 1992, Guo and Allen 1994a). In the United States, the majority of the US oyster industry has now converted to triploids (Hudson and Murray 2015).

The onset of triploidy begins when an animal retains an extra set of chromosomes during meiosis thus resulting in a triploid state (3-N). This is naturally seen in some animals (Longwell and Stiles 1996). In oysters, triploidy is achieved through artificial induction or fertilization of natural diploid and artificial tetraploid gametes (Longwell and Stiles 1996; Guo et al. 2009), by treating diploid gametes with hydrostatic pressure or cold/head shock or chemical exposure during meiosis I or II (Piferrer et al. 2009). Chemical and physical treatment are the most popular means to induced triploidy. However, survivability of the offspring is inversely proportionate to concentrations used (Piferrer et al. 2009). Some of the chemicals used to induce triploidy include:

cytochalasin b, polyethylene glycol, 6- dimethylaminopurine (6-DMAP), caffeine, and colchicine (Piferrer et al. 2009).

Triploid organisms are usually characterized by complete sterility (Allen et al. 1986; Lee 1988; Komaru and Wada 1989), which gives them advantages over their diploid counterparts (Guo et al. 2009). Advantages in growth potential and survivability in oysters is mainly due to the extra energy available for growth that diploid oysters have to use for reproduction. (Bayne and Newell, 1983; Hawkins et al., 1989; Widdows and Hawkins, 1989). Extensive growth advantages are seen after the first year of growth, as triploids continue to grow rapidly in comparison to the diploids (Stanley et al., 1984; Allen and Downing, 1986; Barber and Mann, 1991).

Disease infection in North America was a major restriction for their oyster industry. The co-existence of the industry and diseased waters called for some potential solutions to this problem. Two major pathogenic disease MSX and Dermo caused by protozoan parasites; *Haplosporidium nelsoni* and *Perkinsus marinus* respectively, restricted the potential for growth within the industry (Burreson 1991; Dégremont et al. 2012). MSX disease was well known for its abrupt mass mortalities of the Eastern oysters on the eastern coast of North America (Ford and Haskin 1982). Delaware Bay and Chesapeake Bay were recognized as enzootic areas. Oyster losses amounted to 50% of adults along the Gulf of Mexico and South Atlantic as a result of endoparasite *Perkinsus marinus* (Hofmann et al. 1995).

Triploidy became the solution to this problem. With faster growing oysters, younger marketable oysters were now possible (Matthiessen and Davis, 1992). Reduction in potential loss due to MSX and Dermo disease infection became evident (Anderson 2000). In addition, young oysters aren't susceptible to *H. nelsoni* in its infective state, minimizing the parasite's impact on growth and survival (Farley 1965; Andrews 1979a; Ford and Haskin 1982). Similar resistant

effects were also seen in various publications on juvenile oyster susceptibility to Dermo (Mackin, 1951; Ray, 1954; Andrews and Hewatt, 1957).

The problem of producing marketable oysters during spawning season was an on-going issue within the industry, as spawning was associated with inferior tasting oysters (Ogle 1979). Consistency in better tasting oysters were now possible with the production of triploids due to their reduced gonadogenesis during the warmer months (Ogle 1979). By 2015, Triploid oysters amounted to 90% of all farmed Pacific oysters in Virginia and had now taken over North American oyster industry as the main species of production in comparison to diploids (Hudson and Murray 2015).

1.5.2 Triploidy in Eastern oysters

The concept of general meiotic division in eukaryotes is entirely applicable to the Eastern oyster. The Eastern oyster, like most other multicellular organisms, is a true physiological diploid; however, having 10 pairs of chromosomes is unique to this bivalve species (Longwell 1987a). Chromosome manipulation through various artificially-induced techniques is achievable within hours' post-fertilization (Newkirk 1996). One popular means of chromosome manipulation is polyploidy. Manipulation during zygotic development can be controlled by restricting normal development at a specific temperature and time. If done correctly, the diploid offspring are converted to triploids

Triploids can be created through chemical, thermal and physical shock of fertilized eggs (Longo 1972; Chourrout 1984; Quillet and Panelay 1986). Each method of induction can provide a high percentage of triploid offspring; however, this is only achieved through proper timing of the polar bodies extruded as the fertilized egg develops into larvae. Immaculate induction is highly unlikely when conducting these procedures as there are many factors influencing the outcome

(Stiles et al. 1983; Stanley et al. 1981). The newly spawned eggs are treated at either phase I or II of meiotic division (Stanley et al. 1984). One of the first successful triploid inductions was achieved through chemical induction using Cytochalasin B (Stanley et al. 1981). Characterized by fast growth rates, sterility, high glycogen storage and in some cases diseases resistance, triploid oysters are desirable for both the producer and consumer (Allen 1987; Allen and Downing 1991).

Allen and Downing (1991) have documented improved meat quality in triploid oysters. Better quality products, less mortality and improved growth during the spawning season was also documented in triploid oyster stocks (Allen et al 1993). Increased growth leading to shortened production cycles was also achieved through triploid production (Matthiessen and Davis 1992). There are also indirect benefits to the production of triploids (Anderson 2000). Apart from becoming marketable in a reduced time period, triploids are produced in a hatchery setting hence less exposure to the open water. Less exposure adds to the reduced risks of diseases and predation (Anderson 2000)

1.5.3 Triploidy in other aquaculture species

Natural polyploidy in many species is achieved through infrequent abnormal development during meiosis (Beatty 1957). Natural occurrences usually involve an egg with excess chromosomal content that fuses with a regular haploid sperm resulting in a triploid offspring (Longwell and Stiles 1996). The successful growth and development of such offspring can lead to the creation of tetraploids through artificial means (Longwell and Stiles 1996). Ploidy manipulation in respect to triploids is most frequently induced artificially in marine species. Since the first introduction of this technique by Swarup (1959) in stickleback, modified versions of this practice have been conducted in many other marine species. It has served to solve many issues in respect to product quality (Piferrer et al. 2009). A wide range of successful studies involving

triploid induction have been documented in some species such as the European flat oyster (*Ostrea edulis*)(Grizel 1990), Pacific oyster (Allen and Downing 1986, 1987; Allen et al. 1986), *Ruditapes philippinarum* (Dufy and Diter 1990), *Pecten maximus* (Beaumont 1986); *Mytilus galloprovincialis* (Scarpa et al., 1994), *Pinctada (fucata) martensii* (Jiang et al. 1987; Wada et al. 1989; Durand et al. 1990), *Argopecten irradians* (Tabarini 1984); *Mytilus edulis* (Yamamoto and Sugawara 1988; Desrosiers et al. 1993; Gérard et al. 1994a), *pectinid Placopecten magellanicus* (Desrosiers et al., 1993), *S. glomerata* (Nell et al., 2004), *Mya arenaria* (Allen et al. 1982; Mason et al. 1988), *Haliotis discus hanai* (Arai et al. 1986), *Chlamys varia* (Baron et al. 1989), *Chlamys nobilis* (Komaru et al. 1988) and *Tape semidecussatus* (Beaumont and Contaris 1988; Gosling and Nolan 1989).

The unique benefits of triploidy can be seen with varying species. In triploid bay scallops, an excessive growth almost 50% increase in adductor muscle size was among the advantages observed (Tabarini 1984). This would translate to longer shelf life post-harvest and would allow the bivalve to retain moisture for longer periods (Tabarini 1984). Pacific oysters in Hiroshima were seen to have reduced moisture content and increased glycogen storages in triploids. Less moisture and more energy reserves in the form of glycogen translated to a stronger oyster for farmers and a more enticing oyster to consumers (Akashige and Fushimi, 1992). Among the benefits, many research showed that triploid have reduced gametogenesis (Allen et al. 1986; Allen and Downing 1990; Tabarini 1984, Barber and Mann 1991, Allen and Downing 1986, Allen and Downing 1990, Guo and Allen 1994b). Ultimately, this translates to faster growth in comparison to the regular diploid. An 8.6% increase in shell height was documented in triploid Sydney rock oysters (*Saccostrea commercialis*) when compared to the control diploids. In warmer climates, such as the Gulf of Mexico, long spawning seasons were no longer associated with non-marketable oysters

so oyster sales were now possible all year round (Anderson 2000). In a paper reported by Akashige and Fushimi (1992), general mortality during open water culture was reduced by half in triploid Pacific oysters in comparison to their diploid counterparts.

1.5.4 Polyploidy detection

There are many ways of testing for polyploidy. Two of the most effective methods used in shellfish research are direct chromosome counting (Nell et al. 1996) and flow cytometry (Allen 1983). Direct chromosome counting is fairly efficient; however, it is time-consuming when analyzing large sample groups. Also, challenges may arise when trying to produce clear and concise readable slides for counting (Allen, 1983; Zhou et al., 1999; Ding et al., 2000).

Flow cytometry is most frequently used as it is more accurate and time efficient (Allen 1983). Flow cytometry is the main method for polyploidy detection in this study. Using fluorescent stained cells in liquid suspension, DNA or RNA content within each cell can be quantified by measuring their reflective intensity when passed through the sensing zone of a cytofluorographic analyzer within the machine (Allen 1983). The flow cytometer can relate the intensity to the level of ploidy within each cell/ sample (Chaiton and Allen 1985). Somatic cells of varying tissue types pass through the machine's detector system. Somatic cells in the form of gill, mantle, siphon, or foot samples are usually provided for assay when checking ploidy levels. When larval species are being used, usually the whole larvae at approximately 1000-5000 larvae are used to get clear readings depending on their current size. Larvae as small as 250 μm can be read by the flow cytometer; these sizes correspond to the trochophore larval size in oysters (Downing 1989b). The diameter of this tube is very similar to the cell sizes being read which allow for individually- based analysis (Hawley and Hawley 2004; Jahan – Tigh et al. 2012). Fluorophores such as propidium

iodide (PI) and 4,6-diamidino-2-phenylindole (DAPI) are used for staining nucleic acid within the cells prior flow analysis (Chaiton and Allen 1985; Utting and Child 1994).

Within the sensing zone and upon exposure, the stained cells are transferred to an excited state where they fluoresce and the intensity is measured by a photometer. Precursors are usually set to various numerical figures to correlate this quantified signal (Hawley and Hawley 2004). The machine then records each output and represents it with various peaks representing the level of intensity read. This value representing the emission intensity in triploid cells, should be 1.5 times this emission intensity of a diploid cell (Nell 2002).

Microfluorometry (Komaru et al. 1988; Uchimura et al. 1989; Durand et al. 1990), nuclear sizing (Child and Watkins 1994; Gardner et al. 1996) and image analysis (Ge´rard et al. 1994a) are among the many techniques for analysis ploidy levels; however, they are less frequently used in comparison to the two mentioned above. Microfluorometry follows the same concept as flow cytometry; however, it is slightly modified with the involvement of microscopic imaging of the targeted sample. Image analysis involves the use of electromagnetic radiation or light transmission to analyse stained nucleic acid. Finally, nuclear sizing is a less conclusive means to determine ploidy levels as it can be very subjective. Using this technique, the diameter of the nucleus is measured through a compound microscope and counted using a Coulter counter. The diameter of triploid nuclei is 1.5 times larger than diploids.

1.5.5 Chemical induction

Many studies have shown the use of various chemicals varying in toxicity to inhibit the release of first (I) and second (II) polar body (PB) during meiotic development. The two chemicals mainly used in bivalve triploidy induction are Cytochalasin B (CB) and 6-dimethylaminopurine (6-DMAP). However, other studies have shown successful use of other chemicals such as

polyethylene glycol, caffeine, and colchicine (Piferrer et al. 2009). Cytochalasin B, a fungal metabolite is the most popular chemical used for polyploidy induction in oysters. This is achieved by its ability to inhibit cellular micro-filament construction in cells (Copeland 1974). Studies have shown 100% induced triploids using this chemical (Stanley et al., 1981; Downing and Allen, 1987; Barber et al., 1992). Chemical treatment is very well known to be more successful in inducing triploids (Downing, 1987). These chemicals cause microtubule dysfunction during cytokinesis preventing the cells from extruding its polar body correctly, hence producing an offspring with an extra set of chromosomes. To optimize induction rates, unified cellular development should be prioritized and targeted.

In regular maturation division, polar bodies are formed at two stages, meiosis phase I and II. During these stages of polar body extrusion, the egg expels a portion of its chromosomal content. CB prevents the construction of a cleavage furrow hence retaining the set of chromosomal content that should have been expelled (Longo 1972). Cells exposed to CB while undergoing mitosis show no cytoplasmic cleavage furrow nor micro-filaments which are necessities for effective cytokinesis (Copeland 1974). Dimethyl sulphoxide (DMSO) is usually used alongside CB to optimize survivability of treated larvae. After treatment, DMSO is used to dissolve the residual amounts of CB as it remains hydrophobic in pure water. Using concentrations amounted to 0.01 – 0.1 in filter seawater for approximately 15-20 minutes removes residual CB from the eggs.

Polyploid studies using chemicals for induction have successfully generated triploids in a wide range of marine species, including *Crassostrea virginica* (Stanley et al. 1981, 1984; Matt and Allen, 2014), *Saccostrea glomerata* (Nell et al. 2004), *Crassostrea madrasensis* (Mallia et al. 2006), *Crassostrea gigas* (Allen and Downing, 1986, 1987; Allen et al. 1986; Quillet and Panelay, 1986; Desrosiers et al., 1993; Guo and Allen, 1994; Guo et al. 1996; Hubert et al. 2009), *Ruditapes*

philippinarum (Dufy and Diter 1990), *Pecten maximus* (Beaumont 1986); *Mytilus galloprovincialis* (Scarpa et al., 1994), *Pinctada (fucata) martensii* (Jiang et al. 1987; Wada et al. 1989; Durand et al. 1990), *Argopecten irradians* (Tabarini 1984); *Mytilus edulis* (Yamamoto and Sugawara 1988; Desrosiers et al. 1993; Gérard et al. 1994a), *pectinid Placopecten magellanicus* (Desrosiers et al., 1993), *S. glomerata* (Nell et al., 2004), *Mya arenaria* (Allen et al. 1982; Mason et al. 1988), *Haliotis discus hanai* (Arai et al. 1986), *Chlamys varia* (Baron et al. 1989), *Chlamys nobilis* (Komaru et al. 1988) and *Tape semidecussatus* (Beaumont and Contaris 1988; Gosling and Nolan 1989). Although the methods in these studies were successful in inducing polyploidy, they also caused high mortalities. In chemical induction, it is very well known that toxicity is highly correlated with mortalities and also have detrimental effects of both the operator and species of choice, hence the development of other means to induce polyploids (Guo and Allen 1995).

1.5.6 Physical induction

Physical treatment during cellular development is another means to induce polyploidy (Ding et al. 2007). Treatment using this method is virtually harmless to humans in comparison to the use of chemicals. Polyploid induction using physical treatment is mostly seen in the form hydrostatic pressure and thermal shock (Piferrer et al. 2009). Thermal shock is presented in the form of either heat shock with temperatures ranging from 25 to 38°C or cold shock, 0-5°C (Beaumont and Fairbrother 1991). The efficiency of this method is most dependent on the thermal tolerance of the species rather than the temperature itself (Allen 1987). Research has shown heat combined with chemical treatment as a means to induce polyploidy (Yamamoto et al. 1990). This procedure operates similar to chemical induction; however, the physical effect of pressure and temperature fluctuations disrupt meiotic cytokinesis and brings about change in the cell's density (Piferrer et al. 2009). This ultimately produces the same effect, producing polyploid offspring.

Using hydrostatic pressure, the fertilized eggs along with water is encaged in a pressure resistant chamber. Pressure acts upon the water in the chamber thus creating a hydrostatic environment ultimately applying indirect pressure to the eggs within the water. Similar to the effect of various concentrations used in chemical induction, pressure intensities and duration of treatment affect the level of success in producing polyploids. With increasing concentrations and intensities beyond the optimal regimes, mortality increases. The release of pressure post-treatment allows for egg and zygote development to resume. This form of artificial induction has not been as extensively studied as chemical induction; however, a few studies have documented its use. When comparing both physical treatments, hydrostatic pressure proves to be easier to apply than thermal shock. In fishes and amphibians, physical induction has been documented by Goudie et al. (1995), Lou and Purdom (1984), Tompkins (1978), Chourrout (1984), Onozato (1984), and Cassani and Caton (1986). Shellfish-related polyploidy induction using these techniques have been also documented by Shen et al. (1993); Chaiton and Allen (1985); Allen et al. (1986); Arai et al. (1986); Komaru and Wada (1989) and Ding et al. (2007).

1.5.7 Tetraploidy

Tetraploid induction in oysters created a revolutionary form of culturing oysters in the United States. Initially documented by Guo et al. (1996), this successful publication led to the shifting of priorities from artificial induction to a more sustainable means to create triploids (Allen and Guo 1998; Hershberger & Hostuttler, 2007). By suppressing meiosis phase (I) during egg development, tetraploidy could be induced using hydrostatic pressure or thermal shock. Greater survival using chemical induction is seen when creating tetraploids with the aid of CB (Allen and Guo 1998). Tetraploids can also be created through artificial induction using partially fertile triploids in Pacific oysters (Longwell and Stiles 1996). Finally, natural tetraploid offspring can be produced by mating two tetraploids to create 2nd generation tetraploids (Guo et al. 1996)

Utilizing tetraploid male stocks to produce triploids by mating with diploid females proves to be more efficient, as a 100% induction from diploid to triploids have been consistently produced. Other benefits are seen where selection of economic traits for both parents can be used to produce even more superior triploids for culture (Hershberger & Hostuttler, 2007).

