REDUCING THE ENVIRONMENTAL IMPACT OF OYSTER CULTURE BY SELECTING FOR SPAT THAT MOST EFFICIENTLY USES NATURAL RESOURCES

by

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Dedicated to my parents, ***Stephen*** *and* ***Eileen***
Whom have been my biggest supporters and source of inspiration

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ABSTRACT

The main objective of this study, was to discover SNPs associated with absorption efficiency in the Eastern oyster, which is a component of feed efficiency, and to use marker-assisted selective breeding to improve growth and efficiency. Absorption efficiency was measured, by collecting water/feed samples and fecal samples, and filtering on to pre-combusted, weighed, glass-fiber filters. Individual absorption efficiency were calculated by comparing the fraction of organic matter in fecal matter versus feed samples. After the trial, all oysters were euthanized, tissue samples were collected to extract DNA and RAD-seq analysis occurred. Bioinformatic analysis was conducted using the software Stacks, analysis was conducted *de novo.* 17 significant SNPs were discovered in this study that were correlated to high and low AE. SNPs were successfully applied using marker-assisted selection in a broodstock generation and spawned

LIST OF ABBREVIATIONS AND SYMBOLS USED

Abbreviation	Definition
A	Aquaculture
AE	Absorption efficiency
AFLP	Amplified fragment length polymorphism
CFIA	Canadian Food Inspection Agency
CI	Condition index
DFO	Department of Fisheries and Oceans
EST	Expressed sequence tag
FET	Fisher's exact test
FISH	Fluorescence in situ hybridization
F_{ST}	Fixation index
GWA	Genome-wide association studies
IPN	Infectious pancreatic necrosis
M	Million
MAS	Marker-assisted selection
MT	Metric Tonnes
NCBI	National Center of Biotechnology Information
NGS	Next generation sequencing
OR	Odds ratio
QTL	Quantitative trait loci
Rad-seq	Restriction enzyme associated DNA sequencing
SH	Shell height
SNP	Single nucleotide polymorphism
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptors
US	United States Dollar
USD	United States Dollar
W	Wild

~	Approximately

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CHAPTER 1: INTRODUCTION

1.1. Introduction

Globally aquaculture is an important industry and is currently the fastest growing food sector in the world (Subasinghe *et al.* 2009). The human population is increasing at such a rate that, food production from land-based agriculture and capture fisheries cannot meet future demands (Subasinghe *et al.* 2009; Godfray *et al.* 2010). This means that the demand for food will continue to increase, and aquaculture has been identified as having the potential to help meet these global food demands (Godfray *et al.* 2010). Canada has a great capacity for aquaculture development due to its abundant coastal regions. According to the Department of Fisheries and Ocean (DFO), in 2014 Canada produced ~133,583 metric tonnes (MT) of farmed food fish that was valued at $664M (USD). The shellfish aquaculture sector is responsible for 29% of the total Canadian production at 39,927MT valued at $75M (USD). Oysters are an important part of the shellfish industry in Canada. However, climate change (Shackell & Loder 2012), pollution (Cauwenberghe & Janssen 2014; Cole & Galloway 2015; Sussarellu *et al.* 2016), and diseases (Ford & Tripp 1996; McGladdery & Stephenson 2005; Bower 2014) are threatening this industry. It is therefore extremely important to conduct research to better understand genomic and physiological processes of oysters so that techniques and breeding programs can be implemented to produce oysters with optimal efficiency and resilience. The following chapter is a comprehensive literature review of the history, the challenges and the evolution of the oyster industry in context to the research objectives of this project.

1.2. Oyster Industry

1.2.1. History of the oyster industry in North America

Since 1608, when Samuel de Champlain first discovered and tasted oysters from the Gulf of Saint Lawrence, they have been an important economic shellfish species in North America (Lavoie 2005; Lavoie, 1978). During the early years, oysters were not typically farmed but rather collected from the wild through a series of hand picking, raking and dredging techniques, which eventually developed into a commercial fishery. This method continued until the late 1700's and early 1800's, when demand was steadily increasing but

oyster beds were becoming depleted and degraded due to overexploitation. In the early to mid-19th century, Chesapeake Bay began exporting live oysters to many depleted bays. It became common practice to export oyster spat in the spring to other bays (MacKenzie 1996; Ingersoll 1881). The spat would be grown on-bottom during summer months, and then sold in the fall (MacKenzie 1996; Ingersoll 1881). Up until the 1950's, the United States (US) was the top producing country for oysters. However, due to pollution, disease outbreak and overexploitation, oyster culture began to drastically decrease. In 1965, Japan became the top oyster producing country and in 1995, Japan fell to China as the top producing country. This rapid decline in oyster production in North America led to the experimentation of off-bottom aquaculture in the region, which was proven to be successful in other regions (Matthiessen 2001; Shaw 1970). Oyster aquaculture first began in the 1600's in Hiroshima Bay, Japan. During this time, bamboo fencing was used to enclose sections of a bay, and a series of bamboo stakes and rocks were used as a substrate for oyster larvae to settle and grow inside the enclosed area (Matthiessen 2001; Shaw 1970; Dean 1902). This method was used until the 1920's, when there was a shift to using a hanging raft as an oyster culture method. Growing oysters in hanging rafts was advantageous as it, increased growth rate, reduced predation, and increased grow-out area (Matthiessen 2001; Imai 1978). After raft culture was established, the longline oyster culture method was developed in 1947 in the Miyagi Prefecture, Japan. The longline method, was adapted for locations that were less sheltered and exposed to winds (Matthiessen 2001; Bardach *et al.* 1972). In North America, experimentation with raft culture did not start until 1965 in the US (Shaw 1970). Today, off-bottom aquaculture is the main form of oyster production in North America.

1.2.2. Oyster aquaculture in Canada

Due to the abundance of coastal areas, and estuary habitats on the Atlantic and Pacific coasts of Canada, oyster production is high and is focused on two main species: The Eastern oyster/American oyster (*Crassostrea virginica*) on the Atlantic coast and the Pacific oyster (*Crassostrea gigas*) on the Pacific coast (DFO 2013). According to DFO (2014), ~12,600MT of oysters were produced in Canada at a value of $26 M. (USD). The Maritime Provinces (New Brunswick, Prince Edward Island and Nova Scotia) are the primary

producer of the Eastern oyster in Canada and in 2014, Prince Edward Island produced 3,321 MT, New Brunswick produced 847 MT, and Nova Scotia produced 314 MT (DFO 2013). In the past, oysters were mainly grown on-bottom, however, there has been a major shift to off-bottom oyster culture (Transport Canada 2007; Doiron 2008). Although Prince Edward Island produces the greatest amount of oysters in the Maritime Provinces, their transition to off-bottom culture has been slower compared to the other Maritime Provinces, such as New Brunswick, which has been the leading province to implement the off-bottom technique (McDonald 2010). In addition, although the Eastern and Pacific oyster are both produced in aquaculture, the spat collection method differ. In Atlantic Canada, Eastern oyster growers rely almost entirely on wild spat collection, by setting spat collectors during the wild population's natural spawning season. However, in British Columbia, the Pacific oyster has the majority of its spat supply coming from hatchery production in Washington, Oregon, Hawaii, Chile, and Alaska (Leask *et al.* 2008; DFO 2015).

1.3. The Eastern oyster (*Crassostrea virginica*)

1.3.1. Natural distribution range

The Eastern oyster, has a natural distribution ranging from the Western Atlantic from the Gulf of Saint Lawrence in Canada, to the Gulf of Mexico, the Caribbean and coasts of Brazil and Argentina (Carriker & Gaffney 1996). The distribution of oysters within this range depends on larval recruitment. The level of larval recruitment is unpredictable due to highly variable spatial and temporal recruitment from year to year. It is believed that nutritional and environmental stress leading to reproductive variability is partially the reason for the variability (Nelson 1905; Helm *et al.* 1973; Kennedy 1996.). Larval recruitment is also partially governed by ocean currents and their ability to survive in different locations (Shumway 1996). In estuaries that have high-tidal currents, recruitment is usually low but consistent. However, in estuaries with low circulation and more freshwater influx, there is usually a higher recruitment with more variability from year to year (Kennedy 1996).

1.3.2. Production in North America

The Eastern oyster is predominately cultivated along the Atlantic coast of North America, with ~98,193MT produced globally at a value of $108,660,000 USD (FAO 2014). In 2014, ~4,496MT of this production was produced in Atlantic Canada; however, due to the cold water temperature associated with the region, production is limited compared to other parts of the world, such as the Eastern US (Eastern oyster biological review team 2007). In 2014, the Eastern US produced ~93,697 metric tonnes of the Eastern oyster, which is ~95% of global Eastern oyster production (FAO 2014).

1.4. Features and anatomy of the Eastern oyster *(Crassostrea virginica)*

1.4.1. General anatomy

The Eastern oyster consists of two valves that are asymmetrical, with the left valve being larger and more cup shaped then the right valve. During the settlement stage of their life cycle, oysters attach themselves on the left valve to a substrate (Galtsoff 1964; Quayle & Newkirk 1989; Eble & Scro 1996; Wheaton 2007). The two valves enclose the oysters and protect its soft organ systems. The two valves are attached by a hinge ligament. The shell is predominantly composed of calcium carbonate crystals in a protein matrix and is divided up into three layers (Galtsoff 1964). The first is a thin outside layer known as the periostracum; the second (central layer) is thick and chalky; and the third layer (nacreous layer) is thin and shiny (Quayle & Newkirk 1989). Oyster shells are highly variable in shape. Oysters grown in softer substrate tend to grow more elongated with a thinner shell, and oysters grown on a hard substrate tend to be more rounded with the left valve being more cupped with a thicker shell. The shell shape and length of an oyster is largely determined by handling and the environment in which it is grown. In high densities, oysters will develop into clumps (i.e. reefs), and will be extremely hard to separate (Galtsoff 1964; Quayle & Newkirk 1989; Wheaton 2007).

Internally, the oyster contains a series of soft organ systems called the visceral mass. The adductor mussel is a large organ, which lies in the posterior region of the body. Its function is to contract and close the shell, or relax and allow the oyster to gape. The adductor muscle

also attaches the visceral mass to the shell (Galtsoff 1964; Quayle & Newkirk 1989; Eble & Scro 1996). The adductor muscle is comprised of two divisions. The first being a large beige coloured region that is responsible for the opening and closing of the shell, and the second is a smaller white region that is a catch muscle, which allows the oyster to hold its valves in a certain position for a period of time (Quayle & Newkirk 1989).

Surrounding the soft organ system and lining the inner shell valves is the mantle, which is a fleshy tissue that has many different functions in the oyster, including protection of surrounding organ systems (Galtsoff 1964; Eble & Scro 1996). The mantle also aids in inflow of water, sensory, shell formation, glycogen and gamete storage (Quayle & Newkirk 1989; Beninger & Veniot 1999). The large central cavity inside the mantle, is known as the pallial cavity (Galtsoff 1964; Eble & Scro 1996) and it is in this cavity that most organ systems are held. The gills and labial palps are ventrally located and the rectum lies dorsally. The heart of the oyster, is located within a pericardial sac that is located on the anterior side of the adductor muscle. Gonadal lobes are located on both the left and right side of the visceral mass and are diffuse between the labial palps and the pericardial sac (Kennedy & Battle 1964; Galtsoff 1964; Quayle & Newkirk 1989; Eble & Scro 1996).

The main organ involved in the respiratory system in the oysters are the gills, which consist of four folds known as demibranchs. The gills are extremely important for many reasons, but especially for the transport and removal of metabolites, including transporting oxygen to cells, food to the mouth and excretion of waste products of metabolic activity. The gills are constantly pumping water by a contraction of lateral cilia within the demibranchs. The adductor muscle, mantle, and gill muscles all play a large role in the regulating the current of water through the gills (Galtsoff 1964). The oyster also contains an open circulatory system that consists of a heart covered by a pericardial sac and a series of partial vessels and sinuses. The heart pumps haemolymph into surrounding tissues, however, haemolymph cells are not contained into vessels, but rather diffused and collected in the sinuses that bathe major organs (Galtsoff 1964).