Even though this technology is relatively new, documentation of its use in research and industry has been numerous due to its known potential. Numerous attempts to create tetraploids have occurred. In oyster-related induction, attempts were made by Guo et al. (1994) and Nell et al. (1998); however, successful artificial induction was achieved by Guo and Allen (1994b), Supan (2000) and He et al. (2000) in *C. gigas*, *C. virginica* and *Pinctada martensi* (Dunker, 1850) respectively. Protocols for artificial chemical induction for tetraploid production were refined showing a clear procedure for induction (Eudeline et al. 2000a, b). Successful use of this technology has been seen in the commercial production of triploid rainbow trout and Pacific oysters via tetraploid stocks (Chourrout et al., 1986; Guo & Allen, 1994; Guo, et al. 1996; Hershberger & Hostuttler, 2007).

1.6 Project Rationale

For over ten years, there have been no major improvements to the oyster industry in Cape Breton. *Haplosporidium nelsoni* still actively causes mortalities in oysters within the Bras d'Or lake. Using the knowledge and experience from various research facilities and research groups within United States on their polyploidy protocols (Allen and Downing 1986; Haskin and Ford 1979; Guo et al. 1994; ABC 2010) and implementing them in our native Eastern oyster stocks will allow for the re-building of the Bras d'Or lakes oyster industry. Understanding how triploidy in our oysters perform in MSX-prone waters will show their potential in alleviating this on-going issue. This can present itself in the form of increased growth rates thus reducing the time taken to

produce marketable oysters. A shortened production period also translates to later quantities of oysters being produced within the said period. This form of technology also has the potential to rejuvenate and even expand on the present oyster industry throughout the Bras d'Or lake watershed and across the Maritime Provinces.

1.7 Objectives

- Through implementing hydrostatic pressure treatment on the Eastern oyster, optimization will be assessed. Pressure intensity and pressure duration, influencing optimization will be tested at three targeted levels each. Pressure intensity at 6000, 7000, and 7500 psi; treatment durations of 3, 5, and 10 minutes and fixed PB (I) – 60% treatment initiation timing will be used to strategically assess induction potential in the Eastern oyster using this method.

- Following the induction assessment, settled diploid and triploid spat will be deployed using the optimal treatment in Bras d'Or lake watershed which is currently prone to MSX. Both replicated groups of oysters will be compared in respect to their growth potential after one year of growth.

CHAPTER 2: INVESTIGATING THE EFFECTS OF PRESSURE INTENSITIES AND PRESSURE DURATIONS ON TRIPLOID INDUCTION RATES OF THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*)

2.1 Introduction

While the Eastern oyster naturally exists in a diploid state, the production of triploid oysters has become an important tool in oyster aquaculture to increase growth rates, reduce exposure to disease and have a high quality product available 12 months of the year (Stanley et al. 1984; Allen and Downing 1986; Allen 1987; Allen and Downing 1991; Mathiessen and Davis 1992). Triploidy in oysters can be achieved by inhibiting the expulsion of the first (I) or second (II) polar body (PB) during meiotic development. This results in the retention of an extra set of chromosomes creating some triploid larvae (3-N). In oysters, triploids are either artificially-induced or produced through natural diploid- artificial tetraploid crosses (Longwell and Stiles 1996; Guo et al. 1994; Guo et al. 2009). Means to artificially-induce triploids are chemical, thermal and physical shock of fertilized eggs (Longo 1972; Chourrout 1984; Quillet and Panelay 1986). Chemicals inhibit proper functioning of microtubule fibers during cytokinesis which prevent the formation of a cleavage furrow. Cytochalasin B (CB) is the most popular chemical means to artificially induce triploidy in oysters. CB is a cell-permeable mycotoxin that when used at the right concentrations can effectively inhibit polar body extrusion. Various studies on Eastern oysters have shown 100% induction rate in oysters using CB (Stanley et al. 1981; Downing and Allen 1987; Barber et al. 1992). However, immaculate induction using artificial means is not common (Stiles et al. 1983; Stanley et al. 1981). Various other chemicals have been shown to successfully induce triploids such as: 6-dimethylaminopurine (6-DMAP), polyethylene glycol, caffeine, and colchicine to induce polyploidy in marine species (Piferrer et al. 2009).

Triploid induction using physical treatment is mostly conducted using hydrostatic pressure and thermal shock (Piferrer et al. 2009). Even though this technique has proven to have lower induction rates, it is more desired as it is virtually harmless to human beings in comparison to the use of chemicals. Physical pressure at various intensities prevents the physical expulsion of PB instead of disrupting cellular activity through chemical influences (Piferrer et al. 2009). Shellfish-related polyploidy induction using hydrostatic pressure has occurred in a few species including the pearl oyster (*Pinctada martensii*) (Shen et al. 1993), Pacific oyster (*Crassostrea gigas*) (Chaiton and Allen 1985), soft-shell clam (*Mya arenaria*) (Allen et al. 1986), Pacific Abalone (*Haliotis discus Hanni*) (Arai et al. 1986), noble scallop (*Chlamys nobilis*) (Komaru and Wada 1989) and sea cucumber (*Apostichopus japonicus*) (Ding et al. 2007). Hydrostatic pressurization has been used mostly on other marine species to induce triploidy: African clawed frog (*Xenopus laevis*) (Tompkins 1978), rainbow trout (*Oncorhynchus mykiss*) (Chourrout 1984), rainbow trout, cherry salmon (*Oncorhynchus masou*), and chum salmon (*Oncorhynchus keta*) (Onozato 1984), grass carp (*Ctenopharyngodon idella*) (Cassani and Caton 1986).

There is industry demand for triploid Eastern oysters due to their desirable traits including disease tolerance, faster growth, reduced mortalities and shortened production cycles (Farley 1965; Allen 1987; Ogle 1979; Andrews 1979a; Ford and Haskin 1982; Allen and Downing 1991; Matthiessen and Davis 1992; Allen et al. 1993). These are all advantageous traits to build up in a seed source to strengthen the Canadian oyster industry. Dégremont et al. (2012) showed this technology being used coupled with selective breeding to produce genetically superior oysters that have the ability to perform better in both disease-infested and disease-free waters. Since protocols to induce triploidy in the Eastern oyster using hydrostatic pressure have not yet been developed, this study examines the impact that two major factors may have on induction rates with this

species; pressure intensity (PI) and pressure duration (PD). Pressure intensity, in respect to artificial induction in oysters, is the quantified physical force that is indirectly inflicted upon fertilized eggs suspended in seawater that are enclosed in a pressure resistant chamber. Pressure duration is the length of time that fertilized oyster eggs are exposed to targeted pressure intensities.

When hydrostatic methods are used to induce triploidy, fertilized eggs suspended in water are placed in a pressure chamber. With the release of air within the chamber, a hydrostatic environment is created. Regulated pressures of various intensities and durations are used to treat the fertilized eggs. The release of pressure allows the treated eggs to resume cellular development. Timing of treatment is essential for optimal results using this method. To optimize this procedure, 60% PB (I) initiation timing should be used (personal communication with Dr. Allen 2016). A pressure intensity of 6000 psi in our pilot studies produced 30% triploids when duration was 3 minutes. Pressure intensities of 7200 psi and 6000 – 8000 psi have produced 60%, 57% triploids on *Crassostrea gigas* and 76% on *Pinctada martensii* in previous pioneering studies conducted by Allen et al. (1986), Chaiton and Allen (1985) and Shen et al. (1993) respectively. These three papers used 10 minutes duration to achieve these induction rates; however, our pilot study used 3 minutes and achieved 30%. Also in our pilot studies, pressure intensities of 8000 psi and above ended in 100% mortality. After observing all these parameters, pressure intensities of 6000, 7000, and 7500 psi and durations of 3, 5, and 10 minutes were the best points to test triploid optimization in the Eastern oyster using this method as they have proven to be successful in similar bivalve species.

2.2 Objective & Hypothesis

The objectives of this study were to measure the impact of different pressure intensity and pressure duration treatments on optimizing triploid induction rates in the Eastern oyster. Three levels of pressure intensity were tested (6000, 7000, and 7500 psi) along with three treatment durations (3, 5, and 10 minutes). Treatments will be applied at a fixed PB(I) percentage of 60% for treatment initiation timing. The hypothesis for this experiment is that triploid optimization will be achieved using one of the targeted pressure treatment combination.

2.3 Materials and Methods

2.3.1 Broodstock conditioning

A total of 60 Eastern oysters ranging from 4 to 7 years old were randomly selected from Big Island oyster farm (Robinson Cove, Nova Scotia GPS: N45°39'31.382''; W62°24'42.538'') for conditioning. These oysters were transported April 27th, 2017 on ice to the Dalhousie University Aquatron facility in Halifax, NS in approximately 2.5 hours. To initiate conditioning, the oysters were placed in rectangular 50 L fiberglass holding tanks with artificially mixed seawater (SW) similar in temperature and salinity to conditions in Robinson Cove (Temp: 11°C; Salinity: 22ppt).

Broodstock conditioning lasted for 6 weeks, during which oysters were fed a consistent mixture of four microalgae species: *Tetraselmis sp.*, *Isochrysis galbana* (T-ISO), *Pavlova lutherii*, and *Chaetoceros mulleri*. All microalgae were live reared on site at the Aquatron Facility. A concentration of 100,000 cells of microalgae per liter of seawater was fed twice per day; this concentration was conducive to the stock's feeding rate and biomass requirements (Helm et al. 2014). The water temperature was elevated by 1°C/day until 18°C was reached. At 18°C, the oysters were held for an additional 2 weeks to allow for optimal gametogenesis. Fresh seawater

at 18 °C was supplied every two days to maintain a clean environment. Once the oysters reached optimal gonad condition, the water temperature was lowered to 14°C to prevent spontaneous spawning prior to initiation of the multifactorial experiments.

2.3.2 Spawning & Fertilization

The physical characteristics of all broodstock were recorded, including shell height, width, and wet weights. All broodstock were also photographed prior to spawning. Manual strip-spawning was used to separately harvest all the eggs and sperm. Strip-spawning was used for this experiment instead of applying a heat shock to induce the release of gametes as it prevents mixture of gametes and unwanted fertilization which are important factors to optimize triploid induction. (Allen et al. 1989). Ten Eastern oysters with mature gonads were selected for each block of the experiment. Each oyster was shucked and sexed and their gametes visually assessed under a microscope (Olympus CH30 compound microscope, Model: CH30RF100). A scalpel was used to remove the gametes residing above and around the digestive organs with periodic washes between incisions. Fresh SW at a constant temperature of 25°C, was used to wash the gametes into their respective labelled beakers (Downing and Allen 1987).

Harvested eggs were visually graded using a numeric scale from 1- 3 based on quality and quantity; with 1 being very poorly shaped and low fecundity and 3 being perfectly shaped and high fecundity. The best quality eggs were pooled, counted and divided into 9 treatments and 2 control groups with each containing approximately 500,000 eggs. These treatment and control groups were replicated 3 times over a three-week period. Despite choosing the best eggs among the group, egg quality appeared to be sub-optimal. This may have been due to the sudden change in environment. Similarly, individual sperm samples from each male were tested for quality using a scale of 1 to 5 with 1 being completely inactive and 5 being very active. The best sperm samples

were pooled after separating excess tissue using a 20- μ m nitex screen. Low quality sperm samples were discarded. Both sperm and eggs were of average quality prior to fertilization and use in induction trials.

Prior to fertilization, the harvested gametes were held in 25°C and 24 ppt SW in separate containers in different areas based on sex to prevent premature fertilization (Downing and Allen 1987). This incubation period allowed for increased synchronicity during larval development post-fertilization. All 9 treatment groups were fertilized at 15 minutes' intervals to track the PB (I) extrusions. A double hand tally counter (Light of Night™), Sedgewick raft counter (Pyser-SGIS50™) and microscope (Olympus CH30 compound microscope™, Model: CH30RF100) were used to track percentage PB (I) extrusion to 60%. This was seen when 60% of the fertilized egg samples showed PB (I) formation. Assistance by other members within the lab was needed at this point to allow for accurate tracking of PB (I) while fertilizing eggs every 15-minutes for all 9 treatments. This procedure involved three individuals; one fertilizing a new treatment every 15 minutes, another tracking PB development in all fertilized treatments, and the last person initiating pressure treatment under one minute after approximately 60% PB(I) was seen within samples (Personal Communication with Dr. Allen, 2016). Using the double hand tally counter, eggs that hadn't reached PB (I) were recorded with one click on the first hand and a corresponding click was recorded on the other representing eggs that completed PB(I) formation. Periodic observations of all egg samples were conducted similarly, tallied individually and a percentage of PB (I) completion within the sample was calculated. Each treatment was defined according to their interactions between pressure intensity and pressure duration. All the respective control diploid groups remained untreated and were placed in their individual tanks after PB(I) was observed.

2.3.3 Hydrostatic Pressure Treatment

The hydrostatic apparatus used was constructed using a 12-ton Powerfist[®] hydraulic press, 700 ml stainless-steel cylindrical pressure chamber and glycerine filled pressure gauge with a maximum pressure tolerance of 10,000 psi. In preparation to receive fertilized eggs from each treatment, the pressure chamber was disinfected using Virkon[™] and partially filled with 25°C SW. The fertilized eggs were then placed in the partially filled pressure chamber and additional SW was added filling the chamber 75% full. The chamber was then closed using a piston-like lid and a valve located on the lid was opened to release excess air from within chamber. A hydrostatic environment was created after all excess air was removed and the valve closed. By pressing a lever on the hydraulic press, the targeted pressure intensity was passed into the stainless steel cylinder and acted on the fertilized eggs. All pressure treatment intensities were reached within one minute of pressure initiation.

After the treatments were administered, the fertilized eggs from each group were placed in separate cylindrical 90 Litre TAMCO[™] polyethylene tanks containing fresh SW at 25 °C and 22 ppt for further development and ploidy testing (see figure 1). Control groups remained untreated and were placed directly into their respective tanks after first polar body extrusion was observed. This was sequentially conducted batch by batch to ensure accuracy when initiating each treatment. The experiment was replicated using three temporal blocks, treatments were randomly chosen to minimize bias (Table 1). After 24 hours, the larvae were drained and a sub-sample packaged and sent to VIMS for ploidy testing using flow cytometry.



Figure. 1. Larval tanks used to rear oyster larvae for 24 hours' post-pressure treatment. Nine tanks were used, each for their treatment regime along with two diploid tanks as control groups. Samples were shipped within 24 hours to VIMS for 48 hr flow cytometry

2.3.4 Test variables

The experimental approach was designed to detect the effects of pressure intensity (PI) and pressure duration (PD) at various levels on triploid induction rates. Pressure intensity levels were: 6000, 7000 and 7500 psi. These intensities were strategically chosen based on results from our pilot study along with literature-based results. Our findings found that using 6000 PSI for 3 minutes on fertilized eggs of the Eastern oyster produced 30% triploids at 60% PB(I) initiation timing (personal communication with Dr. Allen 2016). In a paper by Allen et al. (1986), hydrostatic pressure was used to induced triploidy on a very similar species to the Eastern oyster; the Pacific oyster (*Crassostrea gigas*). With a treatment regime of 7200 psi for 10 minutes, 60% triploids were induced 10 minutes' post-fertilization. During this experiment, temperature remain at a constant 29°C. In addition, Chaiton and Allen (1985), showed a maximum proportion of 57% triploids induced using 6000 – 8000 psi in the Pacific oyster. Both studies used 50% PB (I) timing to initiate pressure treatments. Using this knowledge, we targeted the unknown optimal pressure intensities and pressure durations for the Eastern oyster to optimize induction rates using the information attained from other similar studies.

Similar to pressure intensity, the pressure duration was also strategically chosen based on previous experiments on other species (Allen et al. 1980; Shen et al. 1993) and from our own pilot study results on the Eastern oyster. From our pilot study we used 3 minutes along with 6000 psi which resulted in 30% triploids induced. However, in similar research a standardized pressure duration of 10 minutes produced 57% and 60% triploids in the Pacific oyster (Chaiton and Allen 1985; Allen et al. 1980) respectively and 76% in Pearl oyster (*Pinctada martensii*) (Shen et al. 1993). After observing all these parameters, 3, 5, and 10 minutes were chosen as the best points to test triploid optimization using this method as they have proven to be successful in similar bivalve species. Also, an elevated pattern in induction efficiency is seen as time increases to 10 minutes (Shen et al. 1993). Our pilot studies also showed 100% mortality when eggs were treated for longer than 10 minutes and with pressures at 8000 psi and above.

2.3.5 Fixed variables

Fixed variables in this experiment were parameters that remained at one level while other test variables changed based on the specified treatment regimes targeted. Various fixed variables included water temperature, water salinity, egg incubation temperature, timing of polar body extrusion, and gamete quality (Allen et al. 1989; Huergo and Zaniboni-Filho 2006). These variables were controlled and closely monitored to minimize their overall effect on the experimental outcome. An artificial mixture of SW was constantly provided by the Dalhousie University Aquatron facility. This SW mixture was regulated to 22-24 ppt which was consistent with the broodstock's natural environment. However, temperatures were changed for various parts of the experiments and fluctuated based on external influences such as time of day. Even though salinity remained constant, during conditioning, water temperatures were elevated by 1°C/ day until 18°C was reached. Harvested gametes were incubated pre- and post- fertilization at 25°C to

allow for synchronicity in meiotic development (Downing and Allen 1987). This temperature was maintained throughout all the experiments.

All treatments used were targeted to be initiated when 60% PB (I) was seen within the sample. Even though in the literature it states that hydrostatic pressure treatments should be initiated when 50% PB (I) is seen (Allen et al. 1989), technical advice of targeting 60% PB (I) was given to improve accuracy in PB timing (personal communication Dr. Allen 2016). Microscopic observation was used to assess the quality of sperm and eggs prior to use in experiments. Averaged quality gametes were kept and incubated for 10 minutes prior to experiments while obvious bad quality ones were discarded.