1.4.2. Digestive system

The digestive system of the Eastern oyster is located throughout the visceral mass, beginning ventrally with the labial palps. The palps are triangular in shape and form a posterior pointed peak (Shaw & Battle 1957). Cilia in the gills, create a water current that move food particles to the labial palps to be actively sorted. The labial palps consist of two pairs of flaps, with each pair consisting of an inner and outer palp. The two outer sets of palps join together above the mouth of the oyster. The inner set of palps join together under the mouth (Galtsoff 1964; Quayle & Newkirk 1989; Eble & Scro 1996). The function of the palps, is to sort and direct food particles into the opening of the mouth. The mouth is directly attached to a small esophagus, which transports food to the stomach. The stomach is a sac that is completely surrounded by the digestive diverticula, which is a series of green-brown tubules (Shaw & Battle 1957). The stomach is divided into two chambers, the anterior and posterior chamber. The anterior chamber, is attached to the base of the esophagus and functions as a ceca. This chamber also gives rise to two ducts that lead to the digestive diverticula. The posterior stomach is attached to the intestine which brings food from the stomach to the rectum. Associated with the intestine is the crystalline style sac, which aids in digestion, however, it is not often observed in the oyster because it is only present when the oyster is feeding. The crystalline style is resorbed into the gastrointestinal lining when it is not being used. The midgut attaches the stomach to the rectum, which runs dorsally over the adductor muscle and ends at the anus (Galtsoff 1964; Quayle & Newkirk 1989; Eble & Scro 1996).

Oysters are considered to be suspension feeders which are organisms that feed on material such phytoplankton that is present in the water column. The Eastern oyster will consume phytoplankton ranging between 1–30µ (Quast *et al.* 1988; Newell & Langdon 1996) and have the ability to filter at a rate of 1.5–10.0$Lh^{-1}g^{-1}$ (Stanley & Sellers 1986; Newell & Langdon 1996). Suspension feeders are faced with two main challenges, regulating the amount of material that is ingested and sorting of edible phytoplankton versus non-edible inorganic matter, poisonous or low nutritional value sources of phytoplankton. The challenge of active sorting of food resources leads to the rejection of certain particles, called pseudofeces (Sierzen *et al.* 1992; Beninger *et al.* 1999). The process of accepting

and rejecting certain particles for digestion, is primarily carried out by the labial palps. The mechanism by which the labial palps decides what should be ingested versus what should be rejected is not completely known. In the past, it has been hypothesized that the labial palps used a series of chemical receptors (chemoreceptors) to specifically detect certain particles (Newell & Jordan 1983). However, more recently, there has been research conducted by Pales Espinosa *et al.* (2009) that hypothesizes that lectins, which are found in the mucous on the labial palps and feeding organs, are able to recognize carbohydrate compounds on the cells surface of phytoplankton, and use this recognition to determine if the particle should be accepted or rejected.

1.4.3. Reproduction

The Eastern oyster is a protandrous hermaphrodite, meaning that they, usually, mature as males but change into a female if triggered to do so by population and environmental cues (Coe 1943; Thompson *et al.* 1996). Larger oysters are usually female, even in their first year of development (Coe 1934). In late summer, the oyster undergoes a period of energy storage in the form of glycogen. Oysters must store sufficient amount of glycogen in the late summer and fall, before winter, for gametogenesis. In the spring, water temperatures above 10ºC stimulate gametogenesis (Kennedy & Battle 1964; Kennedy 1991; Thompson et al 1996; Abgrall *et al.* 2010). Gametogenesis will occur until gonads are fully ripened and are ready for spawning. When water temperatures reach ~22—23 ºC, spawning is stimulated (Galtsoff 1964). In the Maritime Provinces, this period is usually between June–August depending on the year and the location (Sullivan 1948; Kennedy & Battle 1964; Abgrall *et al.* 2010). The Eastern oyster is an external spawner, meaning that fertilization occurs externally and the offspring develop in the water column. (Thompson *et al.* 1996; Eastern Oyster Biological Review Team. 2007). The fecundity of the oysters can differ between individuals based on age, weight, nutrient reserves, habitat, and environmental condition. Females, can release from 5—20 million eggs during a spawning event (Thompson *et al.* 1996; Abgrall *et al.* 2010). After spawning, oysters go through a dormant phase, during which any undischarged eggs and sperm are reabsorbed along with the gonads. Outside of their active reproductive phase, male and female oysters cannot be distinguished from each other (Eble & Schro 1996; Thompson *et al.* 1996).

1.4.3.1. Larval lifecycle

Gametes and sperm are released into the water column where fertilization occurs. If developing at a water temperature of 22—24 ºC, the first polar body is extruded between 25—50 minutes post-fertilization. After this, a second polar body is formed and is extruded within 45—70 minutes post-fertilization (Galtsoff 1964). Once the first and second polar bodies are extruded, cell division forms a blastula approximately four hours post fertilization. After ~4—6 hours post-fertilization, a gastrula develops along with a band of cilia, known as the prototoch. The gastrula continues to develop for another 8—9 hours, and is then considered to be a trochophore larvae, which has the ability to swim. The newly hatched trochophore, is ~ 40—50µ in diameter. During the trochophore stage, a band of cilia is formed at the ventral side, and the digestive system develops. After 24—48 hours, the trochophore develop into the veliger stage (D-shape) (Galtsoff 1964). During the veliger stage, a velum which aids in movement and feeding, develops. Other organs, such as the mouth, stomach, crystalline style, and intestine also develop. During the pediveligar stage, development continues and a well pronounced hinge and umbo are formed. In addition, a foot and byssal gland, which are eventually reabsorbed, are formed (Galtsoff 1964; Baker & Mann 1994). Swimming behaviour occurs throughout the larvae stage until approximately two weeks, when veliger's reach their final stages and begin to develop an eyespot and are light sensitive (Nelson 1926). At this point, they begin to metamorphose and settlement occurs (Coon *et al.* 1990). During the settlement phase, the oyster larvae attaches to a substrate by secreting cementing fluid from the byssal gland, and the velum and foot are reabsorbed (Nelson 1926). The newly attached juvenile oyster (spat) are no longer motile. After ~6—10 weeks post-settlement the Eastern oyster will begin to develop gonads, and are considered to be an adult (Galtsoff 1964).

1.4.4. Environmental tolerances and growth

The Eastern oyster can grow in a variety of different environmental conditions and on a variety of different substrates, however, they are most suited for the sub-tidal or intertidal zone on reefs, rocky coastlines and semi-firm mud bottoms. They are not well adapted for softer substrates, such as sand and soft mud (Galtsoff 1964). They exhibit a wide tolerance

to temperature (-2—36ºC) and salinity (5—40‰) (Galtsoff 1964; Carriker and Gaffney 1996) with their optimal temperature range being from 20—30ºC (Stanley and Sellers 1986), and salinity between 10—27‰ for larvae (Calabrese & Davis 1970) and 14—28‰ for adults (Moore 1900; Butler 1949; Chanley 1958; Galtsoff 1964). They commonly grow in estuaries where water currents are calm, non-turbulent, but with enough circulation that fecal and gaseous wastes are carried away (Galtsoff 1964). In addition, oysters feeding rates are maximized between 5—10mg/L concentration of diatoms and phytoplankton. After this point, higher concentrations of seston results in pseudofeces release (Higgens 1980). Feed availability stress has been found to occur at, food concentrations of 6% bodyweight in hatchery conditions (Helm *et al.* 2004).

Growth rate depends on many factors including: temperature, food availability and life-stage. In warmer climates, such as in the Gulf of Mexico, oysters can reach market size (~75mm) in 18—24 months, however, oysters in cooler climates take 3—5 years (Shumway 1996). It has been found, in the first six months oysters grow on average 10mm/month, however, after this stage growth rate decreases (Quast *et al.* 1988). Another study on the Eastern oyster in Chesapeake Bay, suggest that growth rate decreases linearly from 28.97—0.85mm/year from age 0—5 years and found it took ~3 years, on average, to reach market size (Coakley 2004). Growth rate is controlled by many factors, including genetics and physiology. For instance, Sydney rock oysters (*Saccostrea glomerata*) that were selectively bred for growth exhibited a higher feeding rate, and had a lower metabolic rate and cost in terms of growth than non-selectively bred ones (Bayne *et al.* 1999; Bayne *et al.* 2000). In addition, they gained weight in terms of protein deposits, whereas, the non-selective bred oysters gained weight in terms of lipid deposits, which appeared to be a trade-off in terms of reproduction, as lipid deposits are beneficial in gametogenesis (Bayne et al 2000). Another study linking the correlation of growth, studied filtration rates, absorption efficiency and metabolic rates in the Pacific oysters. Physiological differences were compared between spat bred for growth versus slow growing spat at three food concentrations (0.5, 1.5 and 3.0mg/L). Higher food concentrations caused higher energy gain rates; however, absorption efficiency was similar between all concentrations. Similar to the study carried out with the Sydney rock oyster, the Pacific oyster spat selectively bred for growth had higher energy gains, lower metabolic rates and energy costs (Tamayo *et al.*

2014). Furthermore, Pernet *et al.* (2008) discovered that the presence of a polyunsaturated lipid membrane and the presence of lower metabolic rate increased the growth rate of the Eastern oyster.

1.5. The Eastern oyster aquaculture production

1.5.1. Oyster culture in the Maritime Provinces

1.5.1.1. Wild spat collection

Spat can be collected from the wild during spat fall period or purchased from a hatchery (Doiron 2008). In Atlantic Canada, the most common method for obtaining spat is through wild spat collection. In the past, spat was collected on Chinese hats; however, recently growers have been using plastic tubes, which are inexpensive and easier for spat removal. During wild spat collection, structures are hung in the water column. Eyed larvae, then settle and metamorphose on the collectors and grow to 5–15mm before they are transferred to a grow-out rearing system (Doiron 2008).

1.5.1.2. Grow-out methods

Off-bottom culture is conducted in the water column, rather than on the ocean floor, which increases food availability and allows oysters to grow to market size in a shorter time period (3—5 years, rather than 5—8 years on-bottom) (Bastien-Daigle *et al.* 2007). There are two methods of suspended culture commonly used. The first is a floating bag system, which uses a series of oyster bags that are maintained at the surface of the water. A set of cylindrical buoys are attached to each side of the bag, which helps keep it afloat The second method is the long-line method, which consists of two rows of ~50 bags, which are held together using a series of ropes and rigid crossbars. The system is usually 60m long and 2.4m wide and has cylindrical buoys attached to the bags to keep them afloat (Doiron 2008). The density of the oysters in the bags is a key component of growing oysters efficiently in suspended aquaculture. The density of oysters in a bag is decreased as the oysters grow, since oysters are subject to their environment, and high densities cause oysters to clump and grow in odd shapes (Galtsoff 1964; Quayle & Newkirk 1989; Wheaton 2007). Generally the density starts out as 1,000 oysters per bag in the first year,

and decreases to 500 oysters per bag in the second year. In year three, the oyster density decreases to around 200 oysters per bag to ensure overcrowding does not damage the shells before oysters reach market size. In Atlantic Canada, Eastern oysters are considered to be market size at ~75mm (Doiron 2008).

1.5.2. Hatchery production methods

1.5.2.1. Feed production

Algae production is one of the most important activities in a hatchery. In order to successfully condition broodstock and rear larvae, it is imperative that large quantities of high quality algae be produced. Production begins with the master/stock algae culture, this is usually contained into a small (250mL) Erlenmeyer flask containing a monospecific free-living culture of algae (Helm *et al.* 2004). The master stocks are used to inoculate the next stage of culture, which are usually grown within a 4L Erlenmeyer flasks (Helm *et al.* 2004). After inoculation, the algae will be grown to an appropriate density and then transferred to a 20L Carboy tank where it grows to an appropriate density (Helm *et al.* 2004). The final stage, is to transfer culture into a larger reservoir, usually a Kalwall tube that can hold over 50L of algae. The algae is further grown, until it reaches a suitable density for harvesting and then is used to feed broodstock and larvae (Helm *et al.* 2004; Doiron 2008). At all stages, algal culture require additional nutrients which is a mixture of nitrates, phosphates, and essential trace elements (Helm *et al.* 2004).

1.5.2.2. Broodstock conditioning

Broodstock conditioning is the process, in a hatchery setting, where gametogenesis is stimulated. Conditioning involves many aspects of culture including optimal tank set up and feeding regimes, stimulating gametogenesis, and spawning methods (Helm *et al.* 2004). Broodstock, which are either collected from the wild or are grown from the larval stage, are kept in tanks which contain optimal water conditions, with either a flow through systems at no more than 5kg biomass to 150—120L or recirculating system at low oyster density 2—3g biomass per 1L (Helm *et al.* 2004). In a recirculating system, water is changed at least twice a week. Broodstock tanks are usually shallow tanks that contain

trays supported off-bottom. The seawater in the broodstock environment, is most optimal when not filtered, as it contains nutrients that wouldn't be present if filtered. The Eastern oyster is conditioned ideally at a salinity of 25‰, temperature 18—20ºC, and flow rate of ≤25mL/min (Helm *et al.* 2004). In addition, it is important that the broodstock conditioning area be in a quiet location, without frequent disturbances such as vibrations which cause the oysters to shut their valves and cease feeding or spawning (Helm *et al.* 2004).

The most common species of algae and diatoms fed to oyster broodstock during conditioning are *Tetraselmis sp., Isochrysis galbana, Monochrysis lutherii, Chaetoceros muelleri, Thalassiosira pseudonana, T. weisfloggi* and *Skeletonema costatum* (Helm *et al.* 2004). Using a mixture of these species during the conditioning process, is optimal to ensure a balanced diet. Oysters are optimally fed between 2—4% of their dry body weight daily, not exceeding 6%. Having too many particles in the water can cause sorting stress to the oyster. Preconditioning is started early in the season when water temperatures are colder and before gametogenesis. Oysters are fed 4—6% of dry body weight for ~4—6 weeks, in order to stimulate increased glycogen reserves (Helm *et al.* 2004). After this, water temperature is increased 1—2ºC per day to 18—20 ºC and oysters are fed at 2—3% dry body weight concentration. The oysters remain at this temperature for an additional 28—42 days to further stimulate gametogenesis (Helm *et al.* 2004).

After the conditioning period, spawning is conducted. There are a two main methods to induce spawning. The first is manual strip spawning, which involves killing the broodstock and then manually removing the gametes (Helm *et al.* 2004). Broodstock are shucked open, and the flat shell is removed. Gametes reside over the digestive gland and develop towards the umbo and over the adductor muscle (Helm *et al.* 2004). There are two methods of removing gametes from the oyster. The first is to make continuous cuts using a scalpel into the gametes and then washing using filtered seawater into a beaker (Helm *et al.* 2004). The other method is to use a Pasteur pipette to draw the gametes out and then are placed into a bucket containing filtered seawater (Helm *et al.* 2004). It's important during this method, to avoid rupturing the digestive gland, which could cause bacterial contamination of the eggs and sperm (Helm *et al.* 2004). In addition, eggs and sperm are kept separate during this method; therefore, before stripping the oysters need to be identified as male or female

using a microscope under 40 or 100X magnification. During this step, broodstock are shucked, and a small gonad sample is viewed under the microscope and identified as male or female. Once identified, the flat shell is laid on top of the oyster while waiting to be stripped (Helm *et al.* 2004).

The second method to spawn oysters through induction, by manipulating environmental parameters. A variety of different stimuli can be used to stimulate spawning; however, the most successful method is using temperature stimuli. The first step in this method, is to take oysters out of the broodstock tanks and clean to remove any fragments or fouling organisms (Helm *et al.* 2004). After cleaning, the oysters are placed into a spawning tank. The spawning tank is usually a shallow trough, so that the oysters can be easily viewed. The bottom of the trough is ideally black to aid in visibility of gametes when they are released (Helm *et al.* 2004). Spawning is induced using this method by creating rapid temperature changes. The first step is placing oysters in water at ~12—15ºC for ~30— 40 minutes. After this time, water is drained and warmer water is added to the tank at (25—28ºC). Again, oysters are left in this water condition for ~30—40 minutes (Helm *et al.* 2004). In both cycles, food is added into the spawning tanks to stimulate the oysters to open up. This cycle of warm and cold water can continue for up to 3—4 hours. In the summer months, oysters are usually induced to spawn within an hour of induction; however, early in the season it may take the full 3—4 hours (Helm *et al.* 2004). If oysters have not been stimulated by this time, then it is best to return them to the broodstock conditioning tanks for an additional week. In addition, other stimuli, such as sperm, can be added into the water column. Male oysters naturally spawn first, and this stimulates females to spawn (Helm *et al.* 2004). Once spawning has been induced, it is important to quickly remove spawning individuals and place them into individual containers to keep gametes separate. Gametes are identified from each oyster as male and female and then are marked. Spawning will occur for no longer then 40—60 minutes, and after this time the broodstock should be removed from the gametes, as the adults will filter feed the gametes if both are left together in the same container (Helm *et al.* 2004).

After either method of spawning (strip spawning or induction), eggs should be separated by washing them through a 90μm nylon sieve to remove debris and then through a 20—

40μm nylon sieve to retain the eggs. Sperm from the various males are pooled together and 1—2mL of sperm is added to each container holding eggs. Once the sperm is added to the eggs the mixture sits for 60—90 minutes, before the contents carefully placed into the larval rearing tanks (Helm *et al.* 2004).

1.5.2.3. Larval rearing

Larval rearing is conducted in circular or semi square, flat or conical bottom tanks, made out of polyethylene or fibreglass, and set up with a static water flow. After ~60—90 minutes, post-fertilization larvae are stocked into these larval tanks at a density of ~15,000—20,000 Larvae/L (Helm *et al.* 2004). The newly fertilized eggs are left for 24—48 hours to develop into D-stage larvae (Helm *et al.* 2004; Seafood Industry Training Organization 2006). After ~24—36 hours small amounts of algae is added to the tank for the first feeding (Helm *et al.* 2004). Approximately 48 hours post-fertilization, larvae tanks are drained using a series of nylon mesh sieves, including a 60μm sieve over a 40μm sieve. The arrangement of sieves ensures that grading of different size larvae can occur, while also protecting the fragile larvae from drying out (Helm *et al.* 2004).

After the initial filtration, larvae will be feeding and have specific dietary needs. In general, *Chaetoceros calcitrans, Chaetoceros muelleri* and *Thalassiosira pseudonana* are the most commonly fed diatoms and *Isochrysis galbana, Monochrysis lutherii* and *Tetrasemis* species under 120μm, are the flagellate species most commonly used (Helm *et al.* 2004). Larvae should be fed daily with rearing tanks cleaned every 2—3 days. It takes ~15—20 days for the Eastern oyster to develop an eyespot and undergo metamorphosis (Helm *et al.* 2004; Seafood Industry Training Organization 2006). Oyster larvae are free-swimming organisms; however, when they are getting ready to undergo metamorphosis feeding activity slows and larvae tend to spend more time at the bottom of the tank. There are two phases of settling. Firstly, when larvae settle to the bottom of the tank and use their foot to crawl around and look for an appropriate substrate. Secondly, when metamorphosis occurs and substantial physiological changes occurs to create a newly attached, sessile spat (Helm *et al.* 2004). In a hatchery, the eyespot is a major indication that settling and metamorphosis

will soon occur. At this stage, there are two options to provide oyster growers with eyed larvae for remote setting or continue settling in a hatchery (Helm *et al.* 2004).

1.5.2.4. Spat rearing

At the time of settling, oysters larvae are ≥290µm in size (Wallace *et al.* 2008). The first step at this stage, is to sieve out the larvae in the tanks and grade based on size. Oyster larvae that pass through an 180µm (254µm diagonal opening) sieve are returned to the larval tank. The larvae that are retained on the 180µm sieve, are sieved again through a 210µm (296µm diagonal opening) sieve (Wallace *et al.* 2008). Again, the larvae that pass through the screen are put back into another larval tank. The retained larvae are greater than 296µm, and ready for settling (Wallace *et al.* 2008). At this stage, eyed larvae are usually given a cultch substrate to settle on, such as old PVC pipes, shell chips (250—500µm), aged clean shells attached to gear or ropes, cement-coated or plastic Chinese hats.

Chemical induction is also possible to induce settlement of individual oysters (Helm *et al.* 2004). Typically, settled individual oysters are preferred by growers. This can be obtained using shell chips as a cultch. Settlement can occur in a downweller system, which consists of a bucket with a fine mesh bottom. Water is set to flow down through the meshed bottom and shell chips are placed in a layer over the fine mesh bottom. During settlement, tanks are covered with a black tarp, as darkness encourages settlement behaviour (Wallace *et al.* 2008). Larvae retained in the 210µm sieve are ready to be placed in the downweller. At this stage, oysters are stocked at a density of 100 larvae per square centimeter. Larvae are kept in the downweller until settlement. Metamorphosis occurs usually within 48h (Wallace *et al.* 2008). Once the larvae are settled, the downweller is converted to an upweller, whereby the flow of water passes up through the fine screen (Wallace *et al.* 2008). Spat can be grown in an upweller until they reach 5mm shell length, at which time they are then ready to be transferred to a grow out system (Doiron 2008).

1.6. Production issues with oyster aquaculture industry

1.6.1. Environmental impacts of oyster aquaculture

The aquaculture industry is currently undergoing a major assessment to ensure it is environmentally sustainable (Fisheries and Oceans Canada 2010; Troell *et al.* 2014; Byron & Costa-Pierce 2013). In Atlantic Canada, oyster production is a fairly natural process with very few inputs into the water, except for infrastructure—long line systems, buoys, cages etc. (Bastien-Daigle et. al.2007; Doiron 2008). The addition of oysters in high densities is raising concerns about the potential for negative effects to the water column and the related interactions, such as carrying capacity, organic composition, population dynamics, hydrodynamics, fouling organisms, predators, and biodiversity (Bastien-Daigle *et al.* 2007). A risk assessment conducted by Bastien-Daigle *et al.* (2007) for Department of Fisheries and Oceans considered oyster aquaculture to be of low impact due to the scale at which it is occurring in the region, and most concerns could be minimized through proper site selection (Bastien-Daigle *et al.*2007; Guyondet *et al.* 2005, 2013).

1.6.1.1. Exceeding carrying capacity of an ecosystem

Carrying capacity of an environment was defined by Kaiser and Beadman (2002) as the maximum production a species or population can support in relation to the available resources. In Atlantic Canada, the risk of exceeding carrying capacity on oyster culture sites is considered low (Bastien-Daigle *et al.* 2007). Due to the current market demand for oysters, the industry will most likely continue to grow (Chopin 2015), which increases the risk of exceeding carrying capacities in certain bays. This risk can be minimized through proper site selection, where food renewal is high (Guyondet *et al.* 2005, 2013). Moreover, since no feed is added to the environment, other filter-feeding organisms in the ecosystem might be competing for the same food source.