2.3.6 Triploid detection

Three blocks of triploidy experiments were conducted over a period of 3 weeks with each block of the experiment lasting 1 week. At approximately 24 hours' post-fertilization, larvae from each treatment (9 treatments in total) were collected individually using a 20 µm nytex meshed screen. A sub-sample of 1000-5000 larvae was packaged with ice packs in a Styrofoam container and shipped overnight for flow cytometric analyses. All treatment groups along with their controls were sent to ABC Flow Cytometry Laboratory in Aquaculture Genetics and Breeding Technology Centre at Virginia Institute of Marine Science (VIMS) for flow cytometric polyploid testing. The flow cytometry techniques used were conducive to those described by Downing and Allen (1987). Samples were run on a Sysmex-Partec Cy Flow Space flow cytometer. Flow cytometry has been frequently described in both shellfish and finfish research as an efficient means to detect polyploidy (Allen 1983; Stanley et al. 1984; Thorgaard et al. 1982; Utter et al. 1983). Using fluorescing stained cells, flow cytometry measures the amount of DNA content within each cell by their fluorescing intensity. The intensity is then quantified and categorized based on individual

cell's ploidy level (Chaiton and Allen 1985). These cells travel in a straight stream through the machine's detector system so they can be individually analysed (Hawley and Hawley 2004; Jahan – Tigh et al. 2012). Fluorophores used for staining nucleic acid in such analysis are Propidium Iodide (PI) (Utting and Child 1994) and 4, 6-diamidino-2-phenylindole (DAPI) (Chaiton and Allen 1985). DAPI was used as the staining medium for this experiment. Numerical precursors of 50, 100, 150, and 200 were set to represent haploid, diploid, triploid and tetraploid cell peaks respectively. These precursors were used to identify which peaks were being detected within each sample based on the level of ploidy present.

2.3.7 Mini-experiment on survivability of diploids only

To assess diploid larvae survivability after 24-hours of rearing, four tanks labelled a, b, c, and d were used. These cylindrical 90 Litre TAMCO™ polyethylene tank were also used for triploidy experiments (see figure 1). An initial population of 500,000 larvae were stocked in each tank which was conducive to the stocking density used in each tank for each block of the triploidy experiment. A small portion of broodstock (3 females and 1 male) were used for the survivability testing of the untreated diploids. Prior to strip-spawning and fertilization, water quality assessment within each tank was conducted to ensure all water parameters were in optimal ranges for Eastern oyster larval rearing (Helm et al. 2004). These parameters were similar to those of the triploidy induction experiments. The same fertilization, rearing and harvesting techniques used in triploidy experiments were conducted. The surviving population for each tank at 24-hr post fertilization was recorded.

2.3.8 Statistical design

To optimize triploidy induction using the hydrostatic pressure method, a 3 x 3 multifactorial statistical design was used (Table 1). The two main factors of interest in this experimental approach were PI and PD. Nine treatment combinations of PI and PD of 6000, 7000

and 7500 psi and 3, 5, and 10 minutes respectively were constructed. Treatment timing of 60% PB(I) extrusion remained fixed among all treatments. This allowed for the testing the two main factors' effects, their interactions and their influences on two responses: induction rate (%) and survival rate (%). Factorial designs are efficient in studying the effects of multiple factors as it takes into account the individual effects of each factor and all possible factor combinations at every level contributing to a certain response (Montgomery 2013). There are two types of effects in factorial experiments, the main and interaction effects (Montgomery 2013). The main effects are responses influenced by a change in level of the factor however, the interaction effects are a response between the levels of one factor that are not the same at all levels of other factors (Montgomery 2013). All nine treatment combinations were randomly tested in three blocks each lasting for 1 week. Blocking was used to facilitate replications as the induction process is time sensitive.

In factorial designs, the total variability could be due to main effects, interaction effects and uncontrollable variables (Montgomery 2013). Since our experiment is a 2-factor factorial design, the model used was: $Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$; Where, $i = 1, 2, \dots, a$; $j = 1, 2, \dots, b$; $l = 1, 2, \dots, n$, Y_{ijk} = response variable, μ = overall mean, α_i is the main effect of PI, β_j is the main effect of PD and ε_{ijk} is the error term. The interaction effects include: $\alpha\beta_{ij}$ - interaction between pressure intensity and pressure duration (Table 1). Each treatment was defined in accordance to the assigned pressure intensity, pressure duration and the interaction between them (Table 1).

Table 1. Multifactorial experimental design showing the randomized order of controls (C) and treatments (Trt) within their respective blocks. Each treatment is assigned an identity tag to simply distinguish among type of interaction between Pressure Intensity (PI-psi) and Pressure duration (PD-mins). The targeted timing of Polar body extrusion (PB) remained fixed at 60% among all treatments used.

Trt: PI vs PD	Block 1 (Week 1)	Block 2 (Week 2)	Block 3 (Week 3)
Trt 1: 6000 vs 3	Trt 1	Trt 3	Trt 3
Trt 2: 6000 vs 5	Trt 7	Trt 2	Trt7
Trt 3: 6000 vs 10	Trt 9	Trt 4	Trt 8
Trt 4: 7000 vs 3	Trt 8	Trt 5	Trt 9
Trt 5: 7000 vs 5	Trt 6	Trt 7	Trt 6
Trt 6: 7000 vs 10	Trt 4	Trt 6	Trt 1
Trt 7: 7500 vs 3	Trt 3	Trt 9	Trt 2
Trt 8: 7500 vs 5	Trt 5	Trt 1	Trt 5
Trt 9: 7500 vs 10	Trt 2	Trt 8	Trt 4
C1 : Diploids	C2	C1	C2
C2 : Diploids	C1	C2	C1

2.3.9 Statistical analysis

The results were statistically analyzed using Minitab 17™ using Analysis of Variance (ANOVA) and Tukey’s pairwise comparison to test the significance of the differences among the mean triploid induction rates produced by both the individual treatment effect and treatment interaction effects. The statistical model used to govern this experimental approach was: $Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$, Where $i = 1, 2, \dots, a$; $j = 1, 2, \dots, b$. In this experiment, Y_{ijk} – mean triploid induction rate (%), μ - overall mean, α_i - main effect of pressure intensity, β_j - main effect of pressure duration, $\alpha\beta_{ij}$ – interaction effect of pressure intensity and pressure duration and ε_{ijk} – random error associated with the experiment. Several hypotheses were used to assess the result produced from the ANOVA and Tukey’s pairwise comparison (Table 2). All the necessary assumptions for normality on residuals, homogeneity of variance and independence were verified prior to running the ANOVA tests.

To ensure normal distribution, the normal probability plot (NNP) of residuals was executed (Montgomery 2013). Normal distribution was achieved when the residual plots fall along a standardized line and passes the pen test where a standard size pen covers all plots on the NNP (Montgomery 2013). In addition, the p-value produced from the normality test can be used; if it is equal or below 0.1 then normal distribution on the error terms is confirmed (Montgomery 2013). Homogeneity of variance was confirmed by plotting the residual versus fits graph and visually assessing the distribution of the plots (Montgomery 2013). Confirmation of homogeneity of variance occurs when the plots show a horizontal band across the graph and appeared to be randomly distributed, however if the data are pattern oriented, violation is confirmed (Montgomery 2013). The assumption of independence among samples is verified through randomization of treatments, blocks and treatment combinations (Montgomery 2013).

Table 2. Summary of hypotheses tested from the ANOVA. These hypotheses were inclusive of both the main effects and interaction effects of PI and PD on the mean triploid induction rates produced.

Null Hypotheses (H_0)	Alternative Hypotheses (H_a)
<u>Main Effects</u>	
$H_0 = \alpha_1 = \alpha_2 = 0$	$H_a =$ At least one of the $\alpha \neq 0$
$H_0 = \beta_1 = \beta_2 = 0$	$H_a =$ At least one of the $\beta \neq 0$
<u>Two-way Interaction Effect</u>	
$H_0 = (\alpha\beta)_{ij} = 0$	$H_a =$ At least one of the $(\alpha\beta)_{ij} \neq 0$

Note. Significance level: $P = 0.05$

When testing these hypotheses, each main and interaction effect with their related mean square is divided by the mean square error to calculate the F values (Montgomery 2013). The F value is then used to find the p-values which is determining factor for accepting or rejecting the null hypotheses. The sum of square, degrees of freedom, mean squares, expected mean squares, F and P values are all presented in the ANOVA table.

Multiple means comparison (MMC) was used to make further conclusions on the results of the ANOVA test in the case of the null hypothesis being rejected. Rejection of the null hypothesis means that a significant difference was seen among variables of interest. The method of MMC used would be based on its appropriation to type of experiment being conducted and the experimental error allowance (Montgomery 2013). In this experiment, the MMC method used was the Tukey's pairwise comparison when significant differences were seen ($p < 0.05$). This method is known to be conservative accommodating a low error rate however it is powerful in distinguishing differences in means. The experiment was conducted in a controlled hatchery-based laboratory setting. Depending on the nature of the results, other applicable MMC methods may be used which may be more effective in distinguishing differences among means.

2.4 Results

2.4.1 Percent survival

The highest mean survival of specific treatment rates over the three blocks of experiment were: 1 and 5 which produced mean percentages of 5.1 and 3.1 respectively (see figure 2). No treatment was statistically better than the other in respect to survival rate ($p > 0.05$).

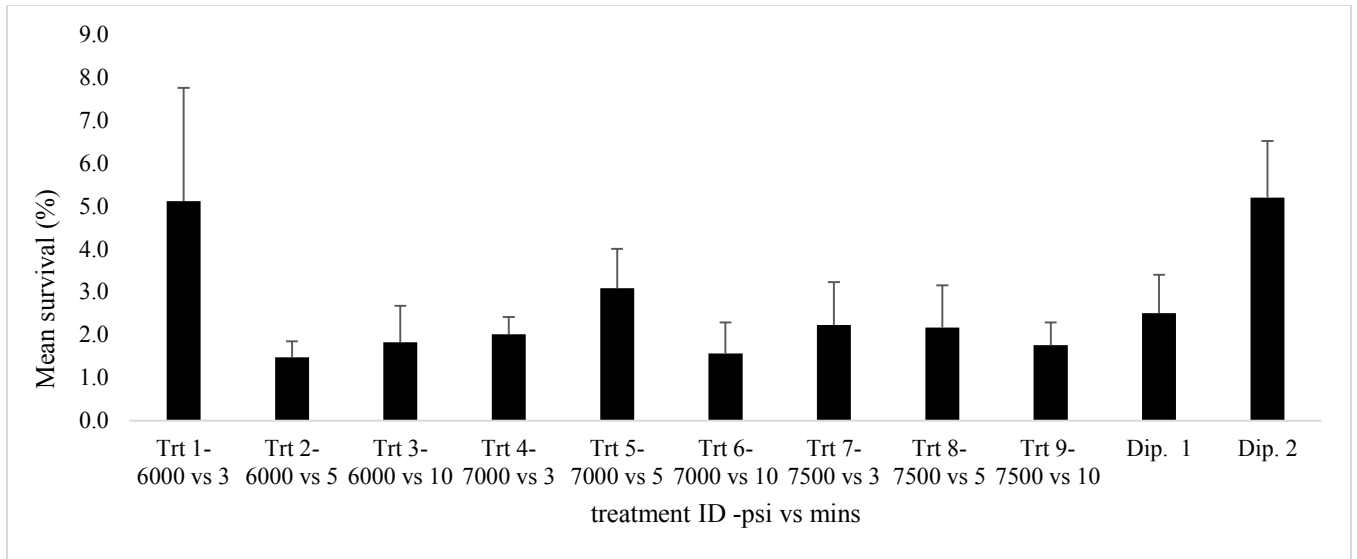


Figure. 2. Comparison of mean (+SE) survival (%) of both treated and untreated groups for 3-blocks of triploid induction experiments ($p>0.05$). Nine (9) pressure treatments were used in each block, 1000-5000 individual 48-hr larvae were analyzed by flow cytometry weekly (per block) for 3 weeks.

2.4.2 Mini-experiment on survivability of diploids

Survival in diploid larvae at 24-hrs among all 4 untreated tanks ranged from 18.4 – 24.5%.

Water quality assessment showed parameters: water temperature: 21°C, salinity: 24 ppt, Nitrate:

1.6 mg/L, Nitrite: 0 mg/L, Ammonia: 0 mg/L, and pH: 7.89.

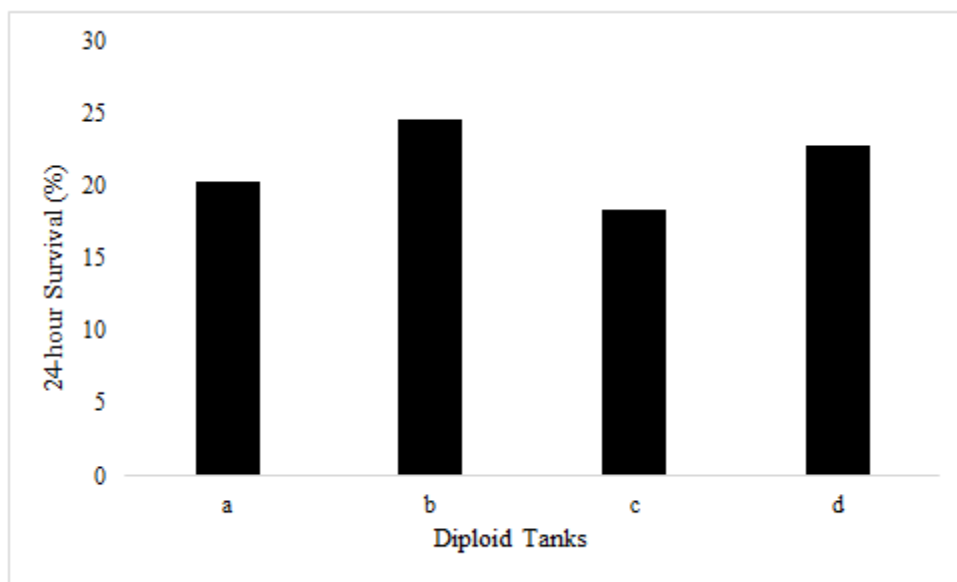


Figure. 3. Survivability after 24 hour among untreated diploids reared in the same conditions and using gametes from the same broodstock used in triploidy experiments. Four untreated diploid tanks (a, b, c and d) with initial populations of 500,000 larvae each were used to assess survivability of diploids in comparison to treated triploid and diploid control for the triploidy experiments.

2.4.3 Percent triploidy

Treatments 1-7 produced various percentages of triploids however, 8-9 yielded no triploid larvae. The highest triploid percentages induced over the three blocks of experiment were: 25, 23 and 25 % seen in treatment 3, 4, and 5 respectively (see figure 4). No treatment was statistically better than the other in respect to triploid induction rates ($p > 0.05$).

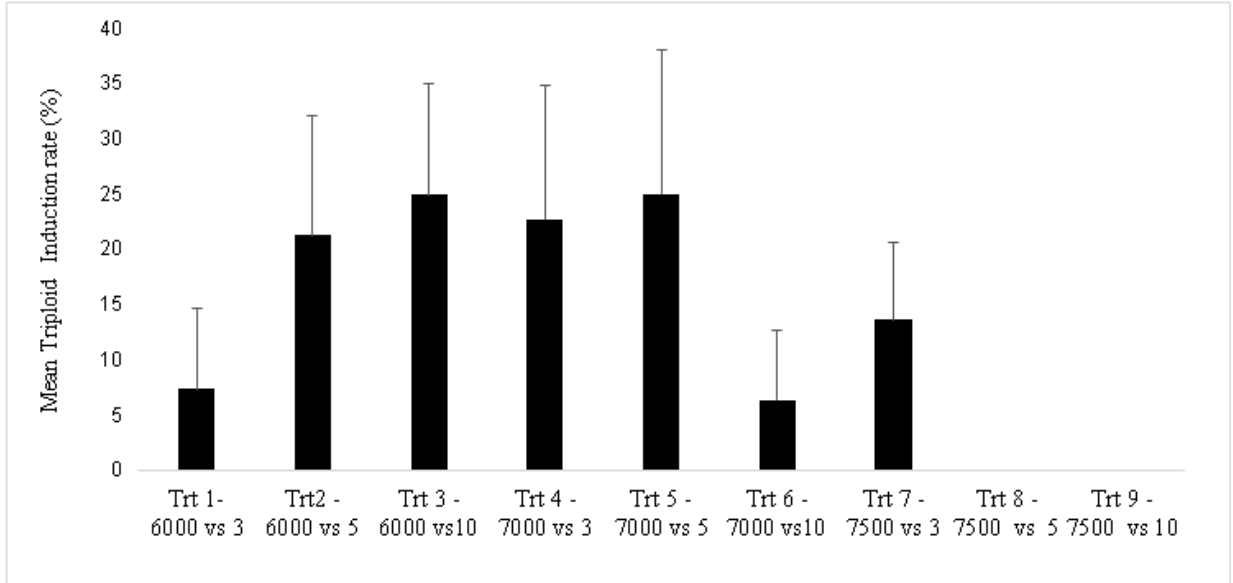


Figure. 4 Comparison of mean (+SE) triploid proportions induced (%) for 3-blocks of triploid induction experiments ($p > 0.05$). Nine (9) pressure treatments were used in each block, 1000-5000 individual 48-hr larvae were analyzed by flow cytometry weekly (per block) for 3 weeks.