1.6.1.2. Biodeposits: Implications for benthic environment

Oysters create waste products in the form of fecal matter and pseudofeces as direct products through feeding and digestion. When producing large quantities of oysters, such as at an aquaculture site, accumulation of these waste products form biodeposits, usually under or

around the lease (Newell & Mann 2012; Gallardi 2014). There is concern related to the effect of biodeposits on the ecosystem. These concerns are often focused on the effects of the increased organic load on the ecosystem, which has been shown to ultimately cause an anaerobic environment to develop. This ultimately leads to a higher bacteria load, higher prevalence of benthic invertebrates, and decrease of macrobenthic infaunal abundance (Cranford *et al.* 2003; McKindsey *et al.* 2006; Callier *et al.* 2009; Gallardi 2014). In such a case, it is extremely important to reduce the biodeposits by modifying farm practices or production to allow the ecosystem to recover (Newell &Mann 2012). However, these effects are variable, site specific, and also cannot be applied to all situations. Biodeposits can be processed by the ecosystem in many ways by bacteria and metazoan; however, how these biodeposits are processed depends on the location and the environmental conditions (Newell &Mann 2012). As discussed, there is evidence that supports that negative effects due to biodeposits is occurring, especially with other species of shellfish such as mussels (Kaspar *et al.* 1985). In contrast, there is also evidence to support that shellfish aquaculture has minimal effect (Crawford *et al.* 2003). The concerns for the benthic environment are usually in sheltered locations, or where tidal and water currents may not be adequate to appropriately disperse the biodeposits; therefore, these effects could be mitigated by proper site selection (Newell & Mann 2012).

1.6.2. Spat collection variability

The quantity of oyster spat that is collected from the wild can fluctuate greatly from year to year. Fluctuations are due to environmental conditions that can either be beneficial or detrimental to fecundity and early larval survival (Doiron 2008). In Atlantic Canada, there are many sites that are known to consistently have highly successful spat collection and other sites where spat production fluctuates. An example of this, is Bouctouche Bay and Caraquet Bay in New Brunswick. Bouctouche Bay is known to have excellent spat collection from year to year, whereas, Caraquet Bay is known to have fluctuating yields of spat collection (Doiron 2008). Méthé and Leger (1994) assessed oyster spat, growth and collection in Bouctouche Bay from 1987 to 1993 and although great spat yields, there was some fluctuations between years. Caraquet Bay spat collection was also assessed from 1979—2014 and showed similar results, with most years having great spat collection but

there have been great fluctuations, especially 1983—1992, when lower spat collection occurred more frequently (Doiron 2014). This variation in accessibility to spat, can make it difficult for oyster growers to maintain a constant supply of oysters on their culture site.

1.7. Review of genomic information known on the Eastern oysters

1.7.1. General genetic information known

The Eastern oyster is considered to be a non-model species and therefore does not have a reference genome. One of the first genetic studies conducted on the Eastern oyster, discovered that they exhibit standard meiotic divisions, and have 10 pairs of chromosomes (Longwell *et al.* 1967). In addition, they do not exhibit sex chromosomes, which is indicative of protandrous hermaphrodites. All sets of chromosomes appear to be very similar in size, with the exception of the largest and the smallest set (Longwell et al 1967). Having basic knowledge of chromosomal structure, is an important part of understanding Eastern oyster genomics. The first attempt at mapping chromosomes was conducted by Wang *et al.* 2005(a), who characterized chromosomes using P1 bacteriophage identification by using fluorescence in situ hybridization (FISH). During this study 21 P1 clones were used and provided clear FISH signals, and 9 P1 clones were assigned using karyotyping and co-hybridization to seven chromosomes. Five of the 9 P1 clones in this study were sequenced. Since this study, the use of FISH has continued to be used to develop cytogenetic maps. Currently there are several loci identified including 9 P1 clones (as mentioned above), major 18s—5.8S—28s and minor 5s rRNA, and an unknown sequence (Wang et al 2005(a); Wang et al 2005(b); Guo et al 2008).

Other research to develop a linkage map for the Eastern oyster was developed by Yu and Guo (2003) using amplified fragment length polymorphisms (AFLPs), microsatellite and Type I markers. Since this time, functional genes have been mapped, with the use of Type 1 markers. Currently there are 47 type 1 markers that have been mapped, these markers consist of 17 single nucleotide polymorphisms (SNP) and 30 expressed sequence tags (EST) (Guo *et al.* 2008; Zang & Guo 2010).

1.7.2. Genetic markers

1.7.2.1. Disease markers

Diseases such as Dermo (*Perkinsus marinus*) and MSX (*Haplosporidium nelsoni*) have been a major issue in the Eastern US since the late 1950's (Ford and Haskin 1982; Ford & Tripp 1996). Disease resistance is not something expressed phenotypically, without the disease present in the environment; therefore, it is extremely important to be able to discover genetic markers that are associated with it. A study was conducted by Yu and Guo (2006), to discover quantitative trait loci (QTL) that were associated to disease resistance for Dermo and MSX. Amplified fragment length polymorphism markers were discovered in the genome of the Eastern oyster before and after exposure to Dermo. After the exposure, there appeared to be a shift in genotypes in the population. It was discovered that markers that were associated with this genotype shift were closely linked on a genetic map, which indicates that disease resistant genotype was not random. In this study, 12 putative QTLs were discovered in male and female oysters that showed resistance, indicating that it was a candidate for further analysis (Yu & Guo 2006).

Another study conducted by Yu *et al.* (2011), looked at the *cvSI-1* gene which belongs to the serine protease inhibitors family. This gene was determined to potentially have a link with resistance to Dermo. This study looked at polymorphisms occurring in the *cvSI-1* gene, to determine the association between these polymorphisms in either resistance or disease induced mortality. A total of 12 SNPs were identified but the SNP198, with the C allele, appeared to be greatly upregulated in the population after disease induced mortality occurred by Dermo and potentially MSX. It was determined that SNP198 (C allele) may have an association to disease resistance due to a potential link between the SNP and a function polymorphism (Yu *et al.* 2011).

1.7.2.2. Growth markers

Other research has been conducted to discover growth markers in the Eastern oyster. One study conducted by Singh and Zouros (1978) found that there were significant differences between growth rate of one year old oysters from Nova Scotia with the genotype *Got-1*

*(*glutamate oxaloacetate transaminase), which is synonymous with *Aat* locus (aspartate oxaloacetate aminotransferase). Oysters that were homozygous 4/4 genotype, were larger than oysters with 2/4 heterozygotes and the 2/2 homozygotes (Singh and Zouros 1978). However, two years later, another year old cohort was tested from the same population and it was found that the 2/4 heterozygotes genotype and 4/4 homozygous genotypes appeared to be similar in size and 2/2 were smaller (Zouros *et al.* 1980). In addition, aminotransferase has also been found to be associated with size in the Pacific oyster (Sugita and Fujio 1982) In this case, oysters that were homozygous for B/B at the *Aat* locus were 50% larger than A/B.

In addition, Zouros and Singe (1978) and Zouros *et al.* (1980) gave rise to significant information on the relationship between multilocus heterozygosity and fitness related traits such as growth and viability. Singh and Zouros (1978) found that heterozygosity in four loci considered in this study, resulted in faster growth rate. Moreover, using a larger sample size and two additional allozymes gave the same results indicating that multilocus heterozygosity was positively correlated to growth rate (Zouros 1980; Koehn and Gaffney 1984; Gentili and Beaumont 1988; Koehn *et al.* 1988; Zouros *et al.* 1988; Alvarez et al 1989). However, studies by Beaumont *et al.* (1985) and Gaffney (1986, 1990) did not yield similar findings. In addition, to growth, multilocus heterozygosity also appears to be correlated with the viability of young oysters. In fact, oysters that exhibit heterozygosity, have been found to have a higher survival rate at two weeks post settlement and after three years of growth (Zouros *et al.* 1983).

Koehn and Shumway (1982), discovered that there was a correlation between oysters that were multiloci heterozygous and the amount of oxygen consumed. Oysters that were heterozygous at multiple loci used 10—20% less oxygen then homozygous oysters. There also appeared to be an additive affect for each locus in that the more heterozygous the oyster the more substantial difference in oxygen consumption.

1.7.2.3. Population genomics

There has been a great debate regarding the genetic diversity of the Eastern oyster from the Atlantic Coast of Canada to Gulf of Mexico. In the past, it was believed that genetic

diversity was evenly distributed. This is due to its long pelagic life stage which allows it to be carried to new locations, and the fact that it has been physically transferred to many bays since the 1800's (Eastern oyster biological review team. 2007). This was confirmed initially by Buroker (1983), who used protein-gel electrophoresis to examine 19 different population of oysters from the Atlantic region, to the Gulf of Mexico. Genetic similarity was determined using 32 structural loci and found that there was a low genetic diversity from Cape Cod, MA to Corpus Christi, TX. It was estimated that these populations had 99% similarity, while the Brownsville population of Lugana Madre was found to be highly genetically different with only a 93% similarity to other populations. Other studies found similar conclusions (McDonald *et al.* 1996; Small & Chapman 1997). However, there are major flaws in the majority of the studies that conclude only one population exists. After rerunning the studies to include more populations, two genetically distinct populations were discovered (Eastern oyster biological review team. 2007). In addition, there is a great deal of evidence to suggest that there are two or more genetically distinct populations from the Atlantic Coast to the Gulf of Mexico. In 1990, Reeb and Avise conducted a study using restriction site enzymes in mitochondrial DNA, to survey genetic diversity between populations of oysters in the Gulf of St. Lawrence and Brownsville, TX. This study determined that diversity was high, indicating that the northern and southern populations of the Eastern oyster showed very different genetic similarities. The transition between the two populations was found to be along Florida's mid-coast. The current consensus amongst the scientific community is that Eastern oyster populations are two distinct populations, (Eastern oyster biological review team. 2007; Karl & Avise 1992; Cunningham & Collins 1994; Hoover & Gaffney 2005; Varney *et al.* 2009, 2016).

1.8. Development of a hatchery-based selective breeding program

1.8.1. Existing selective breeding programs

Selective breeding for the Eastern oyster has occurred both unintentionally, by grading based on size, and intentionally, for specific traits through selective breeding programs. The Eastern US, more specifically, have been selective breeding for specific traits such as disease resistance. This is mostly due to disease outbreaks, since the 1960's, after the first

outbreak of MSX in Delaware Bay and Chesapeake Bay, US. Selective breeding programs relied heavily on traditional methods of selective breeding, through family-based lines that exhibit desired phenotypes (Allen *et al.* 1993; Ford & Haskin 1982; Ford & Tripp 1996). Rutgers University in New Jersey, through the Haskin Shellfish Research Laboratory was the first research group to begin selective breeding for disease resistance. After years of breeding, five lines of MSX resistant oysters were developed (Haskin and Ford 1979; Guo *et al.* 2008). By 1990, two issues became apparent in oyster populations in Delaware Bay. First, an outbreak of a disease known as Dermo, and, second, inbreeding amongst MSX resistant oysters (Guo *et al.* 2008). As a result, in 1992 the breeding program at Rutgers University started selecting for Dermo disease resilient strains of the Eastern oyster. Rutgers also increased the diversity in the stock, by crossing MSX resistant oysters from mid-Atlantic and Northeastern region. This cross produced a new strain of Eastern oysters, the Northeast High Survival line (NEH) that had dual resistance to dermo and MSX (Allen 1993; Guo *et al.* 2008; Guo *et al.* 2003).

Meanwhile, companies such as the Frank M. Flower's Oyster Company of New York were interested in developing faster growing oysters for cold water conditions and strains resistant to Roseovarius oyster disease (ROD) (*Roseovarius crassostreae*). As a result, a line of oysters with superior growth and resistance to ROD was developed, and named the Flower's strain (Farley et al 1998). Limited research has occurred since, due to the fact that this can be achieved through triploidy and tetraploidy. Growing triploid and tetraploid oysters has become a common practice in the US. Triploid oysters were first developed by Stanley *et al.* (1981) by stopping the extrusion of the polar bodies directly after fertilization by using a chemical called cytochalasin B. Triploid oysters exhibit a higher growth performance because they are sterile animals that put all their energy into producing muscle tissue instead of gametes. In addition, more success has come from using tetraploidy, which was first discovered by Guo and Allen in 1994, using the Pacific oyster at Rutgers University. Tetrapoidy was developed by successfully crossing a triploid with a diploid oyster.

1.8.2. Marker-assisted selective breeding programs

Although there has been a lot of success using traditional methods, there are also several limiting factors associated with this technique. The first, is that it is a time consuming method that relies heavily on phenotypic characteristics to be evaluated, and doesn't consider other factors associated with phenotype, such as environment and genomics. In other instances, traditional selective breeding programs have caused a loss of genetic variation from inbreeding (Guo *et al.* 2008). Due to these limitations, the use of marker-assisted selection (MAS) has gained in popularity. MAS uses sequencing technologies, which give the ability to detect markers that are correlated with traits of interest. As our knowledge of the Eastern oyster genetics increases, our ability to develop marker-assisted selective breeding also increases. Several studies have already begun, such as the development of the linkage maps and identification of QTLs associated with specific traits such as disease resistance (Yu & Guo 2003; Yu & Guo 2005). The use of DNA markers for genotyping, has also been occurring. There are two key markers types that are promising in MAS, including SNPs and microsatellites. Microsatellites are a type of polymorphisms associated with tandem repeats in a DNA strand, whereas, SNPs are single mutations at a specific nucleotide location in a DNA strand. Although marker-assisted selective breeding programs are becoming more popular as an alternative method for traditional selective breeding programs, there are no marker-assisted selective breeding programs currently being used, to knowledge, for the Eastern oyster (Guo *et al.* 2008; Yue 2013).

1.8.2.1. RAD-seq analysis: a tool for marker discovery in non-model species

Restriction site associated DNA sequencing (RAD-seq), is a new technology that uses Illumina sequencing technology to identify many genetic markers throughout the entire genome of target organisms (Baird *et al*. 2008). This technology, has major advantages compared to other marker discovery technology, especially for non-model organisms such as the Eastern oyster where a reference genome is not available. Using technologies such RAD-seq, thousands of SNPs can be found that are correlated to specific desirable phenotypic traits. In this method, restriction enzymes are used to cut at specific locations

along the DNA strand. Illumina based Next Generation Sequencing (NGS) technology, is then used to sequence these restriction enzymes cut site locations (Davey & Blaxter 201; Davey *et al.* 2011). The product of RAD-seq are fragments called RAD-tags, which can be analyzed using available software, including Stacks (Catchen *et al.* 2011; 2013). RAD-seq can be used to compare different sequences at restriction sites that are present or absent of SNPs, allowing for a comparison of the marker prevalence between individuals. If there is no reference genome available, the data can be analyzed *de novo*. Using this method, identical sequences can be matched and treated as potential alleles which allows for SNPs to be found within (Davey & Blaxter 2011). Using this method, it is possible to compare individuals exhibiting phenotypic traits and discover thousands of SNPs correlated to them. These SNPs can then be identified in a broodstock, and used as a method of MAS (Yue 2013).

1.8.3. Importance of development of a hatchery industry in Atlantic Canada

With wild spat collection being a variable source to an industry already having difficulty in keeping up with product demand, it has become apparent that a hatchery model that produces a stable and high quality spat source needs to be developed. Globally, many shellfish aquaculture sectors have started switching from wild-caught spat to hatchery spat. In North America, receiving spat from a hatchery is standard practise for the Pacific oyster (*Crassostrea gigas*) in British Columbia, and for the Eastern oyster in the US (Flimlim 2012; Center for Shellfish Research 2008). New Brunswick and Nova Scotia have also begun the development of a hatchery system for their oyster aquaculture sector (Aquaculture Association of Nova Scotia 2012; Doiron 2008; Aquaculture Association of Nova Scotia 2009). Implementing a hatchery source gives growers a consistent supply of spat from year to year. In addition, it means there is an opportunity to improve spat quality by selective breeding using MAS of desired traits, such as increased growth, resiliency to environmental condition and disease. Furthermore, it may be argued that the importation of spat from the Eastern US could be a feasible solution, since they already have selective bred oysters for many of the traits of interest. However, due to the presence of many pathogens not present in Atlantic Canada (Ford & Tripp 1996; McGladdery & Stephenson 2005; Bower 2014), and the fact that the oysters have been found to be genetically different

(Reeb & Avise 1990; Eastern oyster biological review team. 2007; Karl & Avise 1992; Cunningham & Collins 1994; Hoover & Gaffney 2005; Varney *et al.* 2009, 2016), the risk of transport is too high to be considered by industry regulators.

1.8.4. Important traits for a breeding program

1.8.4.1. Stress and disease resiliency

There are many stressors present in the Eastern oyster's ecosystem due to climate change (Shackell & Loder 2012), emerging diseases (Ford & Tripp 1996; McGladdery & Stephenson 2005; Bower 2014) and environmental pollutants (Cauwenberghe & Janssen 2014; Cole & Galloway 2015; Sussarellu *et al.* 2016). This means oysters need to exhibit resiliency. Selective breeding programs in the US have had great success in developing disease resistant oysters. Diseases, such as MSX and Dermo, pose a tremendous threat to oyster populations in the Atlantic region. MSX has already invaded the Bras d'Or lakes in Nova Scotia, and Dermo continues to move further North with its closest invasion being in Maine, US (Ford 1996). It is therefore of upmost importance that disease resistance and stress resiliency to environmental factors, be implemented in an oyster breeding program in oysters in Atlantic Canada as soon as possible.

1.8.4.2. Feed efficiency ratio and growth

In the Eastern US, growth has been selected as a trait of interest for the Eastern oyster, however, feed efficiency ratio has not been considered yet. As climate changes occurs, changes to the environment will follow, which will lead to changes in phytoplankton distribution (Shackell & Loder 2012). Wild populations are also recovering from Malpeque disease that occurred in the 1950's (Bastien-Daigle *et al.* 2007). While this is occurring, the demand for oysters exceeds production, indicating that aquaculture oyster production will increase as well (Chopin 2015). It is therefore important to ensure that the carrying capacity of the ecosystems in which we grow oysters is not compromised. Improved feed efficiency and growth in cultured oysters will ensure that they reach market earlier, while at the same time reduce their ecological footprint. Selecting for these traits in a hatchery-

based industry could be beneficial in ensuring the aquaculture production can increase without depleting natural microalgae resources.

Feed efficiency ratio in the most basic terms, is a measurement of an animal's efficiency at converting feed into growth, usually in the form of muscle mass. There are several factors contributing to feed conversion ratio/feed efficiency, one being energy dynamics (Bayne & Newell 1983). Absorption efficiency and net and gross growth efficiency are the main factors that affect energy dynamics. Energy acquired from the environment must be balanced with its maintenance costs in order for somatic and reproductive growth to occur (Bayne & Newell 1983). Feed efficiency ratio/feed and growth has been improved in many other aquaculture species such as in coho salmon (*Oncorhynchus kisutch*), Japanese flounder (*Paralichthys olivaceus*), Rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), etc. (Thodesen *et al.* 1999; Ogata *et al.* 2002; Silverstein *et al.* 2005; Neely *et al.* 2008).

Absorption efficiency is defined as the efficiency at which the ingested food is absorbed (Bayne & Newell 1983). There are many factors that affect absorption efficiency in mollusks. Absorption efficiency is partially controlled by genetic factors (Prudence *et al.* 2006). Other factors involved in absorption efficiency are environmental, such as food concentration and composition. It is known that mollusks being fed algae sources in a hatchery can be up to 80% efficient, whereas oysters being fed natural sea water can be as low as 30—60% efficient (Bayne 1976; Winter 1978; Bayne & Newell 1983). Furthermore, the type of species being fed can also be a factor that effects absorption efficiency. It has been found that flagellates such as *Isochrysis galbana* are more digestible then other phytoplankton sources such as diatoms which contain a thick cell-wall that is hard to digest (Navarro *et al.* 2009 Romberger & Epifanio 1981; Gosling 2004; Ren et al. 2006). Feed concentration and presence of inorganic matter may play another role in absorption efficiency, as higher concentration of both have been found to reduce absorption efficiency (Thompson and Bayne 1974; Widdows 1978; Widdows *et al.* 1979; Griffiths 1980; Bayne & Newell 1983; Kuenster 1988; Gosling 2004).

Although many factors that control absorption efficiency are environmental factors, genetic factors also play a role in absorption efficiency. As a result absorption efficiency is a trait that can be improved in a selective breeding program. Selecting oysters that have a high absorption efficiency means that food resources would be allocated more efficiently. In addition, as discussed in earlier sections, oysters that have a low metabolic rate have been discovered to have a faster growth rate than oysters with a high metabolic rates (Bayne *et al.* 1999; Bayne et al 2000; Tamayo *et al.* 2014). Therefore oysters with a high absorption efficiency and a low metabolic rate, would be ideal traits to select for in a marker-assisted selective breeding program.

1.8.5. Implications of introducing hatchery reared spat into ecosystem

Shellfish industries in many parts of the world do not rely on wild spat supply, but rather instead purchase it from commercial hatcheries. Often, these hatcheries are involved in selective breeding programs, with specific traits chosen based on the economic benefit to the grower. These selectively bred oysters are then moved to a grow-out site where they are placed into an open ecosystem until they reach market size (Doiron 2008). Since oysters are considered sexually mature at after ~6—10 weeks post-settlement and are broadcast spawners (Galtsoff 1964; Thompson *et al.* 1996; Eastern Oyster Biological Review Team. 2007), there is ample time for these oysters to spawn in the water column. This raises a potential concern when oysters are bred for specific traits, and then reproduce naturally amongst the wild population, there is concern regarding possible genetic impacts on wild populations (Camara & Vadopalas 2009). This risk is currently considered minimal in the Maritime Provinces as wild recruitment is still used, and therefore population genetic alterations are minimal (Bastien-Daigle *et al.* 2007). However, in the future, when the industry shifts to a hatchery-based source, these impacts must be weighed.

Although spat supply is obtained from wild, in Atlantic Canada natural oyster stocks, population dynamics have already been altered in many ways. For instance, the outbreak of Malpeque disease wiped out 99% of oysters in PEI leaving only a small subset of the natural population (Needler & Logie 1947; Logie 1958; McGladdery & Bower 1999; McGladdery & Stephenson 2005). In addition, restoration efforts and spat transfers have

occurred in this region by shipping oysters between different locations such as from Bedeque Bay to Malpeque Bay in Prince Edward Island and also to Nova Scotia and New Brunswick after the outbreak in the 1950's (Found & Logie 1957; Morse 1971; MacKenzie 1996; MacKenzie & Burrell 1997). Although restoration efforts generally have good intentions, other potential issues can arise through altering the original genetic structure of the population, which may impact adaptation mechanisms. However, this is not always the case, and often if conducted properly can minimize the risk of population structure and adaptation issues while improving the population of oysters (Camara & Vadopalas 2009). Population genetics frequency has also been altered in Atlantic Canada through selecting for oysters that settle well on collectors and by grading and culling oysters based on size (Bastien-Daigle *et al.* 2007; Doiron 2008; Rubio 2010). This can change the population dynamics through an increase of a specific genotype, which may not be advantageous to the population and or potentially increase inbreeding in the population (Camara & Vadopalas 2009).

The Maritime Provinces are currently poised to take advantage of developing a hatchery-based industry to breed oysters for specific traits. Although a hatchery may solve many of the issues the oyster aquaculture industry faces such as disease outbreaks, climate change and unpredictable spat production, there are potential implications relating to introducing selectively bred oysters to the shellfish aquaculture sector. This introduction could compromise the genetic structure of the wild populations, which could threaten its long-term viability. This is especially true in incidences where wild population contain patterns of inheritance that are well adapted for their current environmental conditions, or if there is enough genetic variation in the population that could respond to future environmental challenges. Therefore, introduction of hatchery reared oysters to an environment could ultimately lead to a reduction of fitness, and or reduce or replace wild genotypes that are well adapted for the environment (Camara & Vadopalas 2009). In contrast, introduction of hatchery reared selective bred oysters could also benefit wild oysters, if the oysters introduced exhibit genetics that benefit long-term viability of the population, such as resistance to climate changes that may not be highly prevelant genetically in the wild population. This is especially true for wild populations that exhibit low genetic diversity

that may have been effected by previous phenotypic-based selection programs that lead to inbreeding (Camara & Vadopalas 2009).