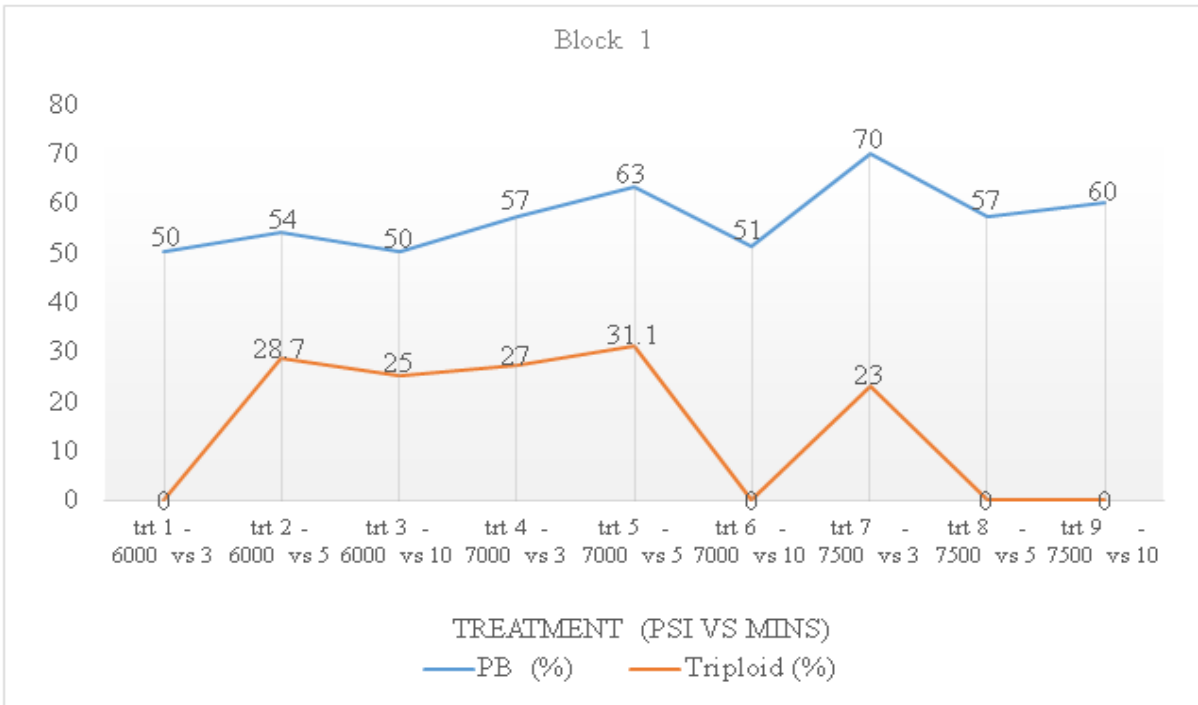


Figure 5. Block 1 of 3 showing percentage of triploid larvae induced at various stages of polar body development (%) within each treatment (trt – psi vs mins) used. A standardized population of approximately 500,000 eggs were fertilized per treatment each week. This study was replicated for three weeks; percent triploidy was determined using flow cytometry.

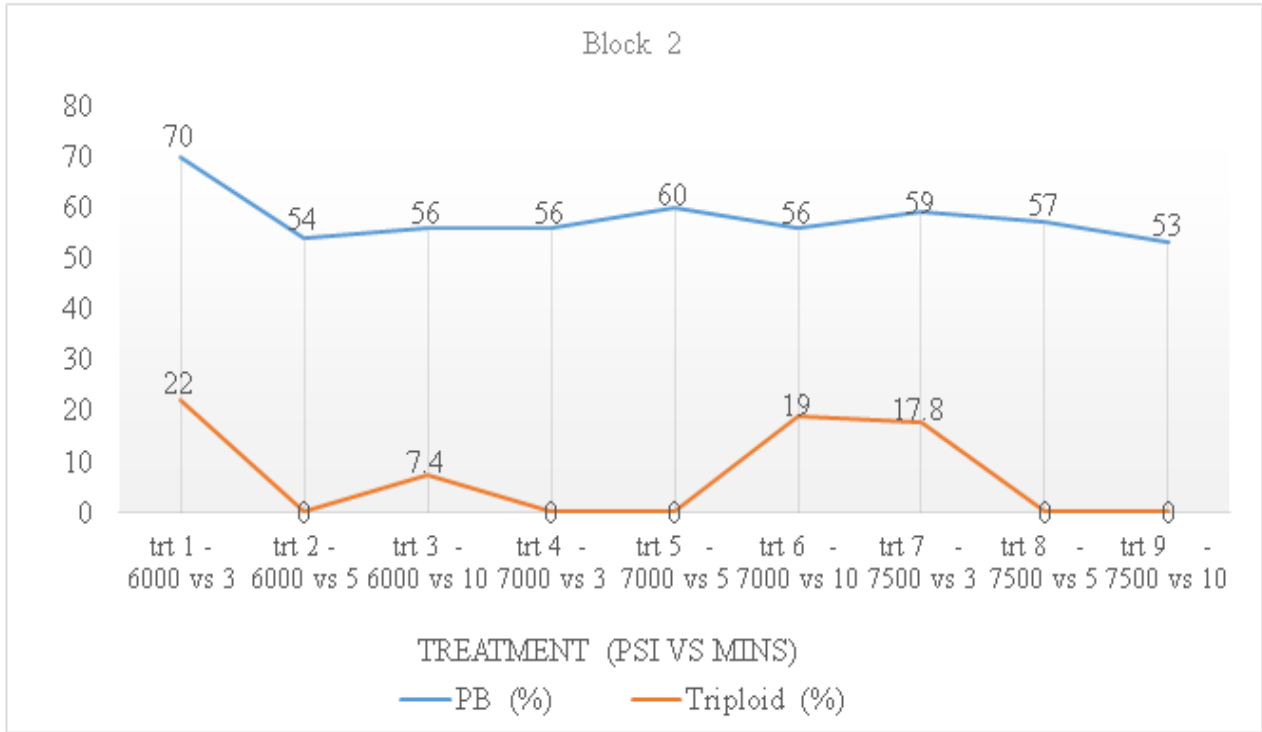


Figure 6. Block 2 of 3 showing percentage of triploid larvae induced at various stages of polar body development (%) within each treatment (trt – psi vs mins) used. A standardized population of approximately 500,000 eggs were fertilized per treatment each week. This study was replicated for three weeks; percent triploidy was determined using flow cytometry

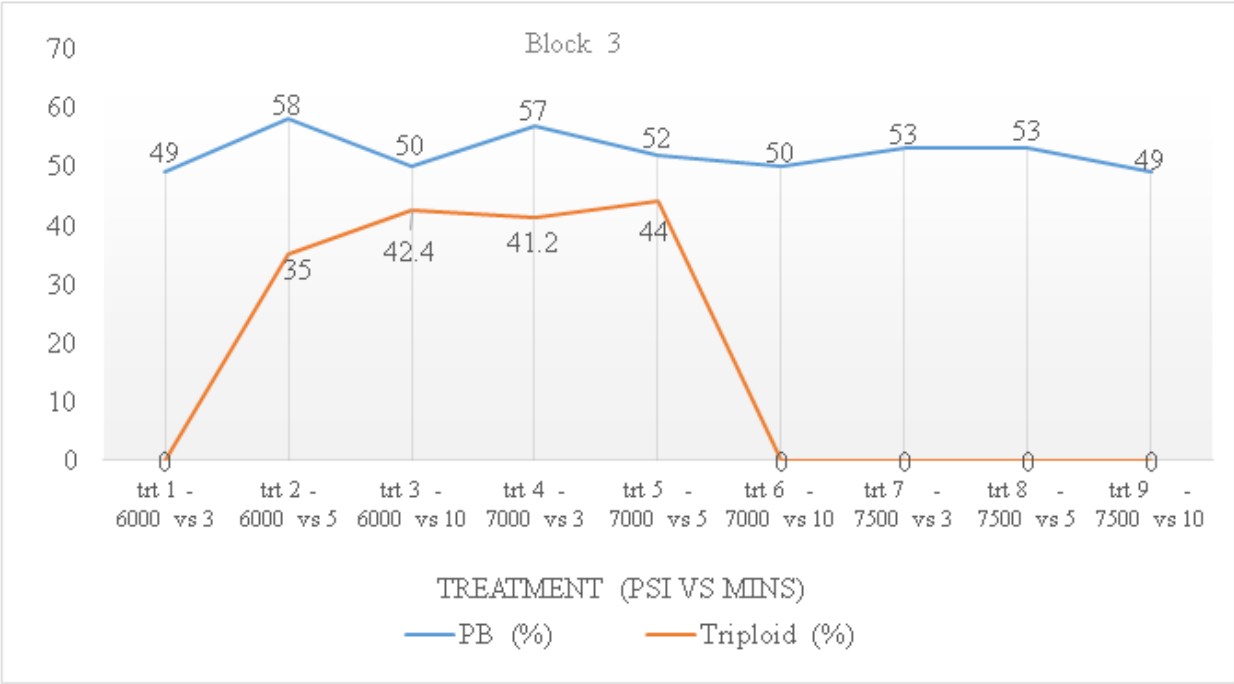


Figure 7. Block 3 of 3 showing percentage of triploid larvae induced at various stages of polar body development (%) within each treatment (trt – psi vs mins) used. A standardized population of approximately 500,000 eggs were fertilized per treatment each week. This study was replicated for three weeks; percent triploidy was determined using flow cytometry

The three highest percentages of triploids induced among replicates in this study were 44% – trt 5, 42.4% – trt 3, and 41.2 % - trt 4 as a result of capturing PB (I) extrusion at 52, 57, and 50% respectively in block 3. Triploid induction percentages varied extensively with PB (I) extrusion timing among all treatments on a weekly basis (see figure 5-7).

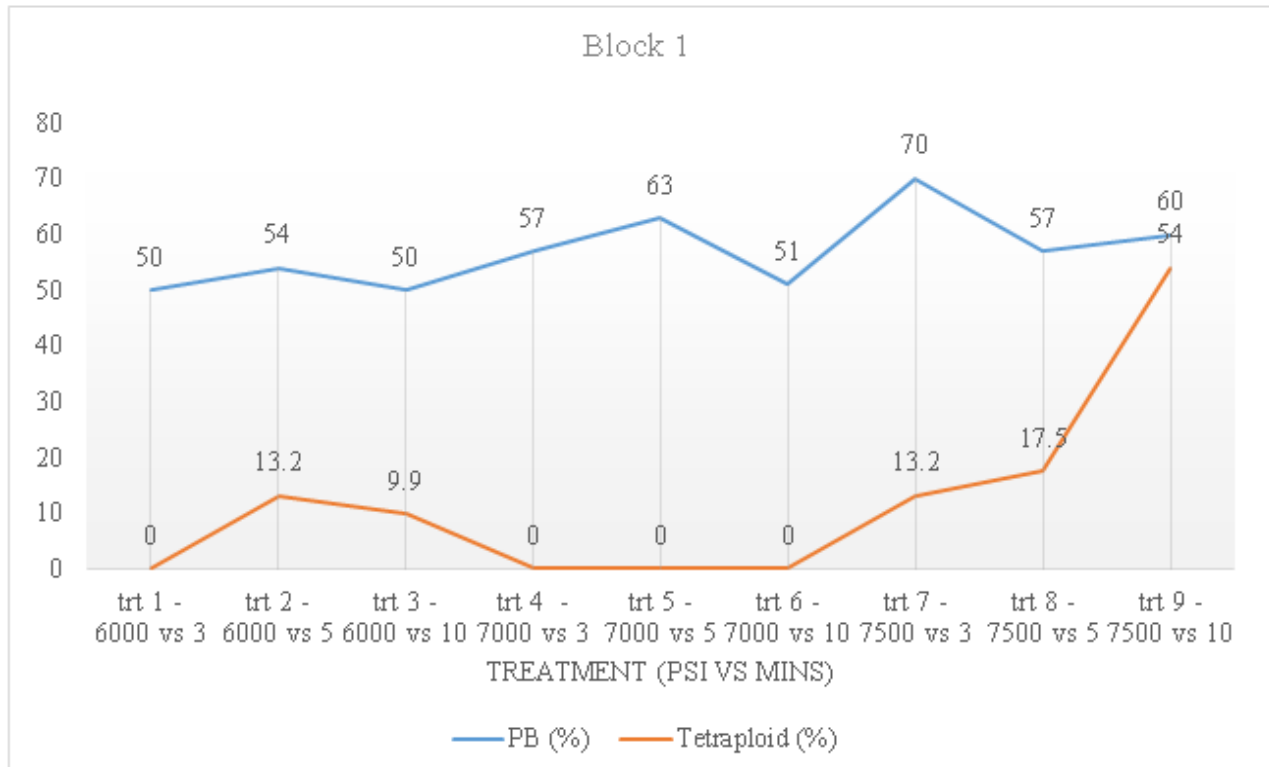


Figure 8. Block 1 of 3 showing percentage of tetraploid larvae induced at various stages of polar body development (%) within each treatment (trt – psi vs mins) used. A standardized population of approximately 500,000 eggs were fertilized per treatment each week. This study was replicated for three weeks; percent tetraploidy was determined using flow cytometry

Unexpected tetraploid induction was only seen in block 1 of the experiment. The three highest induction rates of tetraploids were produced by treatment 7, 8 and 9 producing 13.2, 17.5 and 54 % tetraploids (see figure 8). These tetraploid inducing treatments were initiated at 70, 57 and 60 % PB (I).

2.5 Discussion

2.5.1 Percent survival

In most studies involving artificial triploid induction in both Eastern and Pacific oyster, survivability of larvae post-treatment is usually 10-60% less than their diploid counterparts (Stanley et al. 1981; Tabarini 1984; Downing and Allen 1987; Allen and Bushek 1992). However, different treatment methods have different effects on early larval development (Beaumont and Fairbrother 1991). From our experiment, mean survival of pressure-treated larvae throughout the series of experiments ranged from 1.8 to 8.9% (see figure 2). Diploid controls throughout the three blocks of experiment ranged from 2.5 to 5.2% (see figure 2). ANOVA statistical outputs showed no significant difference in survival rates among all treatments ($p>0.05$). When analysing triploid survival in comparison to diploids, a difference in survival range of 0.7 – 3.7% was seen. This shows better survivability in triploids in comparison to the expected 10-60% survival rate post-treatment (Stanley et al. 1981; Tabarini 1984; Downing and Allen 1987; Allen and Bushek 1992). Despite this, an overall low survivability was seen among all treatments. In addition, the presence of a generally low survival rate, 18.4 to 24.5% was seen among 4 tanks in our ‘diploid only’ rearing experiment showing that survival was being affected by unknown factors apart from our treatments (see figure 3).

In artificially-induced triploid oysters, survivability of the larvae is mainly dependent on treatment timing, egg quality, and larval rearing system (Quillet and Panelay 1986; Allen et al. 1989; Downing and Allen 1987). Physical induction methods have proven to be less successful than chemical as a result of the lessened treatment window of vulnerability associated with this technique (Beaumont and Fairbrother 1991). Consequently, treatment leads to mortalities when this window of opportunity is missed (Guo et al., 1992a; Guo et al., 1992b; Guo et al., 1994). When

blocking PB (I), survival and growth of induced triploids is negatively affected (Guo et al. 1992). One study conducted by Guo et al. (1996) shows survival in artificially induced triploids amounting to 0.2 % when treated with CB; with only one of three CB induced groups surviving to spat stage.

It is hard to compare these technical impacts on survival using different techniques as they impact meiotic development differently (Beaumont and Fairbrother 1991). However, these are the only references currently available to compare oyster-based artificial induction. The induction of Eastern oyster triploids using hydrostatic pressure in Canada has never been documented prior to these experiments. Survival in respect to commercial production is not very significant issue as oysters are a highly fecund species that can be manipulated to produce multiple times a year (Beaumont and Fairbrother 1991).

2.5.2 Mini-experiment on survivability of diploids only

Generally low survival rates were seen in 24-hour larvae. This experiment was conducted using the same techniques and broodstock as per triploidy experiments. A generally low survival rate of 18.4 – 24.5% was seen among all four experimental tanks (see figure 3). This experiment was conducted to assess the unusual low survival rates seen in 24-hour larvae (both treated and diploid controls) prior to shipment of larvae for flow cytometric analysis. Along with the experiment, water quality assessments were conducted to detect any abnormalities responsible for the unexpectedly low survival rate. Water quality assessment showed parameters of: water temperature: 21°C, salinity: 24 ppt, Nitrate: 1.6 mg/L, Nitrite: 0 mg/L, Ammonia: 0 mg/L, and pH: 7.89. All parameters were within optimal range for proper larval rearing in Eastern oysters (Helm et al. 2004). This ruled out the possibility of low water quality affecting general survival. Despite proper broodstock conditioning, egg and sperm quality seemed sub-optimal which is known as a factor that inhibits fertilization success and proper meiotic development (Allen et al., 1989). This

may have ultimately affected triploid induction rates as proper meiotic performance in a synchronized manner is a necessity for optimizing artificial induction in oyster (Allen et al., 1989). Slightly higher survival rates were seen in this diploid only experiment when compared to the triploidy induction series of experiments (see figures 2 & 3). The reduced survival seen in the triploidy experiment was most likely due to the actual induction effect on the oyster larvae; as it is not uncommon to see mortalities associated with PB (I) inhibition treatments (Guo et al. 1992).

2.5.3 Percent triploidy

As demonstrated in this study, the Eastern oyster's response to hydrostatic pressure triploid induction is unique in comparison to other aquatic mollusc species (Chaiton and Allen 1985; Allen et al. 1986; Arai et al. 1986; Komaru and Wada 1989; Shen et al. 2003; Ding et al. 2007). Using the same induction method, Pearl oysters' (*Pinctada martensii*), response shows that longer treatments of 10-15 minutes yielded higher induction rates (Shen et al. 2003). It is important to note that Pearl oysters in the Shen et al. (1993) experiment were subjected to substantially lower pressures; 150 – 300 kg/cm² which is equivalent to 2133.5 – 4267 psi. In our experiments, optimization of triploid induction using hydrostatic pressurization was not achieved (see figure 3). Statistically analyzed pooled data showed that there was no triploid induction performance that was superior among all nine treated groups, this was due to the high variability in induction rates per block of experiment.

Chaiton and Allen (1985) and Allen et al. (1986) used similar pressures intensities, 6000 – 8000 psi on *Crassostrea gigas* in comparison to our treatments used in the Eastern oyster. Induction rates in their experiment amounted to 57% (using 6000 – 8000 psi) and 60% (using 7200 psi) inhibiting PB (II), both for a 10 minutes treatment duration. Different results were seen in our targeted species, *C. virginica*. Optimization of this technique in *C. virginica* was not attainable;

instead, excessive variability in triploidy percentages among all treatments was evident. Synchronicity of egg development may have played a role in this variability, this may be due to sub-optimal egg quality. Among most treatments, PB timing was slightly inaccurate (see figures 5-7). At 25°C, the three highest triploid proportions induced were 44, 42.4, and 41.2 % when treatments were initiated at PB (I) extrusion was 52, 57, and 50% respectively. The ideal timing to initiate triploidy induction is when 60% of fertilized eggs have completed M(I) (personal communication with Dr. Allen 2016). However, due to the rate of meiotic progression at 25°C, accurately initiating pressure treatment at 60% PB (I) was difficult to achieve as seen in figures 5-7. This adds to the notion that when using artificial induction methods, there are many factors affecting general induction success (Stiles et al. 1983; Stanley et al. 1981). Various studies have shown high levels of variabilities of 40 to 70% in producing triploids using CB (Stanley et al., 1981; Tabarini, 1984; Downing and Allen, 1987; Allen and Bushek, 1992). In fact, Guo and Allen (1994b) showed 100, 88 and 33% triploids being produced by CB using the same techniques on the dwarf surf clam (*Mulina lateralis*). Guo et al. (1996) study also used CB on oysters to achieve a 46% induction rate, even though in their previous studies they have produced much higher rates using the same techniques in the said species. Artificial induction as a means to create triploid oyster in commercial hatcheries has proven to be an unsustainable and somewhat a tedious procedure. Hence, the emerging studies on a simpler method of naturally producing triploids through diploid-tetraploid crosses (Guo and Allen 1994b; Guo et al. 1996). However, this technology isn't available in Canada so the use of artificial means to create triploid/tetraploid stock is currently the only option.

Barber et al. (1992) demonstrated that using the Cytochalasin B method of triploid induction on Eastern oysters produces a generally higher induction rate than physical induction

using the hydrostatic pressure. Using a CB concentration of 0.25 mg/L, 96% triploids were produced in their experiment. Downing and Allen (1987) were able to achieved 88% induction in *C. gigas*. Since then, 100% triploids using CB have been achieved in numerous publications. Thus far, the maximum induction rate seen among using hydrostatic pressure induction in oysters was achieved by Shen et al. (2003), producing 76% triploids in Pearl oysters. Without a doubt, chemical treatment is superior to hydrostatic pressure treatment in triploid induction rates in oysters. However, in our study, industry requests were narrowed to physical induction means to prevent unwanted market criticism with the involvement of chemical use in genetic alteration of the oysters.

Tetraploid induction was unexpectedly seen in block 1 of the experiment. These results occurred as a by-product of our triploidy experiments. The three highest rates of tetraploid induction were 54, 17.5 and 13.2% as a result of capturing PB extrusion at 57, 70, 51 and 54% during meiosis phase (I) (see figure 8). This phenomenon has been seen in a few publications where meiosis (I) were targeted using CB treatment and tetraploids were produced as a by-product in *Crassostrea gigas* (Stephens and Downing 1988. Cooper and Guo 1989). Induction rates of 91% and 17% were noted in Stephens and Downing (1988) and Cooper and Guo (1989) papers respectively; however, all tetraploids produced from both experiments died within 72-hours post-fertilization. The percentages noted from our experiment was taken at 48 hrs post –fertilization. Based on the nature and purpose of the experiment all larvae produced were culled after results on triploidy rates were received from VIMS. It still remains unclear how these tetraploids are produced under these conditions (Beaumont and Fairbrother 1991). This result was not within the scope of our experiment hence, very limited attention was allocated to understanding the biological mechanisms that aided in the induction of tetraploids. However, this discovery should be studied

more intensively as a potential technique in creating tetraploids as it could play a key role in creating triploid oyster stocks in Canada.

2.6 Conclusions

Triploid induction using hydrostatic pressure in the Eastern oyster have proven to be successful in our experiments however, its applicability to industry as a commercially sustainable means to create triploids is insufficient at this time due to the high level of variability in induction rates using this technique. The CB method would be more applicable and efficient in producing triploid stock however there are still issues with extensive mortalities post-treatment. Tetraploid - diploid natural crosses have proven to be more superior to artificial induction as it is as simple as natural diploid crosses (Guo et al 1996; Wang et al. 1999). The observation of tetraploid production as a by-product of this experiment have brought the attention of its possibilities with more intensive research. With the absence of this technology in Canada, our only means to creating tetraploid oysters is either through diploid or triploid artificial induction.

CHAPTER 3: ASSESSING GROWTH RATES IN TRIPLOID AND DIPLOID EASTERN OYSTERS AFTER 1 –YEAR OF OPEN WATER BOTTOM CULTURE WITHIN THE BRAS D’OR LAKE

3.1 Introduction

Triploidy in aquaculture bivalve species provides various production advantages, some of which are improved meat quality through glycogen retention, sterility due to retarded gonadal development and superior growth as a result of sterility (Guo et al 1992). While diploids' biological energy is targeted to both growth and reproduction, superior growth achieved in triploids is due to targeted growth rather than reproduction. Polar body retention in diploid eggs leads to the creation of triploid offspring; this instability leads to forced sterility. In oysters, various papers describe superior growth in triploids in comparison to diploids. Faster growth has been documented in CB-induced Pacific oysters (*Crassostrea gigas*) (Allen and Downing 1986; Yamamoto et al. 1988; Hawkins et al. 2000) and Eastern oysters (*Crassostrea virginica*) (Barber and Mann 1991; Mathiessen and Davis 1992). Superior growth in triploids is usually distinguishable after the first year of growth when oysters reaches maturity (Allen and Downing, 1986; Barber and Mann, 1991).

Two types of triploids can be created through artificial induction; Meiosis (I) also known as M(I) and Meiosis (II) also known as M (II) triploids. Meiosis (I) triploids have a higher level of heterozygosity hence are faster growing than M (II) triploids and diploids (Guo et al. 1992). However, despite slower growth in Meiosis (II) triploids, they still have superior growth over diploids (Guo et al 1992). However, the main disadvantage of producing Meiosis (I) triploids through artificial means is the excessive mortalities among treated groups (Guo et al. 1992). Less mortalities occur in the induction of Meiosis (II) triploids (Guo et al. 1992).

In this study, we look to assess the growth potential of hydrostatic pressure-induced M (I) triploid stock within the Bras d'Or lake. These triploids will be produced using the same techniques from our previous study. Both diploid and triploid replicated groups will be deployed into the Bras d'Or lake and assessed after 1-year of growth.

3.2 Objectives & Hypothesis

Hydrostatic pressure-induced triploid spat will be deployed in Bras d'Or lake watershed which is currently prone to MSX. Replicates of diploid and triploid oysters from two year-class (2016 & 2017) will be compared with respect to their growth potential after one year of open water bottom culture. The hypothesis associated with this experiment is that triploid groups will perform better than their diploid counterparts after one year of growth.

3.3 Materials and Methods

3.3.1 Experimental design

Two treatments of 6000 psi and 7000 psi for 3 and 5 minutes were used to induce Meiosis (I) triploids for the growth assessment. From our previous study, these treatments showed no significant differences in triploid proportions produced ($p > 0.05$). Pressure-induced triploids were placed in a commercial hatchery environment until they were 3 mm in size when they were transferred to open water bottom culture until 1-year-old. Eskasoni hatchery and grow out bottom lease site (4115 Shore Road, Islandview, Nova Scotia GPS: N45°57'21.336"; W60°35'8.857") was used for this experiment. Once a location for commercial oyster farming, it is now a dormant oyster hatchery and grow out site along the Bras d'Or lake due to the MSX outbreak in 2002 (Stephenson et al. 2003). Two year-class (2016 & 2017) of diploids and hydrostatic pressure-induced triploid Eastern oysters were created for growth assessment. The experimental design incorporated three replicate groups of triploids with three corresponding diploid control groups

with each own in their individual tanks. One thousand to five thousand larvae were sent ABC Flow Cytometry Laboratory in Aquaculture Genetics and Breeding Technology Centre at Virginia Institute of Marine Science (VIMS) to conduct triploidy testing using flow cytometry. The remaining oyster populations of larvae in each group were grown to 3 millimetre spat using standardized hatchery practises (Helm et al. 2004). Each treatment group of oyster were placed in individual Oystergro™ bags with assigned identification tags and assessed after 1-year of bottom culture within the Bras d'Or lake.

3.3.2 Broodstock conditioning

Eskasoni hatchery provided broodstock for this experiment, from oysters that were collected from the natural wild population residing within the Bras d'Or lake. Broodstock conditioning of these oysters were conducted by staff at the Eskasoni hatchery in preparation for subsequent experiments. Conditioning of the broodstock oysters lasted for 6 weeks during which they were fed a combination of microalgae species. These included: *Tetraselmis sp.*, *Isochrysis galbana* (T-ISO), *Pavlova lutherii*, and *Chaetoceros mulleri*. Feeding regime and concentrations for each batch of broodstock was provided based on their biomass requirements (Helm et al. 2004). Natural seawater from the Bras d'Or lake was heated to the necessary temperatures to allow for proper conditioning of the broodstock (Helm et al. 2004). The water temperature was elevated by 1°C/day until 18°C was reached. At 18°C, the oysters were held for an additional 2 weeks to allow for optimal gametogenesis. This was done for both year classes of broodstock used. Procedures conducive to Helm et al. (2004), bivalve manual was used in respect to their conditioning techniques. Following broodstock conditioning, the parental oysters were place in a refrigerator at a temperature of 4°C to prevent spontaneous spawning prior to the experiments.

3.3.3 Spawning & Fertilization

Physical characteristics such as shell width and height were taken along with the wet weight of all broodstock used. In addition, an individual photo was taken of all the chosen broodstock used in the experiments. Several oysters from the broodstock were opened one at a time, sexed and gonadal material assessed based on quality and quantity. Gametes used in these experiment were retrieved using the strip-spawning method, to prevent pre-mature fertilization or mixing of opposite sex samples (Allen et al. 1989). Approximately 23 oysters were used in both experiments as brood. A scalpel was used remove the egg and sperm residing in the oysters' body cavity. Incisions made in these areas would allow for the release of gametes, which were periodically washed in their respective labelled beakers using fresh SW at approximately 25°C. Ratios of male to female for fertilization were chosen based on the quality and quantity of gametes available. Visual assessment of each oyster's gametes was conducted with the aid of a compound microscope (Olympus CH30 compound microscope, Model: CH30RF100).

A numerical scale from 1-3 was used to rate egg quality and quantity based on their appearance. A highly fecund sample along with pear-shaped eggs would be given 3 while low fecundity and poorly shaped eggs would be given 1 using this scale. Similarly, to egg quality assessment, a numerical scale of 1 to 5 was used to rate sperm samples. A sample observed to be inactive was given a rating of 1 while a high active healthy sample was given a 5. All low quality gametes were recorded and removed from the experiment. The best samples of eggs were pooled together and incubated in 25°C SW prior to fertilization. This was also done for the sperm samples (Downing and Allen 1987). Excess tissue from stripping was removed from both samples using a 20- μ m and 55- μ m nitex screen. The 55- μ m screen was used to catch large extraneous tissue while the eggs passed through and were caught on the 20- μ m screen below. For sperm samples,

extraneous tissue was caught on the 20- μ m screen and the sperm was captured below in its respective beaker. The total volume of pooled eggs was recorded and an estimate of quantity of eggs within that beaker was calculated using a Sedgwick-rater counting cell (Pysen-SGIS50™).

Natural SW from the Bras d'Or lake at approximately 20 ppt was heated to approximately 25°C to be used in the subsequent experiments. Eggs and sperm were gently mixed thoroughly in filtered- natural SW within their respective beakers. All 3 treated replicates were fertilized at 15 minutes' apart to allow ample time to track the PB (I) extrusions during meiotic development. A double hand tally counter (Light of Night™), Sedgwick raft counter (Pysen-SGIS50™) and compound microscope (Olympus CH30 compound microscope™, Model: CH30RF100) were used to track percentage PB (I) extrusion to 60%. Assistance by other involved hatchery staff were needed at this point to allow for accurate tracking of PB (I) while fertilizing eggs every 15-minutes for all treatments. This procedure involved three individuals; one fertilizing a new treatment every 15 minutes, another tracking PB development in all fertilized treatments, and the last person initiating pressure treatment for one minute after approximately 60% PB(I) was seen within samples (Personal Communication with Dr. Allen 2016). Using the double hand tally counter, eggs that hadn't reach PB (I) was recorded with one click on the first hand and a corresponding click was recorded on the other representing an egg that completed PB(I) formation. Random observations of eggs within each sample were tallied and a percentage of PB(I) recorded. All 3 control diploid groups remained untreated and were placed in their individual tanks after PB (I) was observed.

3.3.4 Hydrostatic Pressure Treatment

The hydrostatic apparatus used was constructed using a 12-ton Powerfist[®] hydraulic press, 700 ml stainless-steel cylindrical pressure chamber and glycerine filled pressure gauge with a maximum pressure tolerance of 10,000 psi. The pressure chamber was disinfected using Virkon[™] and partially filled with 25°C SW prior to treatment initiation. The pressure chamber was filled to 75% of its total volume to allow for quick transition from polar body tracking to treatment initiation. Its piston-like lid was used to air-lock the untreated eggs and a valve located adjacent to the top portion of the lid was opened to release excess air from within chamber. By doing this, a hydrostatic environment was created. By pressing a lever on the hydraulic press, the targeted pressure intensity was passed into the stainless steel cylinder and acted on the fertilized eggs. As a result of no optimal treatment distinguished from the prior induction experiment in chapter 2, the two low psi and durations for each treatment regimes were used ($p > 0.05$). Year-class 1 was treated with 6000 psi for 3 minutes while year-class 2, 7000 psi for 5 minutes. This was sequentially conducted for all three replicates until treatments were completed. All diploid replicates remained untreated; however, handled the same way as triploids and placed in their respective tanks after PB (I) was observed in majority of samples.

3.3.5 Triploid detection

A sub-sample 1000-5000 in all three replicate groups were packaged with ice packs in a Styrofoam container and sent for next-day shipping. All treatment groups along with their controls were sent to ABC Flow Cytometry Laboratory in Aquaculture Genetics and Breeding Technology Centre at Virginia Institute of Marine Science (VIMS) for flow cytometric polyploid testing. The flow cytometry techniques used were conducive to those described by Downing and Allen (1987). Samples were run on a Sysmex-Partec Cy Flow Space flow cytometer. DAPI was used as the staining medium for this experiment. Numerical precursors were assigned to each ploidy level for

easy referencing of samples during flow cytometry analysis, haploid cells - 50, diploid cells – 100, triploid cells – 150 and tetraploid cells - 200.

3.3.6 Larval Rearing

Immediately after treatment, the eggs from each group were placed in separate 100 L tanks containing natural SW at 25 °C and 20 ppt for further development and ploidy testing (see figure 9). Both diploid and triploid larvae were reared using hatchery protocol as in Loosanoff and Davis (1963). During this time, the larvae were fed a diet of *Tetraselmis* (*T. chuii*, *T. tetrahele* and *T. suecica*), *Isochrysis galbana* (*T-ISO*), *Pavlova lutherii* and *Chaetoceros muelleri* every 2 days along with water changes according to Helm et al. (2004) larval feeding regimes. Population densities were recorded for each larval tank until pediveliger (settlement) stage was reached. The larvae were then transferred to downwellers and allowed to settle. Subsequently, settled larvae were transferred to upwellers where they were grown to 3 mm in size after 5 weeks. At this size, the larvae were placed in Oystergro™ bags and transferred to the open water and further reared using bottom culture.



Figure. 9. Larval tanks (100 litres) used for rearing until pediveliger stage (pre-settlement) was achieved. Samples were shipped within 24 hours to VIMS for flow cytometry

3.3.7 Growth assessment

Juvenile oysters were transported to the open water environments exposed to MSX to assess survivability and growth between untreated and treated groups. Two year classes of oysters were created for growth comparisons: 2016 year class created on July 2016 and 2017 year class created on July 2017. Juvenile oysters from the 2016 year-class were deployed in December 2016 while the 2017 year class was in stored in an upweller system with exposure to infected waters but not deployed due to overwintering. Three hundred larvae were taken from each group to be assessed for growth. All replicate groups were placed in Oystergro™ bags of appropriate size to prevent loss of oyster stock. All bags were placed on the bottom within the lease on the Bras d'Or lake. Triplicate groups of both treated (triploids and treated diploids) and untreated (diploids) were followed for a duration of 16-months. The treated group comprised of both hydrostatic-induced triploids and surviving treated diploids. Shell-height (mm) and wet weight (g) were recorded for each treated and control group. These oysters were sampled to assess their growth and survival after 1 year and 5 months of growth respectively. Both classes of oysters were sampled on November 2017 and only 2016 year-class on August 2017. On August 2017, oysters from 2016 year class were 13 months old and on November 2017 they were 16 months old. For 2017 year class, during November 2017 sampling, the juvenile groups of oysters were 5 months old.