Another implication that needs to be considered, is the fact that conditions on an aquaculture grow-out site are different than in a wild populations (Camara & Vadopalas 2009). For example, in the wild population, oysters settle on a substrate and are attached for the remainder of their lifecycle, while oysters in hatchery are usually set on cultch as individual oysters. Grow-out sites are usually conducted in suspension, where oxygen and food resources are optimal (Bastien-Daigle *et al.* 2007; Doiron 2008), while wild populations may not have such optimal conditions. It therefore can't be determined if the selectively bred traits would be advantageous in both grow-out and wild environments until further research is conducted (Camara & Vadopalas 2009). Additionally, selectively bred aquaculture oysters, might also exhibit a level of inbreeding, and consequently introduction of these oysters could release deleterious alleles into the wild population. It therefore can be concluded, that a study of the wild population, environment and future environmental conditions should be performed before selectively bred oysters are introduced into the environment (Camara & Vadopalas 2009).

1.9. Project Rationale

The variability in wild oyster spat collection, climate change and overall demand for oysters have led to the need for hatcheries in Atlantic Canada. With the development of a hatchery system, oyster farmers will have more control over annual spat supply and the traits that the oyster spat carry. This project has an industry partner in Bouctouche, NB interested in developing a hatchery for the Eastern Oyster in the Maritime Provinces. The industry partner has identified that oysters showing resiliency to environmental conditions and disease, with accelerated growth due to high absorption efficiency and low metabolic rates is key to the long-term success of their industry. The main goal of this project is to discover genetic markers that will allow growers to select for oysters that absorb food resources efficiently, even in suboptimal conditions.

1.10. Objectives

1. To evaluate absorption efficiency in oysters from two different locations in the Bouctouche River, New Brunswick. The first population will be from an aquaculture site (optimal conditions) and the second will be from a wild population of oysters (suboptimal conditions)

2. Identify SNPs in the oyster's genome that correlate to high and low absorption efficiency in both optimal and suboptimal condition using RAD-sequencing technology.

3. Use efficiency markers discovered by RAD-sequencing to select a broodstock that exhibit high absorption efficiency. The broodstock will then be conditioned, and spawned for future evaluation of the F1 generation.

1.11. References

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CHAPTER 2: COMPARISON OF ABSORPTION EFFICIENCIES BETWEEN WILD AND CULTURED OYSTERS, (*CRASSOSTREA VIRGINICA*) FROM BOUCTOUCHE, NEW BRUNSWICK DURING A THERMAL CHALLENGE

2.1. Introduction

The Eastern oyster (*Crassostrea virginica*) is an important bivalve mollusk in Atlantic Canada. Production of this species is expected to increase in upcoming years due to high market demand (Chopin 2015). However, several factors such as environmental fluctuations due to climate changes (Shackell & Loder 2012), spat supply variation (Doiron 2008), and disease outbreaks (Ford & Tripp 1996; McGladdery & Stephenson 2005; Bower 2014) threaten the long-term sustainability of the industry.

Climate change is inducing deviations in the aquatic ecosystem that could be potential stressors for calcareous organisms such as the Eastern oyster (*Crassostrea virginica*) (Shackell & Loder 2012). It is expected as a result of climate change that, temperature and ocean acidity will increase, while salinity and dissolved oxygen levels will decrease. Other changes, including water currents and phytoplankton resources are also expected to occur (Shackell & Loder 2012). Overall, ocean temperatures in North America have increased on average 1ºC in the past century. It is expected that seasonal changes will occur as well, such as an earlier spring and longer summers, giving more time for ocean temperatures to increase (Shackell & Loder 2012). Although the Eastern oyster does exhibit a wide tolerance to temperature (-2—36ºC) (Galtsoff 1964; Carriker & Gaffney 1996), temperatures of 30ºC and above are known stressors (Loosanoff 1958; Shumway 1996). As ocean temperatures continue to rise, more exposure to stressful conditions could occur. Additionally, as ocean temperature rises, oyster pathogens, such as Dermo (*Perkinsus marinus*), which can only survive in warmer climates (Ford 1996), maybe able to move to Atlantic Canada, which could have devastating effects.

Climate change is also expected to affect phytoplankton resources. Research suggests that phytoplankton will decrease in southern regions, due to increased stratification. As a result, in northern regions, phytoplankton will likely increase through higher water temperatures and longer growing seasons (Shackell & Loder 2012). Furthermore, there could be a poleward shift northward of many diatoms and dinoflagellates found in southern climates

(Barton *et al.* 2016). This increase in phytoplankton, depending on the magnitude, could potentially be a stressor to oysters, as high concentrations of phytoplankton at 6% body weight in hatchery conditions is known to be a stressor (Helm *et al.* 2004). Additionally, another study found communities of phytoplankton historically known to the Maritimes, might be moving away in an eastward direction (Barton *et al.* 2016). Oyster's diet usually consist of phytoplankton and detritus between 1—30µm (Quast *et al.* 1988; Newell & Langdon 1996), however, some phytoplankton species are more digestible then others (Romberger & Epifanio, 1981; Bayne & Newell 1983). That being said, very little information is known about how changes in feed diversity, resulting from climate change, could affect health of the Eastern oyster.

Spat supply is also a limiting factor for the industry. In Atlantic Canada, the most common method for obtaining spat is through wild spat collection (Doiron 2008). However, spat supply can be variable annually, which makes it difficult for oyster growers to maintain a constant supply of oysters on their culture site. In addition, climate change is expected to change water currents (Shackell & Loder 2012). Water currents play an important role in larval recruitment (Shumway 1996); therefore, if water currents are disrupted as a result of climate change, spat recruitment may occur in different locations then growers expect. Furthermore, as oceans become more acidic, a decline in physiological health and viability of oyster larvae, and settlement behaviour may occur (Sanford *et al.* 2014; Gobler & Talmage 2014; Ko *et al.* 2014). This phenomenon has already been occurring in other locations in the world such as in Pacific Northwest US, where major declines in spat occurred as a result of ocean acidification between 2005—2009 (Dumbauld *et al.* 2011; Mabardy *et al.* 2015).

To deal with many of these same issues, many locations world-wide are investing in hatchery-based science including the Eastern US, British Columbia, and Pacific Northwest US, etc. (Allen et al. 1993; Flimlim 2012; Center for Shellfish Research 2008; Dumbauld *et al.* 2011). Initiatives to support a hatchery based industry for the Eastern oyster have begun in Atlantic Canada (Aquaculture Association of Nova Scotia 2009, 2012). A hatchery industry, not only allow oyster growers to have a consistent spat supply from year to year, but also allow for selective breeding programs to be implemented for many

important traits identified by the industry, such as resiliency to disease and climate changes, which is extremely important to the long-term viability of the industry.

With that being said, another important factor that needs to be considered as the industry shifts to a hatchery industry, is the wild population. Currently, the wild population of oysters is still recovering from the outbreak of Malpeque disease that began in the early 1900's and wiped out 99% of the oysters in Atlantic Canada (Needler & Logie 1947; Logie 1958; McGladdery & Bower 1999; McGladdery & Stephenson 2005; Bastien-Daigle *et al.* 2007). If oyster aquaculture is to expand, potential concerns to the ecological footprint from the oyster aquaculture industry must be considered. This concern is mainly through exceeding the carrying capacity of an ecosystem, by growing too many oysters in a bay, and causing competition issues of food resources. However, this risk can be minimized through proper site selection, where food renewal is high (Guyondet *et al.* 2005, 2013), but also having oysters that exhibit a high absorption efficiency (AE).

Absorption efficiency is defined as the efficiency at which the ingested food is absorbed. There are two main methods at which energy is lost from the ingested food. The first, is through heat loss (specific dynamic effect) and the second is from excretion from principal losses of cellular processes such as protein metabolism (Bayne & Newell 1983). In this context, oysters that exhibit high AE would not only use food resources more efficiently, but would have access to more energy that could be converted to growth. Absorption efficiency has been calculated in mollusks by adapting the methods developed by Conover (1966). This method was originally developed as a method to measure AE in zooplankton. Unlike many other methods of calculating AE, this method does not rely on the quantitative collection of feces. Instead the Conover method, relies on creating a ratio of the fraction of organic matter in the food source versus the fraction of organic matter in the feces and is described by the following equation: $AE = \left\{\frac{F'-E-}{(1-E')(F')}\right\} \times 100$, where *F'* is the fraction of organic matter in the feed and *E'* is the fraction of organic matter in the feces (Conover 1966).

Previous studies, have demonstrated that environmental factors can cause AE to vary greatly. In general, AE can be over 80% in mollusks being fed algae in captivity (Bayne

1976; Winter 1978; Bayne & Newell 1983). With that being said, AE can also be as low as 30–60% in mollusks consuming a natural seawater diet (Bayne 1976; Winter 1978; Bayne & Newell 1983). Temperature and feed concentration can potentially effect AE in mollusks (Bayne & Newell 1983). Higher temperatures in *Mytilus sp.* have been found to marginally decrease AE (Bayne 1976; Bayne & Newell 1983). In this study, every 1ºC temperature increase caused a 0.7% decline in AE (Bayne 1976). Higher algae concentrations have also been found to greatly decrease AE in mollusks, especially when in captivity (Thompson and Bayne 1974; Widdows 1978; Griffiths 1980; Bayne & Newell 1983; Kuenster 1988; Gosling 2004). In addition, it has been found that the presence of inorganic matter in the water column can potentially have a dilution effect on the organic matter present and result in a suppressed AE (Widdows et al. 1979; Bayne & Newell 1983). Species of phytoplankton also plays a role in AE. It has been found that AE is high in the Eastern oyster being fed *Thalassiora pseudonana* (72%) and *Isochrysis galbana* (74%) (Romberger & Epifanio 1981). However, many species of diatoms and chlorophytes, which contain hard to digest cell walls are less easily absorbed (Romberger & Epifanio 1981; Ren et al. 2006; Gosling 2004). In fact, the Eastern oyster has been found to only have an AE of 6% for the chlorophyte species *Tetraselmis suecica* (Romberger & Epifanio 1981; Gosling 2004). In addition, genetics appear to have a role in AE between mollusks (Prudence *et al.* 2006). As climate change occurs, environmental modifications are expected to continue to occur, which indicates that the efficiency of mollusks such as oysters could also be affected. Therefore, having oysters that exhibit high AE, even under suboptimal conditions, is an important criteria for future selective breeding programs.

In this study, AE was investigated in Eastern oysters from Bouctouche, NB. Oysters were collected from two locations, with both locations containing highly different conditions. The first was an aquaculture site that used suspended culture techniques. At this location, salinity and temperature ranged from 20—28‰ and 0—26ºC, depending on season and tide. This site was considered to be optimal conditions, as salinity and temperature in this location are considered to be optimal for the Eastern oyster (Galtsoff 1964; Carriker & Gaffney 1996), and suspended aquaculture is known to give better access to phytoplankton resources and oxygen (Bastien-Daigle *et al.* 2007). The second location, was from a wild population of oysters, upriver. The salinity ranged from 0—20‰ depending on tide, and

temperature ranged from 0—24ºC, seasonally. This site was considered to be suboptimal conditions, as this site contained a muddy bottom substrate, where oysters were partially buried, had less access to oxygen and phytoplankton and were exposed to predation. In addition, the salinity can fluctuate greatly at this site, and the lower limits are out of the optimal range for the Eastern oyster, with below 5‰ being a known stressor to the Eastern (Loosanoff 1958; Shumway 1996). The two different locations in this experiment, represent the same population, as wild recruitment is still used in this location. However, the aquaculture oysters are being inadvertently selective bred for growth, by grading oysters based on performance (Doiron 2008; Rubio 2010). Additionally, because the second location contains conditions that are suboptimal, natural selection is occurring, where only oysters that successfully adapt and exhibit resiliency are able to thrive. Therefore, it is likely that there could be genetic differences between these groups of oyster, based on growth and resiliency. These two population were used in this experiment to determine if there were differences in terms of both AE and resiliency between the wild oysters in suboptimal conditions and aquaculture oysters in optimal conditions. In this experiment, AE even under suboptimal conditions will be evaluated by using thermal stress challenge, to determine if one group shows superiority in AE in suboptimal conditions.