3.3.8 Statistical Inference

Minitab version 17 was used to perform all statistical analyses. One-way ANOVA was used to assess the differences in growth rates among the diploid (control) and treated triploid groups. Prior to ANOVA analysis, all assumptions on normality, equality of variance and independence within the samples were verified. To validate normality, a normal probability plot (NPP) on residuals was conducted (Montgomery 2013). Homogeneity of variance was also verified by plotting a residual versus fits graph and a visual assessment of distribution pattern of

all plots were observed (Montgomery 2013). Confirmation of homogeneity was made when the plots show a horizontal band across the graph and appeared to be randomly distributed. Independence among the samples was verified through randomization of treatments, blocks and treatment combinations (Montgomery 2013).

3.4 Results

3.4.1 Triploidy

Triploid induction in 2016 year-class showed 30% (n=6/20), 0.05%(n=1/20) and 0% in pressure-treated groups A, B and C as a treatment regime of 6000 psi vs 3 minutes was used. For 2017 year-class, pressure-treated groups A, B, and C, amounted to 0, 13% (n=3171/20902) and 23% (n=2850/12395) induction rates using 7000 psi for 5 minutes while PB(I) timing was 60%.

3.4.2 Survival

In 2016 year-class, diploid survival to pediveliger staged larvae was \bar{x} =10.1% (n=3) while triploids \bar{x} = 4.3% (n=3). However, pooled data showed no significant differences in survival percentages between treated group and diploid control groups ($p>0.05$) (see fig. 10&11). Survival from pediveliger larvae (pre-settlement) to 13 month old juvenile oysters was greater in diploids (5.1%) than triploids (2.2%). In 2017 year-class, diploid survival to pediveliger staged larvae was \bar{x} =0.4% (n=3) while triploids \bar{x} = 0.1% (n=3). However, pooled data also showed no significant differences in survival percentages between treated group and diploid control groups ($p>0.05$).

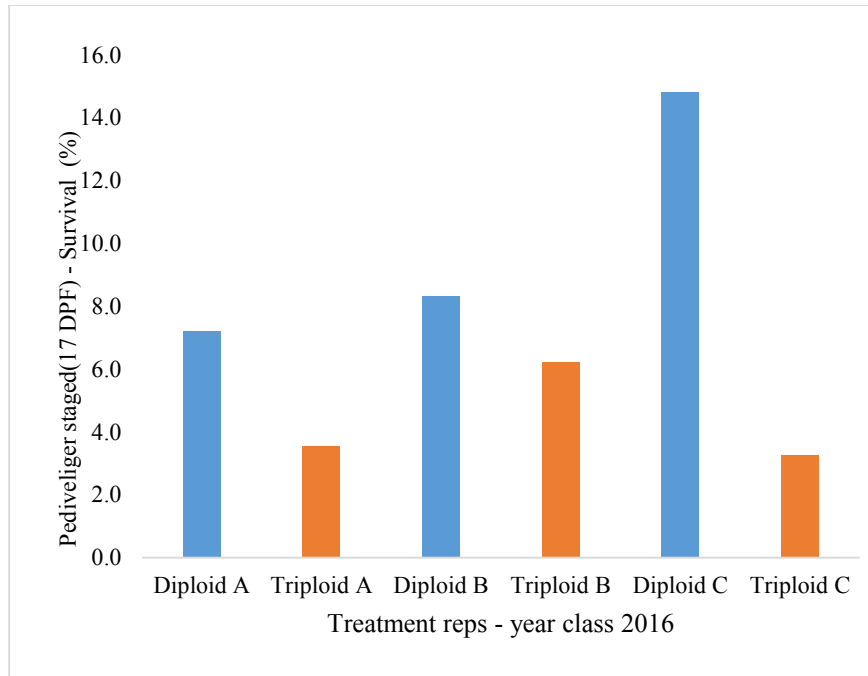


Figure. 10. Comparison of 2016 year class survival (%) of replicate treated and untreated groups at 17-day post-fertilization (DPF) in hydrostatic pressure triploid induction experiment for further growth comparison. All treated groups were subjected to 6000 psi vs 3 minutes at 60% PB (I) treatment. Pediveliger stage larvae began to settle at 17 DPF.

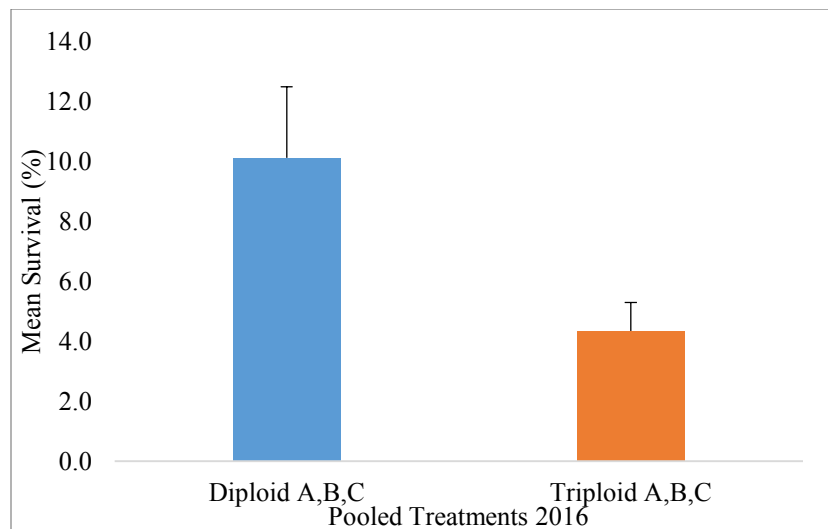


Figure. 11. Comparison of 2016 year class mean (+SE) survival (%) of both pooled treated and untreated groups at 17 days post-fertilization (DPF) in hydrostatic pressure induced larvae growth comparison. All treated groups were subjected to 7000 psi vs 5 minutes at 60% PB (I) treatment. Pediveliger stage larvae began to settle at 17 DPF ($P > 0.05$).

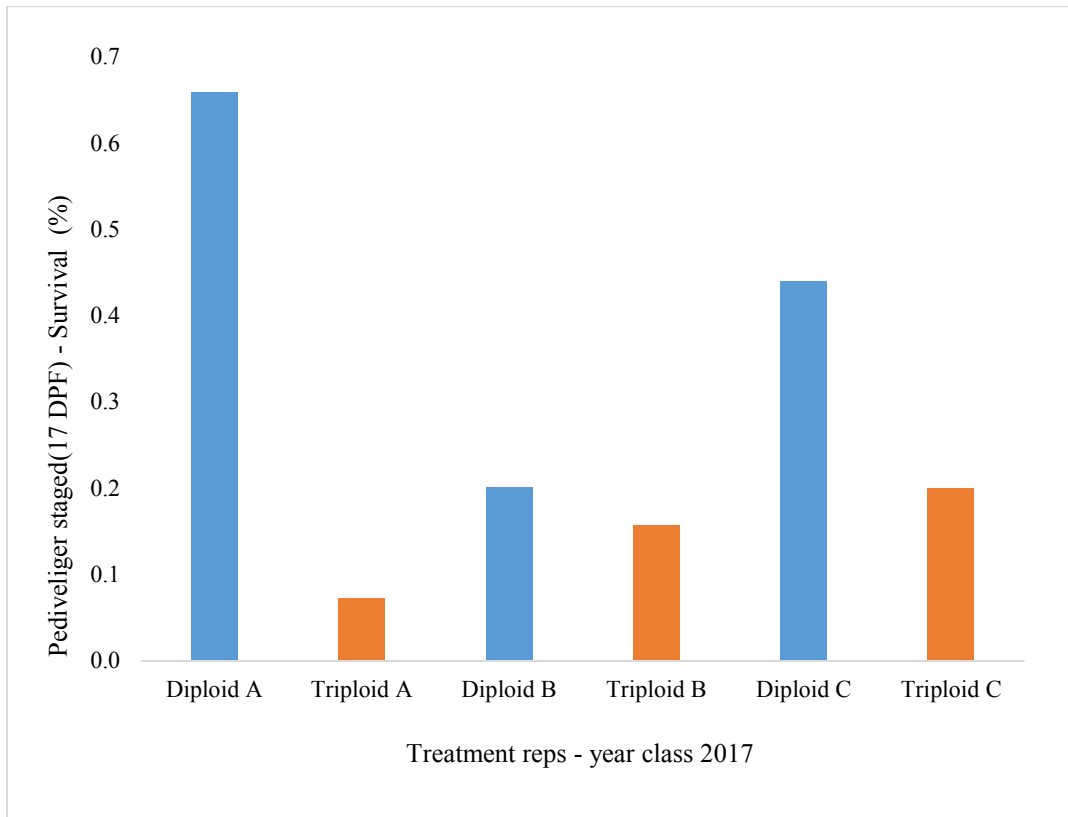


Figure. 12. Comparison of 2017 year-class survival (%) of replicate treated and untreated groups at 17 day post-fertilization (DPF) in hydrostatic pressure triploid induced larvae growth comparison. All treated groups were subjected to 7000 psi vs 5 minutes at 60% PB (I) treatment. Pediveliger stage larvae began to settle at 17 DPF.

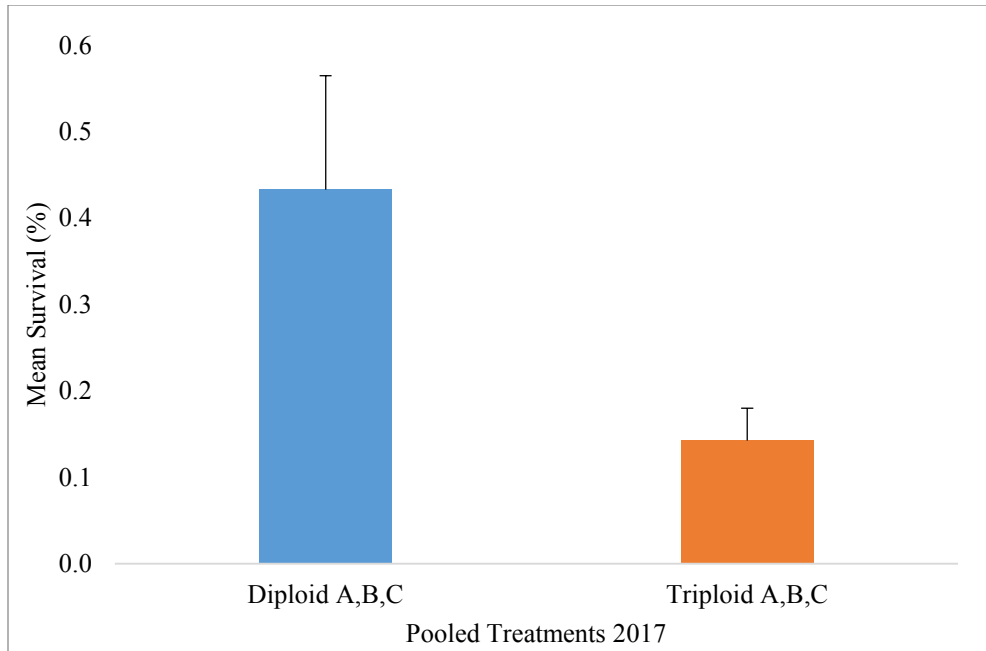


Figure. 13. Comparison of 2017 year class mean (+SE) survival (%) of both pooled treated and untreated groups at 17 days post-fertilization (DPF) in hydrostatic pressure induced larvae growth comparison. All treated groups were subjected to 7000 psi vs 5 minutes at 60% PB(I) treatment. Pediveliger stage larvae began to settle at 17 DPF ($p > 0.05$).

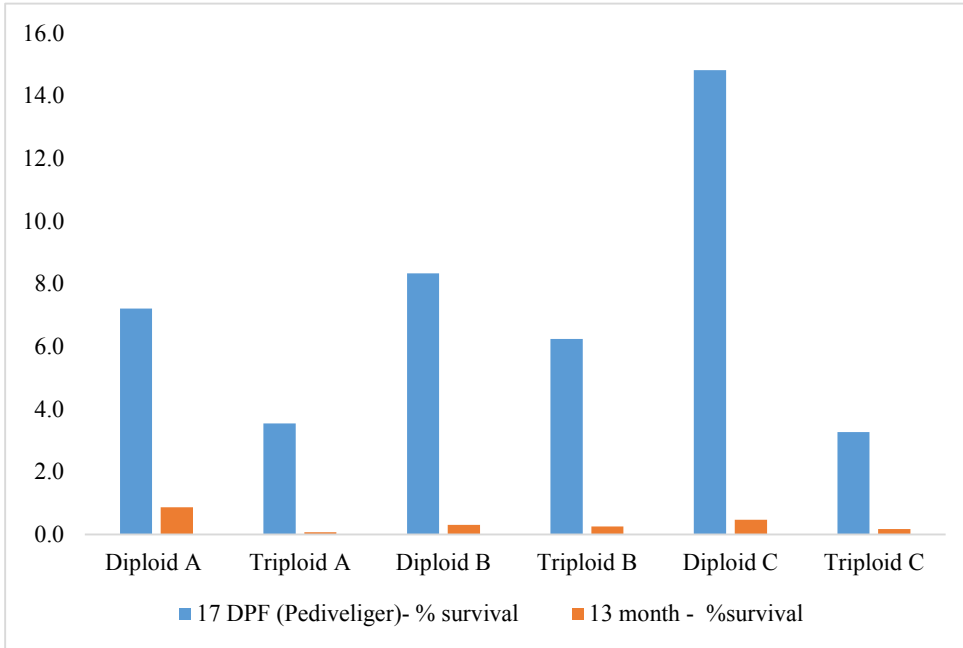


Figure. 14. Comparison of 2016 year class survival (%) of replicate treated and untreated groups from pediveliger stage larvae (post-metamorphosis) to 13 month old juvenile spat. All treated groups were subjected to 6000 psi vs 3 minutes at 60% PB (I) treatment.

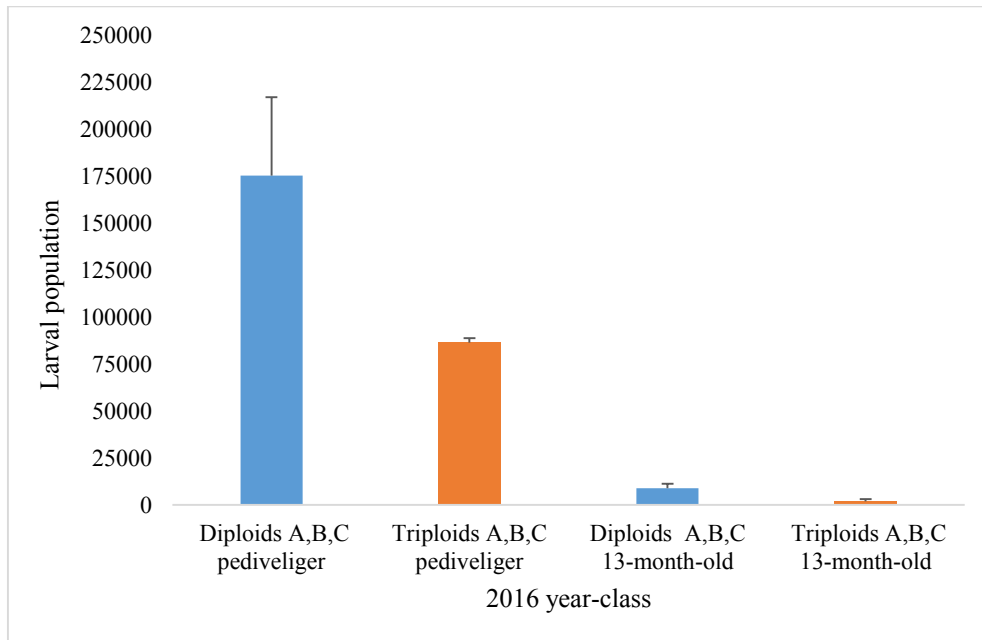


Figure. 15. Comparison of 2016 year class mean (+SE) survival (%) of both pooled treated and untreated from pediveliger staged larvae (pre-metamorphosis) to 13 month old juvenile oysters.