As discussed earlier, a hatchery industry allows for selective breeding programs to be implemented for many important traits identified as necessary by the industry. Oysters that exhibit fastest growth while using the least amount of food resources to grow, has been identified as another important trait in order to minimize the ecological footprint of oyster aquaculture. It has been determined, that oysters that exhibit a low metabolic rate have a higher growth rate (Bayne et al. 1999; Bayne 2000; Pernet et al. 2008). Oysters that exhibit higher AE will also use food resources more efficiently (Bayne & Newell 1983). It has also been identified that resiliency is an important trait for oysters to exhibit, with all the challenges occurring as a result of climate change. This experiment will allow oysters that exhibit high AE even under suboptimal conditions to be discovered. The results of this experiment could aid in implementation of a selective breeding program for oysters that exhibit these traits.

2.2. Objectives

Absorption efficiency is an important physiological criterion for mollusks such as oysters. Having oysters that absorb food resources efficiently, even under suboptimal conditions, could greatly benefit the oyster aquaculture industry. Therefore, the main objectives of this experiment was to evaluate absorption efficiency in optimal conditions and during a thermal stress challenge in two groups of oysters from Bouctouche River, NB. The first group used in this experiment were from an aquaculture site (optimal conditions) and the second from the wild (suboptimal conditions). This experiment aimed to discover: (1) if there were individual differences in AE between oysters; (2) the effects of a thermal stress challenge on AE; (3) if there would be significant difference in AE between the wild oysters in suboptimal conditions and aquaculture oysters in optimal conditions.

2.3. Hypothesis

There were three main hypotheses associated with the objectives of this experiment. Hypothesis (1) was that due to different genotypes between individuals in a populations there would be variations in AE between individuals. Hypothesis (2) was that the thermal stress challenge would significantly reduce the AE in oysters. However, due to genetic differences there would be some individuals that could acclimate to the stress better than others. Hypothesis (3) was that there would be significant differences between wild and aquaculture oysters relating to AE, with the wild population having a lower AE then aquaculture oysters in optimal conditions, but wild oysters having higher AE in the thermal stress challenge. This hypothesis is related to the idea that aquaculture oysters in Atlantic Canada are typically graded based on growth (Doiron 2008; Rubio 2010). Therefore, aquaculture oysters could show genetic superiority in terms of AE compared to wild oysters. However, the wild oysters from suboptimal conditions, could have an advantage over the aquaculture oysters, as natural selection would be occurring and only oysters that adapt would survive in this location.

2.4. Materials and Methods

2.4.1. Oyster collection and acclimation

In May 2015, 60 oysters (~76mm shell height (SH)) were collected from each of the two sites in Bouctouche River, NB. The first site (A), an aquaculture farm, represented optimal growing conditions (at a salinity of 20–28‰; seasonally 0–26 ºC;N46'30"35.35; W64'40"31.33). Oysters were retrieved directly from the culture bags which were suspended in the water column giving them optimal access to phytoplankton, oxygen and stocked at the ideal density. The second site (W), was a wild oyster bed from upriver, which represented suboptimal growing conditions with a muddy bottom, inefficient access to food, oxygen, predation and fluctuating salinity. Oysters were retrieved from a soft muddy bottom (4m depth) using an oyster drag (0–20‰ salinity; seasonally 0–24 ºC; N46'27"20.9; W64'44"42.8). The oysters were partially buried in the sediment. At the time of collection, water temperature was 12ºC at both sites. Salinity was 22.5‰ at site (A), and 14‰ at site (W).

Oysters were transported on ice to the Aquatron facility at Dalhousie University (Halifax, NS) within 24h of collection. Oysters were divided into a treatment and control group. Oysters were randomly distributed into 12 tanks at 10 oysters per tank. There were three replicate tanks per treatment group (source, temperature). The tank system was a flow-through system with a flow rate of 1.6L/min. Water quality parameters such as dissolved oxygen, temperature, salinity, mortality and ammonium concentration were checked daily.

After being introduced into tanks at the aquatron, the wild oyster's salinity was gradually brought up from 14‰— 22‰ over 8 days (increased 1‰/daily) and then was acclimated at 22‰ at 12 ºC for 11 days. The aquaculture oysters underwent an acclimation period at the same water temperature and salinity of their collection site for a total of 19 days. During acclimation, oysters were fed three times a week, with an algae blend of *Isochrysis galbana* (T.ISO)*, Chaetoceros mulleri, Chaatoceros calitrans* and *Monochrysis lutheri* with the final concentration of feed in each tank being l00 cell/µL. At each feeding, water flow was always turned off for approximately one hour to allow oysters to feed.

2.4.2. Thermal stress challenge

During the thermal challenge, oysters in the treatment (stress) group received a temperature of 30ºC and the control (optimal) group received a temperature of 20ºC. After the acclimation period, water temperature in the tanks was increased gradually over 12 days (~1.5ºC/day for thermal challenge and 0.6ºC/day for control group) until the required temperature was reached (20 and 30ºC). Oysters were maintained at the required temperature for 7 days until sampling. They were fed three times daily the same algal blend and, again water flow was turned off during feeding. Thermal stress challenge was conducted using the methods described by Pernet *et al.* (2008).

2.4.3. Absorption efficiency

On the day prior to sampling, debris and fecal matter were removed from the bottom of each tank. Individual oysters were compartmentalized into plastic containers placed on the bottom of each tank. On sampling day, oysters were fed twice: 9am and 2pm, with water flow turned off for one hour. Three one liter water samples were immediately collected from each tank. In addition, three one liter seawater samples were collected from the header tank (blank control). After 24h, feces (not pseudofeces) were collected from individual oysters. All samples were filtered using a vacuum filtration system onto precombusted weighed glass fiber filters (47mm, Whatman, Sigma-Aldrich) and then rinsed with 15mL of 0.5M ammonium formate (ACROS Organics, Cat#AC349896) to ensure no residual salts were present from the water.

Absorption efficiency was calculated for each oyster based on the protocol developed by Conover (1966). Samples collected on pre-combusted weighed glass fiber filters were dried at 80ºC for a minimum of 24h and weighed. Filters were then ashed in a muffle furnace at 450ºC for 12 hours and weighed. The formula $AE = \left\{\frac{F'-E-}{(1-E')(F')}\right\} \times 100$ was used to calculate AE, where F = ash-free dry weight to dry weight (fraction of organic matter) in the food, and E = ash-free dry weight to dry weight (fraction of organic matter) in the feces. Blank control samples were used as a correction factor.

2.4.4. Condition index

Oyster tissue and empty shells were dried at 80ºC for 24 hours and weighed. Dried tissue was put in a muffle furnace at 450ºC, for 12 hours and weighed to determine the ash weight. The ash weight was then used to subtract from the dried weight, to determine organic tissue weight. The relationship between the organic tissue weight and the dry shell weight was obtained. CI = (organic tissue weight *100 /shell dry weight) (Landry et al. 2001). Oysters exhibiting a condition index (CI) less than 1.5 were considered to be in poor condition; between 1.5 and 3, in fair condition; and greater than 3, in excellent condition (Landry et al. 2001).

2.4.5. Statistical analysis

Data was analyzed using a General Linear Model, Analysis of Variance (ANOVA) using Minitab version 17 (Minitab Inc., State College, PA). Analyses was carried out for the response variable in this linear mixed model, AE (%). Oyster population (W or A) and temperature (20, 30ºC) were considered to be fixed first order factors. Tanks were considered to be a random factor due to the hierarchical (nested) structure of the experimental design. The assumptions of normality and homoscedasticity were assessed for the error terms by conducting a normal probability plot of standardized residuals of error terms and equal variance by plotting the residuals of error terms versus fitted values. All values are presented as mean ±SE unless specified otherwise. The significance level was set at $P \leq 0.05$.

2.5. Results

2.5.1. Absorption efficiency

Approximately 20% of the AE values were negative. Therefore, oysters with negative values were assumed to have 0% AE in our analyses. Absorption efficiency ranged from 0–94.8%, with an overall mean ±SE of 35.6±2.6% (Figure 1**)**. Aquaculture oysters from the control (20ºC) group were found to have a mean AE of 39.0±5.3% with the AE ranging from 0–94.8%. Aquaculture oysters from the temperature (30ºC) group had a mean of 48.9±3.2% with a range of AE of 0–72.8%. Wild oysters from the control (20ºC) group

were found to have a mean AE of 30.0±5.4% with AE ranging from 0–85.2% and oysters in the temperature (30ºC) group had a mean of 25.0±5.5% with a range between 0–93.6% efficiency. Figure 1. shows high standard error values and wide ranges within each temperature group, due to the high variability between individual oysters and groups.

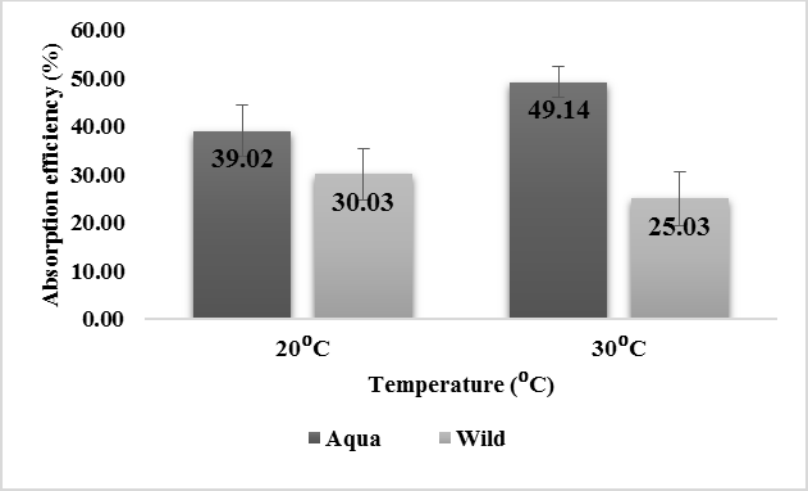

Figure 1: Bar graph of AE between temperature groups (ºC) and source of oyster (wild/aquaculture). Means and standard errors bars are expressed on each bar (n = 118).

Table 1: General linear model ANOVA table for the response variable AE. First and second order terms in the model are shown. Source, Temperature: fixed variables; Tank (Source, Temperature): random variables (n = 118)

	DF	Adj SS	Adj MS	F-Value	P-Value
Source	1	7791.6	7791.6	2.17	0.179
Temperature	1	239.4	239.4	0.07	0.803
Source*Temperature	1	1806.5	1806.5	0.50	0.499
Tank(Source, temperature)	8	28803.3	3600.4	6.60	0.000
Error	106	57855.3	545.8		

Table 1. summarizes the analysis of variance results for the response variable AE. First order and second order responses for source (A or W), temperature (20, 30ºC), and source*temp were found to be not significant. This indicates that there is no difference in AE between wild and aquaculture oysters. In addition, temperature was also not significant

which indicates oysters were able to absorb food at the same level regardless of thermal stress. The random tank effects (first order, random variable with temperature and source nested within) was the only significant variable ($P < 0.001$) and was accounted for in the model and therefore its effects are considered null.