3.4.3 Growth comparison

Mean Shell height (mm)

After 13 months of growth, 2016 year class showed shell heights of \bar{x} = 3.45mm, 3.74mm, and 2.70 mm in diploids group A to C, while \bar{x} = 5.5, 3.96, and 6.20 mm was seen in pressure-treated group A to C. All treatment groups had sample sizes of 40 juvenile oysters ($p < 0.05$). Also, in 2016 year-class, shell heights of \bar{x} = 2.9, 2.3 and 3.5 were recorded in diploid groups A to C during November 2017 sampling after 16 months of growth. Shell height was statistically different between diploid and pressure-treated groups ($p = 0.015$). Pressure-treated groups A ($\bar{x} = 3.0$ mm, $n = 36$), B ($\bar{x} = 3.0$, $n = 40$) and C ($\bar{x} = 1.2$ mm, $n = 7$) appeared to be significantly smaller than diploid A ($\bar{x} = 2.9$ mm, $n = 24$), B ($\bar{x} = 2.3$ mm, $n = 4$), and C ($\bar{x} = 3.5$ mm, $n = 38$) (see fig 17). By this sampling period, majority of the groups died and the remaining survivors were sampled. Replicates within each groups were not significantly different in respect to shell height despite their differences in remaining populations within groups ($p = 0.854$). Pressure-treated group A to C also showed shell heights of \bar{x} = 3.0, 3.0, 1.2 during this sampling period. The remaining sample sizes after 16 months were very low among replicates due to extensive mortalities during rearing showing differences in growth ($p < 0.05$). Diploid groups A to C for year class 2017, showed shell heights of \bar{x} = 0.6, 0.9, and 0.7mm after 5 months of growth. For the pressure-treated groups, shell heights of \bar{x} = 0.4, 0.7, and 0.9 mm were recorded during this sampling period (November 2017). Among the 2017 year-class, after 5 months of growth, no significant differences were seen in shell height between pressure-treated and diploid groups ($p = 0.267$). However, differences in among replicates: pressure-treated groups A ($\bar{x} = 0.4$ mm, $n = 40$), B ($\bar{x} = 0.7$ mm, $n = 40$) and C ($\bar{x} = 0.9$ mm, $n = 40$) and diploid groups A ($\bar{x} = 0.6$ mm, $n = 40$), B ($\bar{x} = 0.9$ mm, $n = 40$) and C ($\bar{x} = 0.7$ mm, $n = 40$) were evident ($p < 0.001$).

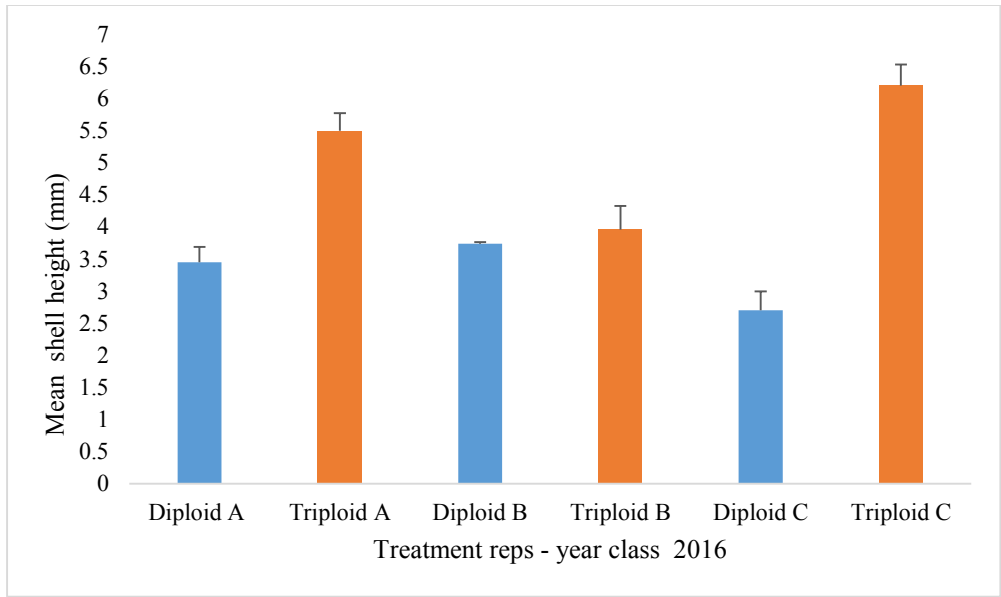


Figure. 16. Comparison of 2016 year class mean (+SE) shell height (mm) of both treated and untreated groups. Sampling was conducted August 2017 after 13 months of growth ($p < 0.05$).

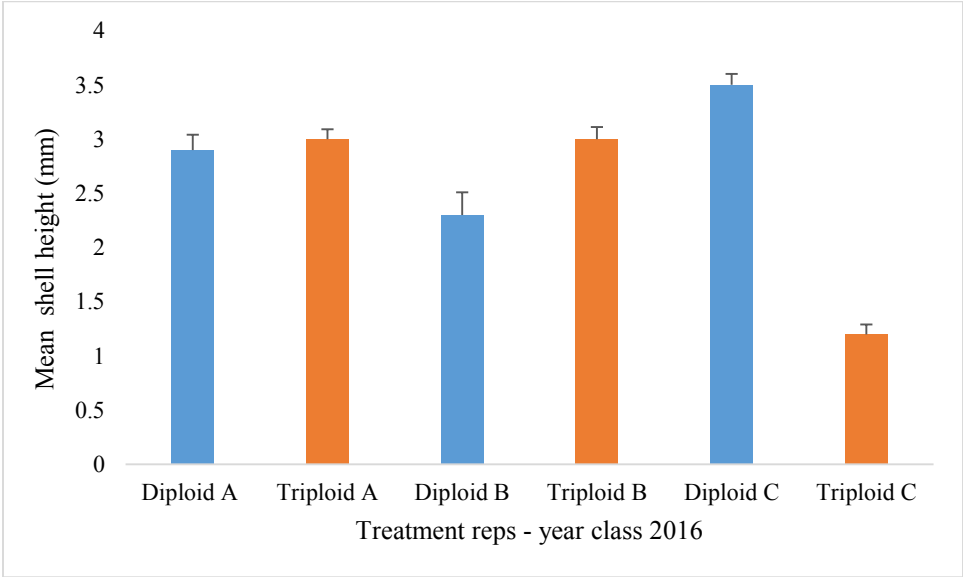


Figure. 17. Comparison of 2016 year class mean (+SE) shell height (mm) of both treated and untreated groups. Sampling was conducted November 2017 after 16 months of growth ($p > 0.05$).

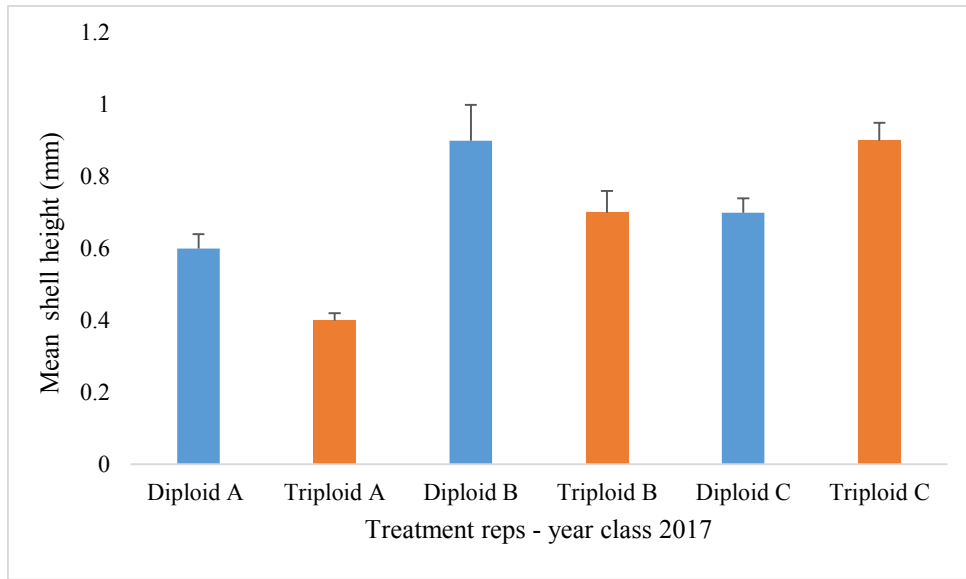


Figure. 18. Comparison of 2017 year class mean (+SE) shell height (mm) of both treated and untreated groups. The 5 month old juvenile Eastern oysters were created on July 2017, sampling was conducted November 2017 ($p > 0.05$).

Mean Wet weight (g)

After 16 months of growth, oysters from 2016 year class showed a significant difference in wet weights between pressure-treated groups ($\bar{x}=2.5\text{g}$, $n=3$) and diploid control groups ($\bar{x}=2.6\text{g}$, $n=3$) ($p=0.009$). Wet weights for diploid A ($\bar{x}=2.4$, $n=24$), B ($\bar{x}=0.8\text{g}$, $n=4$) and C ($\bar{x}=4.6\text{g}$, $n=38$) were significantly greater than pressure-treated group A ($\bar{x}=2.6\text{g}$, $n=36$), B ($\bar{x}=2.5\text{g}$, $n=30$), and C ($\bar{x}=0.3\text{g}$, $n=7$). All groups were low in population size due to extensive mortalities after 16 months of rearing. The remaining oysters among each groups were used for sampling ($p < 0.05$).

For 2017 year class, a total of 40 oysters were weighed in each group. After 5 months of growth, average wet weights between pressure-treated groups ($\bar{x}=0.07\text{g}$, $n=3$) and diploid groups ($\bar{x}=0.07\text{g}$, $n=3$) were highly significantly different ($P < 0.001$). Diploid A ($\bar{x}=0.06\text{g}$, $n=40$), B ($\bar{x}=0.1\text{g}$, $n=40$) and C ($\bar{x}=0.05\text{g}$, $n=40$) wet weights recorded, when pooled together, was

statistically larger than pressure-treated groups A (\bar{x} =0.02g, n=40), B (\bar{x} =0.08g, n=40), and C (\bar{x} =0.1g, n=40). These groups consisted of 5 month old juvenile oysters created in July 2017 and sampled in November 2017.

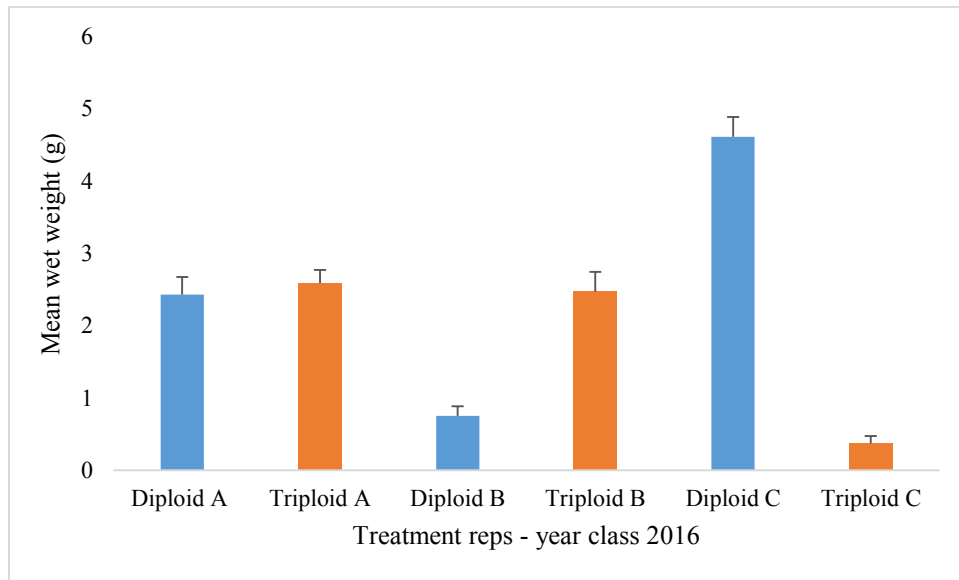


Figure. 19. Comparison of 2016 year class mean (+SE) wet weight (g) of both treated and untreated groups. The 16 month old juvenile Eastern oysters were created on July 2016, sampling was conducted November 2017 ($p < 0.05$).

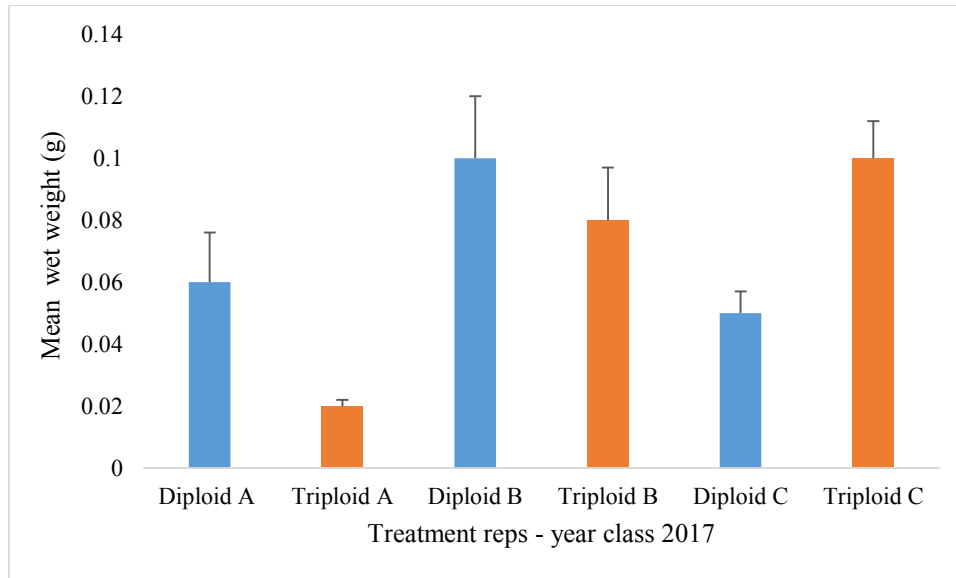


Figure. 20. Comparison of 2017 year class mean (+SE) wet weight (g) of both treated and untreated groups. The 5 month old juvenile Eastern oysters were created on July 2017, sampling was conducted November 2017 ($p < 0.05$).

3.5 Discussion

3.5.1 Triploidy and deployment

Two year classes of pressure-treated oysters, 2016 and 2017, were created along with their diploid controls for growth assessment within the Bras d’Or lakes. All 2016 oyster groups created, provided viable diploid and triploid populations for sampling up 16 months of open-water culture; while, 2017 groups survived up to 5 months of rearing. At 16 months, majority of the deployed oysters died from unknown causes; however, due to the presence of MSX within the Bras d’Or lake (McGladdery and Stephenson 2005; Personal communication with Rod Beresford 2018), this disease is suspected to have contributed to these mortalities. Pressure- treated groups consisted of hydrostatic pressure-induced triploids and treated diploids while untreated groups consisted of only diploid controls.

Prior to deployment of 2016 year class, ploidy assessment showed 30% ($n=6/20$), 0.05% ($n=1/20$) and 0% in pressure-treated groups A, B and C respectively. To induce triploidy in these

treated groups, a hydrostatic pressure treatment regime of 6000 psi vs 3 minutes was used. The remaining percentages within these groups are assumed to be ‘treated diploids’. Triploids produced in this experiment were (MI) triploids due the inhibition of PB (I) during meiosis phase I. Ploidy testing of these 2016 year class oysters were conducted during the ‘spat’ life stage post-metamorphosis due to inaccessibility to flow cytometry machinery at the beginning of these trials. Triploid percentages for 2017-year class showed Triploid A, B, and C, amounted to 0, 13% (3171/20902) and 23% (2850/12395) using 7000 psi for 5 minutes. In contrast to ploidy testing of oyster spat for the 2016 year class oysters, triploid groups for 2017 year class were tested during the larval life stages. In our previous triploidy study, an optimal treatment was not achieved using hydrostatic pressure. These two treatments were both chosen to further test triploidy induction performance despite no optimal treatment distinguished ($p>0.05$). Polar body timing remained fixed at 60% PB (I). Also, the production of M (I) triploids through artificial means leads high mortalities (Guo et al. 1992). This knowledge was taken into account when inducing triploid stocks to conduct these growth assessments. High starting populations of approximately 2 million larvae were produced per group with the intention of only following 300 random individuals per group for the duration of the experiment. However, the pressure triploid induction method used led to excessive mortalities within all treated groups (see fig 10-14) as expressed in the literature (Guo et al. 1992).

A growth period of 16 months was documented in 2016 year class however; excessive mortalities were seen by November 2017 which led to the termination of the experiment. Five month old juvenile oysters from year-class 2017 were placed in upwelling systems to grow during overwintering and will be deployed for further growth assessment in the spring of 2018. These oysters were currently being exposed to waters from the Bras d’Or lake through a flow-through

system which potentially leads to MSX exposure. Sunila et al. (2000) shows the possibility of *H. nelsoni* infecting juvenile oyster spat reared in an upweller system using MSX-infected waters. Mortalities for this experiment amounted to a 19% reduction of the overall population after 11 weeks of culture and eventually reached 80% after 16 weeks. Juvenile oysters from 2016 year class were also held in similar upweller systems until deployment in December 2016 within the Bras d'Or lakes and were subsequently reared using bottom culture for 16 months hence being fully exposed to MSX. Despite MSX being suspected to have contributed to these mortality events, there was no testing to confirm the presence of the pathogen in the oysters; however, through personal communication with Rod Beresford (2018), various levels of MSX disease pressures were recorded throughout the Bras d'Or lake in both 2016 and 2017.

3.5.2 Survival and Growth

Shell height

2016 year class

Survival to pediveliger staged larvae in both diploid and pressure-treated groups showed no differences when statistically analysed (see fig. 10&11). Previous studies on *C. virginica* have shown triploid survival being greater than diploids in MSX-prone waters (Mathiessen and Davis 1992). Another study supporting this idea showed that increased survival in triploids *C. gigas*; a very similar species to *C. virginica*, is due to higher quantities of haemocytes with Concanavalin –A binding sites in comparison to diploids. Increased quantities of haemocytes are translatable to stronger immune-responses to infection and/or diseases with increased phagocytosis (Moore and Gelder 1987). More research should be targeted to understanding the immune responses seen in *C. gigas* to determine whether these responses are influenced by the treatment method used or the species immunocompetence. Both species, *C. virginica* and *C. gigas*

appears to respond differently to triploid treatment in respect to survival; however, the creation of M(I) triploids leads to high variability in mortalities (Guo et al. 1992a; Guo et al. 1992b; Guo et al. 1994) from the induction process. This may add to the lack of consistency seen in triploid survival throughout our experiment (see fig. 10-11). In addition, physical pressure treatment is known to be less successful than chemical treatment due to lessened treatment window of opportunity to induce triploidy; when this window is missed, the treated larvae are killed (Beaumont and Fairbrother 1991).

After 13 months of growth, significant differences were seen between pressure-treated and diploid groups ($P < 0.001$). Pressure-treated groups showed significantly greater shell growth than diploid controls (see fig. 16). A very similar study conducted by Anderson (2000), showed identical results where faster growth was seen in *C. virginica* treated groups than diploids after 12 and 13 months of growth. However, their treated diploid groups showed non-significant differences between both triploid and diploid groups in respect shell height (Anderson 2000). Other studies add evidence that M (I) triploids grow faster than diploids or even M (II) triploids in both *C. virginica* and *C. gigas* (Yamamoto et al. 1988; Hawkins et al. 2000) Increased growth seen in triploids after the first year is mainly due to energy allocation in triploids. Triploid oyster biological energy is targeted to growth rather than gametogenesis after maturity (Stanley et al., 1984; Allen and Downing, 1986; Barber and Mann, 1991).

Various studies on *C. gigas* showed greater survival in diploids than triploids during summer months and when grown in high carrying capacity estuaries (Cheney et al. 2000; Gouletquer et al. 1995). This survival pattern was seen in our experiment as pediveliger larvae (pre-settlement) to 13 months old juvenile oysters was greater in diploids (5.1%) than triploids (2.2%) (see fig. 13 & 14). Survivability of oysters, whether diploid or triploid, is influenced by

many environmental-related factors unique to their growing location (Galtsoff 1964; Shumway 1996). In addition, a study conducted by Sturmer et al. (1993) shows contrasting results as there was no difference in survival between diploid and triploids after 12 months of growth in Florida.

After 16 months of growth, shell height again was statistically different between diploid and pressure-treated groups ($p=0.015$). The pressure-treated groups were statistically smaller than diploid control groups. During this sampling period, majority of the groups died and the remaining survivors were sampled. Replicates within each groups were not significantly different in respect to shell height despite their differences in remaining populations within groups ($p=0.854$). Most papers on triploid oyster growth comparisons shows that superior growth was seen in triploids after 12 months when compared to diploids (Stanley et al., 1981; Barber and Mann, 1991; Matthiessen and Davis, 1992). However, due to the extensive mortalities observed at 16 months of growth, the remaining population showed larger individuals among the diploid groups in comparison to the pressure-treated ones.

Extensive mortality was seen by 16 months of growth which led to 2016 year class growth assessments being terminated. The unusual reduction in sizes of the said oysters in comparison to previous 13th month sampling confirmed that most members within the general population died by this sampling period. Excessive mortalities may be due to MSX exposure (Krantz et al. 1972; Fegley et al. 2003; McGladdery and Stephenson 2005), overwintering or even the triploidy effect (Davis 1988a). Davis (1988a) experiment shows generally lower survivability in triploids than diploids post-maturation. The triploids oysters in Davis (1988a) study showed lower survivability due to greater reduction of glycogen storage in comparison than diploids when starved for 130 days. This is attributed to the triploid effect, due to their genetic instability, growth and survivability performance may be lowered. However, this is not certain, oysters among all the

groups died from unknown causes after 16 months of open-water rearing within the Bras d'Or lake.

2017 year class

Due to the overall non-significance in shell height growth between Diploid and Triploid groups, further insight on the differences among replicates was not necessary. Differences in diploid and triploids oyster growth are not apparent prior sexual maturity (Stanley et al. 1981). Stanley et al. (1981) show no difference in growth rates between triploid and diploid 8 month old juvenile oysters grown in Maine, USA. However, their follow up paper documenting growth post-maturation showed higher growth in triploids over diploid. Furthermore, superior growth in triploids was only seen during spawning season during their experiments (Stanley et al. 1984).

Wet weight comparison

2016 year class

After 16 months of growth, oysters from 2016 year-class showed a significant difference in wet weights between pressure-treated and diploid control groups. Wet weights in diploid groups were significantly greater than in diploid controls. All groups were low in population size due to extensive mortalities after 16 months of culture. The remaining oysters among each groups were used for sampling. Furthermore, the limited surviving individuals for each group varied among samples collected. In Anderson (2000) study, triploids ($\bar{x}=8.7$ g, n=25) and treated diploid ($\bar{x}=7.2$ g, n=4) were statistically heavier than diploid controls ($\bar{x}=6.8$, g n=30) ($p=0.013$). However, treated diploids were not statistically different from triploids and diploid controls ($p>0.05$). In a study conducted by Supan (1997), greater wet weights were again seen in triploids than diploids while conducting growth trials in the Gulf of Mexico. Our unusual results showing diploids being

significantly larger in wet weights may be skewed due to the limited population presented for sampling as a result of extensive mortalities after 16 months of culture within the Bras d'Or lake.

2017 year class

After 5 months of growth, average wet weights between pressure-treated and diploid groups were highly significantly different ($P < 0.001$). In addition, pooled group wet weights recorded on both groups when statistically analysed shows pressure-treated groups being smaller than diploids (see fig. 20). This can be explained by analysing the proportional constituents within each pressure-treated group. These groups consist of both hydrostatic-induced M (I) triploids and treated diploids. Meiosis (I) treatment is known to produce both high mortalities and a high induction of aneuploids (Guo et al. 1992). Aneuploid offspring consist of many ploidy levels that render the organisms highly unstable hence usually resulting in death. The larger portion of these groups are treated diploids which may consist of a variety of aneuploids. Various studies have shown reduced growth in oysters due to aneuploidy (Thiriou-Quievreux et al., 1988, 1992; Zouros et al. 1996; Leitaño et al., 2001). Furthermore, Zouros et al. (1996) explained the inverse relationship between growth and aneuploidy due to deleterious recessive genes expressed in the oyster. In respect to the triploid portions, Mathiessen and Davis (1992) showed faster growth in diploids than triploids during the first growing season. However, triploid growth exceeded diploids within the second growing season and even then growth differences were only evident during spawning season when diploids were disadvantaged due to their reproductive efforts (Mathiessen and Davis, 1992).

3.6 Conclusions

Triploid induction was successful in a few treated groups for both year classes. Hydrostatic pressure-induced triploids and treated diploid and diploid control groups were successful in having viable populations for sampling after 5 months and 1 year of growth in 2017 and 2016 year-classes. Survival to pediveliger stage larvae in both year class showed no significant difference among pressure-treated and diploid groups.

Superior growth was seen in triploids and treated diploids after 13 months of open-water rearing within the Bras d'Or lake. This is most likely due to biological energy-budgeting targeting growth in triploids as a result of infertility. It is known that despite excessive mortalities in creating M(I) triploids, faster growth are seen in these oyster in comparison to M (II) triploids and untreated diploids. After 16 months of growth, majority of the individuals within replicated groups died due to unknown causes. However, MSX exposure was suspected have to played a role in these mortalities. Apart from MSX, other factors may include aneuploidy within treated diploids and potentially low survivability in triploids during overwintering and general rearing. Higher survival in diploids was seen after 13 months of growth. Various studies have shown supporting evidence of diploid having greater survivorship than triploids however, contrasting research have also shown triploids having better survivability.

After 16 months of growth, the few remaining survivors within each group were sampled. This may have led to skewed results due to the limited samples available as a result of excessive mortalities prior to sampling. However, 16th month sampling results show greater growth in diploids rather than triploids. Among these CB groups, mean weight sampling during this period showed diploids having greater wet weights than triploids and treated diploids. For 2017 year class, after 5 months of growth, no statistical differences in shell height between Triploid and Diploid

groups were seen which is confirmable by various other literature publications. This is mainly due to both groups not reaching maturity hence no 'triploid effect' or growth advantage seen in triploids. However, mean weight sampling showed diploids being significantly larger than triploids after 5 months of growth. Studies have shown this in Eastern oysters prior to maturity. However, triploids after maturity tend to out grow their diploid counterparts. There are many benefits to triploidy in oysters and superior growth is even distinguishable in MSX-prone waters of the Bras d'Or lake. However, poor triploid induction rates using this method is an issue. Despite high fecundity in oysters, this method of producing triploids remain unsustainable and should be researched more intensively to improve induction rates. Other means to create triploids such as chemical induction and tetraploid- diploid crosses have proven to be more successful than hydrostatic pressure induction and may be a more practical means to produce triploid oysters for commercial production.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Hydrostatic Pressure Implication

4.1.1 Summary and conclusion

Triploid oysters are produced from artificial induction or through diploid-triploid crosses (Longwell and Stiles 1996; Guo et al. 1994; Guo et al. 2009). Even though diploid-tetraploid crosses are most efficient in triploid production, high inductions rates from chemical treatment has been documented in various oyster-related studies (Stanley et al. 1981; Downing and Allen 1987; Barber et al. 1992; Guo et al. 2009). However, despite its high induction rates in the Eastern oyster (Stanley et al. 1981; Downing and Allen 1987; Barber et al. 1992), toxicity of chemicals used especially with the use of the popularly known CB has potentially detrimental health effects to its user (Cayman Chemical Company 2013). As a result of this, other physical methods such as hydrostatic pressure to induce triploidy in oysters is worth studying. Triploid Eastern oysters have shown superior growth, higher survivability during open-water rearing along with disease resistance (Farley 1965; Allen 1987; Ogle 1979; Andrews 1979a; Ford and Haskin 1982; Allen and Downing 1991; Matthiessen and Davis 1992; Allen et al. 1993). The acquisition of triploid oysters within the Canadian oyster industry could lead to potentially large economic growth.

Hydrostatic pressure method is one of the most studied among triploid induction methods in marine bivalves (Piferrer et al. 2009). However, using this method in the Eastern oyster hasn't be documented. This technique is known to be less efficient than chemical induction; however, it provides the advantage of non-chemical means to induce triploidy (Piferrer et al. 2009). Physical pressure prevents the expulsion of either PB (I) or PB (II) during meiosis inducing triploidy in fertilized eggs (Chaiton and Allen 1985). Using previous studies in similar bivalve species, various targeted pressure intensities and treatment durations were tested to assess induction rates within the Eastern oyster. Pressure intensity of 6000, 7000, and 7500 psi along with three treatment

durations 3, 5, and 10 minutes were tested. Treatments were applied at a fixed PB (I) percentage of 60% for treatment initiation timing.

Triploid induction in the Eastern oyster using hydrostatic pressure was successfully conducted in this study. However, due to the high variability in induction rates among all treatments tested, statistical analyses show no optimal induction performance among the 9 treatment combinations studied ($P>0.05$). As it stands, hydrostatic pressure induction isn't a sustainable means to induce triploidy in the Eastern oyster on a commercial scale. However, using this method to induce tetraploidy in the Eastern oyster as shown in this study may be a more efficient technique to producing triploid oyster stocks. Also, other means through chemical induction and diploid-tetraploid crosses may be more advantageous for producing triploid (Guo et al 1996; Wang et al. 1999) Eastern oyster stock in Canada.

4.1.2 Future directions

As it stands, more research is needed to find ways to improve this method in order for it to be a commercially viable option. Triploid induction through other means such as chemical induction with the aid of Cytochalasin B or through tetraploid – diploid crosses have proven to be more efficient when compared to this method. Using this technique, unintentional tetraploid induction through late polar body extrusion timing was discovered in this project. This novel discovery is very valuable as it may provide the Canadian oyster industry with a more viable option to produce triploid oysters from diploid eggs through artificial induction. Further research in producing tetraploid oysters using this technique is warranted as tetraploid – diploid crosses is known to be the most sustainable means to produce triploids. Application of this technique, especially in Canada, would be very profitable as growers only have diploid oyster stocks available currently.

4.2 Growth Comparison

4.2.1 Summary and conclusion

The growth potential of the oyster industry in Nova Scotia has suffered severely since the first MSX (*Haplosporidium nelsoni*) outbreak in 2002 (Stephenson et al. 2003). *H. nelsoni* effect on the residing Eastern oyster populations within the Bras d'Or lake is still evident to this day (Bagauov et al. 2016). Due to the initial spread of this Haplosporidan pathogen within the Bras d'Or lake, all oyster-related farming has ceased as a result of its potential to spread to other aquatic ecosystems (McGladdery and Stephenson 2005). *H. nelsoni* is a parasitic protistan that hijacks the oysters' body cavity and prevents normal physiological processes within the animal thus leading to compound stress and eventually mortalities (Newell 1985; Vercaemer et al. 2010). Little is known about the life cycle associated with the pathogen which adds to the limited ability to combat its effects on the Eastern oyster (Newell 1985; Savoie – Swan 2012; Burreson and Ford 2004). Adding to this, the environment in which the pathogen resides prevents easy isolation in an attempt to limit its hosts.

MSX effects in the Eastern oyster are known to be more prominent in adults after maturation while it's sporulative stages mostly seen in juvenile oysters (Farley 1965; Andrews 1979a; Ford and Haskin 1982). To add, its detrimental effect which usually ends in mass mortalities, is mostly seen in adult populations (Haskin et al. 1965; Farley 1968). Non-resistant oysters aren't equipped to immunity and tolerance localize and combat MSX infection. However, various methods of building resistance and combatting this pathogen have been developed through breeding programs, triploid technology and genetic selection (Myhre 1973; Ford and Haskin 1987; Dégremont et al. 2012). Using triploid induction, faster growth rates are achievable hence the reduction of exposure to the pathogen and production of younger oysters that are less prone to infection (Allen and Downing 1991; Matthiessen and Davis 1992; Allen et al. 1993).

All treated groups were successful in producing viable populations for growth analyses; however, not all pressure-treated groups produced successfully induced triploids some consisted of only treated diploids. Survival between diploid and triploids groups to pediveliger staged larvae remained statistically non-significant ($P>0.05$). In year class 2016, superior growth was seen in treated groups after 13 months of growth despite survival being higher in diploids than that treated diploids and triploid groups. After 16 months of growth, majority of the MSX exposed oysters were killed for unknown reasons; however, MSX exposure potentially could have assisted these mortalities. Due to the low sample sizes remaining, statistical analyses remain inconclusive and provide skewed results. For year class 2017, no significant growth difference was seen between treated and untreated groups with respect to shell height (mm) ($P>0.05$) after 5 months of growth. However, in respect to mean wet weights (g), diploids had statistical higher wet weights than treated groups. This result has been previously documented by Mathiessen and Davis (1992) however, greater growth in triploids were seen after the first growing season. This is also an expected result for future sampling of 2017 year class.

4.2.2 Future directions

The growth potential of pressure-treated oysters in comparison to diploids after 1 year of culture has been documented throughout this project. The triploids produced in this project were a result of artificial induction through hydrostatic pressurization of diploid eggs during Meiosis phase (I). Further yearly sampling of the surviving populations should be continued to assess survivability and growth within the 2017 year class. To further assess growth potential, triploids produced through tetraploid-diploid crosses could be assessed in the same conditions to see what advantages they may possess in comparison to artificially induced triploids. Furthermore, using

hydrostatic pressure to induce tetraploidy in the Eastern oyster should be more intensively studied as it would provide a more sustainable route to producing triploid stocks in Canada.

4.3 Conclusion

Triploid oysters possess many advantageous traits that can be used to optimize commercial production. These traits included faster growth, sterility, better taste, higher survivability and disease tolerance (Ogle 1979; Stanley et al., 1984; Allen and Downing, 1986; Barber and Mann, 1991; Anderson 2000; Guo et al. 2009). The application of these superior oysters to the Canadian oyster industry could catalyze substantial growth. These results were seen in the US oyster industry where investment in science by developing superior resistant oysters through targeted breeding programs, genetic selection and implementation of triploid technology (ABC 2010; Dégremont et al. 2012) have led to exponential growth.

Triploid induction using hydrostatic pressure in marine bivalves is known to be less efficient than chemical induction. However, due to its benefit of non-chemical means to induce triploidy in oysters using this method, more research and interest has been allocated to studying this technique. Furthermore, Canadian industry requests have been narrowed to physical induction means to prevent unwanted public criticism of chemical use in genetic alteration of the oysters. Eastern oyster hydrostatic pressure induction to induce triploid has not been previously documented. From our project, we have successfully induced triploidy in the Eastern oyster using hydrostatic pressure. However, our attempt to optimize induction rates proved to be unsuccessful due to the high variability seen among all treatments tested. Unintentional tetraploid induction was documented as a by-product of our triploid experiment using this method. This may provide a potentially better technique to sustainably produce triploid oyster stock in Canada if further studied.

Following the induction of triploid Eastern oysters, growth performance of induced triploids and resultant treated diploids were compared to untreated diploids. Two year classes were created in both 2016 and 2017 for growth assessment within the Bras d'Or lake, NS after one year of culture. Year class 2016 showed faster growth in pressure-treated oysters post maturation at 13 months; however, by 16 months of culture most of the individual groups were killed by unknown factors. The remaining population for each group was sampled and faster growth was seen in diploids at this stage of sampling. Also, 2017 year class oyster were sampled after 5 months of growth, this also showed faster growth in diploids. This result has been documented previously by Mathiessen and Davis (1992) studying growth differences in diploid and triploid Eastern oysters.

Despite the successful induction of triploidy in the Eastern oyster using hydrostatic pressure, more research is warranted to find means of optimizing this technique to suit commercial production. As it stands, high variability in induction rates is not sustainable enough to apply to commercial production. Tetraploidy induction using hydrostatic pressure is an exciting discovery and should be more intensively studied as it may be a more applicable technique to the oyster industry as there are currently only diploid oyster stocks in Canada. Advantageous growth in triploids were seen post-maturation and have shown that triploid oysters have the potential to out-perform their diploid counterparts in both Canadian Eastern oysters and environments.

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