2.5.2. Condition index

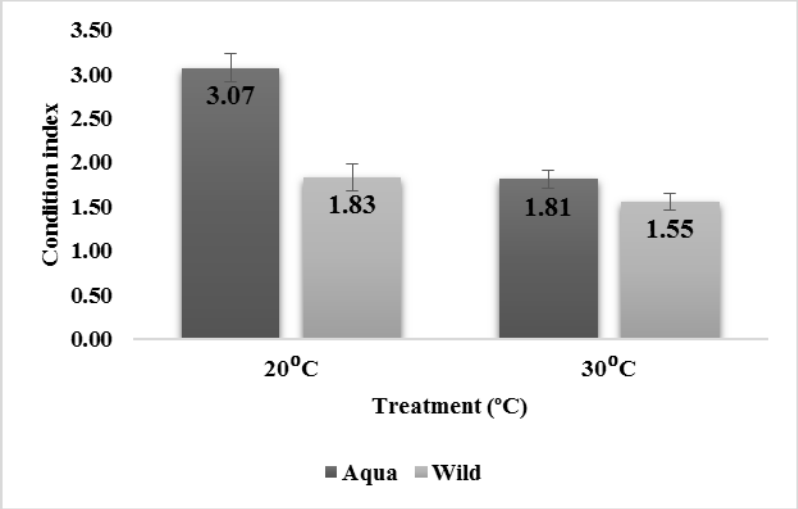

Figure 2: Bar graph of condition index between temperature groups (ºC) and source of oyster (wild/aquaculture). Means and standard errors bars are expressed on each bar (n = 118)

In addition to AE, CI was also used as a general health and stress predictor. In the control temperature group (20ºC), the CI for aquaculture oysters was 3.0±0.16, and for wild oysters 1.81±0.15. In the thermal stress challenge group (30ºC), aquaculture oysters had a CI of 1.83±0.17 and wild oysters 1.55±0.12.

2.5. Discussion

2.5.1. Absorption efficiency

2.5.1.1. Source of oysters

It was discovered that there was no significant differences in AE between the two groups of oysters. This indicates that despite the wild population living in suboptimal conditions and aquaculture oysters living in optimal conditions and being graded based on size

(Doiron 2008; Rubio 2010), that both groups were able to absorb feed similarly. The results of this experiment differed compared to a similar study conducted on the mussel *Mytilus galloprovincialis* by Labarta *et al.* (1997). This study looked at differences in physiological energetics between cultivated mussels and intertidal mussels from Ria de Arousa. The results of this experiment, unlike the present study, suggest that there were significant differences between the cultivated and intertidal mussels in terms of scope of growth. Cultivated mussels exhibited a higher AE, clearance rate, ammonia excretion and ultimately a higher scope of growth. In addition, it has been discovered that the alpha-amylase gene has a role in controlling absorption rate in the Pacific oyster (*Crassostrea gigas*) (Prudence *et al.* 2006; Huvet *et al.* 2008). Two genes coding for amylase mRNA were discovered by Sellos *et al.* in 2003. These two markers were then used by Prudence *et al.* (2006) in a selective breeding program, to find oysters that were heterozygous for the markers to produce five bi-parental families. These oysters were followed for one year and it was found that two of the five families had found to have a significant difference in growth and amylase activity, indicating that the markers discovered might have been a non-neutral response in absorption rate. Therefore, comparing the above two studies to the present study, the results of this experiment could indicate that there was no genetic differences between the two groups of oysters in terms of growth, despite natural selection in the wild and selection for size in aquaculture oysters. However, in contrast, a study by Tamayo (2014) found that Pacific oysters selectively bred for faster growth, compared to control oysters had no significant difference in AE between the two groups. However, this might be a result if only growth being selected for and not feed efficiency. Therefore, further genetic analysis would need to be conducted to determine if there are genetic differences between the two groups of oysters.

There was a lot of variability within groups with the overall range being from 0–94.84%. This indicates that there is a wide variability in the capacity for individual oysters to absorb nutrients in their environment. Absorption efficiency has been found to have a potential genetic component and to differ between groups of mollusks (Labarta *et al.* 1997; Prudence *et al.* 2006; Huvet *et al.* 2008). If this is true, then it is likely that there would be variation between individuals. Genetic variation for growth has been found in wild populations of species such as rainbow trout (*Oncorhynchus mykiss*) (Aulstad *et al.* 1972). In addition,

AE is a component of growth, and in hatchery systems where conditions are controlled, it is often seen that mollusks vary in size at the same age point, due to endogenous genetic factors (Gosling 2015); however, further genetic analysis would be needed to confirm this is true for AE in mollusks as well.

2.5.1.2. Temperature

In this experiment, the thermal stress challenge did not have a significant effect on the AE. Although the Eastern oyster does have a wide temperature tolerance (-2—36ºC) (Galtsoff 1964; Carriker & Gaffney 1996), a temperature of 30ºC, and above, is a known stressor to the Eastern oyster (Loosanoff 1958; Shumway 1996). This is an interesting result as it indicates that despite the stressful condition, oysters were still able to absorb food similarly in both the ideal and the thermal stress group. One potential reason for this result, is that the oysters could have adapted to the water temperature during the acclimation period. The theory of homeoviscous adaptation relates to the idea that the lipid bilayer can change to counteract changes in temperature (Sinensky 1974; Hazel 1995; Hayward *et al.* 2007; Pernet *et al.* 2007; 2008). A study was conducted by Pernet *et al.* (2007) that looked at temperature adaptation in adult Eastern oysters. When temperature was gradually increased to 20ºC, unsaturation index of the phospholipid bilayer decreased in the gills and digestive gland, through changes in 22:6n-3 and 20:5n-3 fatty acids. It was hypothesized that this might offset, disordering effect of increased temperature (Pernet *et al.* 2007). A similar study by Pernet *et al.* (2008), looked at physiological and biochemical traits in differences in growth rate and temperature adaptations for juvenile Eastern oysters. The results indicated, like the previous study, that there was changes that occurred in types of fatty acid distribution during increasing temperature. In addition, slow growing oysters had changes in both 22:6n-3 and 20:5n-3 but fast growing individuals mostly had changes in 20:5n-3 (Pernet *et al.* 2008). Although, temperature was higher in the present study, the results of this experiment appear to be consistent with the results from the experiments by Pernet *et al.* (2007; 2008), and that adaptation could have occurred by slowly acclimating the oysters to the higher temperature.

That being said, very few studies have been conducted on the Eastern oyster, to better understand how thermal stress affects AE. Additionally, there appears to be great inconsistency about what is known relating to the effects of increased temperature on AE in mollusks. One study by Buxton *et al.* (1981) on the European flat oyster (*Ostrea* edulis) looked at the combined effects and exposure of acclimating to temperatures of 5ºC, 10ºC, 15ºC and 25ºC on several factors including AE. The temperature 25ºC was chosen based as the highest temperature that the oyster would face, for periods of time in summer months. There was no significant difference found on AE across all temperature points, including the highest temperature point. A later study by Hutchison & Hawkins (1992) that also evaluated AE relating to temperature in the European flat oyster, echoed the results of Buxton *et al* (1981) and is consistent with the results of the present study. Other studies looked at the combined effects of temperature and salinity. One study by Enriquez-Ocana *et al.* (2012) on the mangrove oyster (*Crassostrea corteziensis*) found that combined effects at different levels of temperature and salinity significantly effected filtration and clearance rates, but did not significantly affect AE. Further analysis using a multiple regression, indicated that temperature was independent of AE (Enriquez-Ocana *et al.* 2012). In addition, a study on the brown mussel (*Perna perna*), which also looked at effects of salinity and temperature on AE, also found an independent relationship (Resgalla *et al.* 2007). In contrast, other studies has been done on the Pearl oysters *Pinctada fucata* and *Pinctada maxima* on the effect of temperature on AE. It was discovered that AE significantly increased with temperature increasing between 18—31ºC for *Pinctada fucata* and 19—31ºC for *Pinctada maxima* (Yukihira *et al.* 2000; Mondal 2006). Furthermore, other research has discovered the opposite that an increase in temperature can decrease AE. Bayne (1976) found that for *Mytilus sp.* increasing temperature decreased AE, for every 1ºC temperature increase caused a 0.7% decline in AE.

2.5.1.3.Other potential factors effecting absorption efficiency results

Approximately 20% of oysters in this experiment had no AE. The presence of these extremely low values ultimately contributed to a low mean AE. The means of this experiment, were much lower than other studies on AE in mollusks being fed algae species, which have reported feed efficiencies of up to 80% (Bayne 1976; Winter 1978). The results

of this experiment, were more similar to what could be expected in a natural seawater environment, which has been reported at 30—60% efficiency (Bayne & Newell 1983). Other studies have also calculated AE to be low, through experimental error due to inefficient separation of feces and pseudofeces (Enriquez-Ocana *et al.* 2012). In many of the negative valued AE, fraction of organic matter in feces (*E'*) was higher than in the feed (*F'*), which could have occurred if pseudofeces was mixed with feces. However, during this experiment, oysters were compartmentalized and true feces were carefully removed to avoid removal of pseudofeces and avoid this error; therefore, it can't be confidently concluded that this is sole cause of the negative values.

That being said, there are many environmental factors that have been found to contribute to AE fluctuation. The algae species fed in this experiment (*I. galbana, C. mulleri, C. calitrans* and *M. lutheri*) are a typical algae species blend used to feed mollusks such as oysters. Research indicates that flagellate species yields a higher level of AE (Ren et al. 2006; Navarro et al. 2009), such as *I. galbana* which is absorbed at ~74% efficiency by the Eastern oyster (Romberger & Epifanio 1981; Gosling 2004). *M. lutheri, C. mulleri, and C. calitrans* have not been studied in terms of effecting AE. However, studies have shown that diatoms and chlorophytes, due to their thick cellular wall are not as easily absorbed (Romberger & Epifanio 1981; Gosling 2004; Ren et al. 2006). Since *C. mulleri and C. calitrans,* are both considered diatoms, this could be a potential reason for the lower AE. In addition, oysters during the AE trial were batch fed at l00cells/µL, which is an extremely high concentration of algae. In a study conducted by Kuenster (1988), an increase of *Thallossiosira weissflogii* from 3—12cells/µL caused a drastic reduction from 90—65% in AE in *Argopecten irradians*. Therefore, it is likely that this level of feed might have been a factor in the lower AE. Other variables that may have impacted the low AE in this trial might include an unidentifiable stressor present, low digestibility in feed (Romberger & Epifanio 1981; Ren et al. 2006; Gosling 2004), presence of high amounts of inorganic molecules (Widdows et al. 1979; Kuenster 1988; Langdon & Newell 1990; Navarro et al. 2009), or too high concentration of feed (Thompson and Bayne 1974; Widdows 1978; Griffiths 1980).

2.5.2. Condition index

In this experiment, CI indicated that aquaculture oysters in the ideal treatment group were in excellent condition, and the thermal challenge group were categorized in fair condition. Additionally, wild oysters in the ideal condition and thermal challenge group were both classified as in ideal condition (figure 2). This result is interesting, as the temperature in the thermal challenge is considered to be a stressor to the Eastern oyster (Loosanoff 1958; Shumway 1996). Temperature stress has been found to significantly decrease CI in the Eastern oyster (Heilmayer *et al.* 2008). One study by Heilmayer *et al.* (2008) looked at the effects of temperature and salinity stress on the Eastern oyster and found that when exposed to high temperatures (>25ºC) for two weeks, body CI was significantly reduced. In contrast, a study by Lanig *et al.* (2006), found that exposure of 28ºC for 20 days did not significantly impact body condition of the Eastern oyster. Interestingly, both studies found that the combination of high temperature with either salinity (<5‰) (Heilmayer *et al.* 2008) or Cadnium (50µg/L) (Lanig *et al.* 2006), had an interactional effect and caused a significant reduction in CI. This indicates that perhaps, temperature is more likely to be a stressor when combined with another stressor. The results of this experiment, seem to be similar to the study by Heilmayer *et al.* (2008); however, the condition of the aquaculture oysters before the experiment is not known, and therefore it can't be speculated if there is a significant difference before and after the treatments. Additionally, it appears that wild oysters in the ideal group had a lower CI then the aquaculture oysters in the ideal group. The wild and aquaculture oysters came from different locations in Bouctouche River, NB, that varied greatly in environmental conditions. One of the key differences in conditions, was the salinity, which fluctuated greatly in the wild site (0—20‰), depending on the tides. Although the Eastern oyster is known, to be very resilient species, the optimal salinity range is from 10—27‰ (Calabrese and Davis 1970) and salinities less than 5‰ (Loosanoff 1958; Shumway 1996) can cause mortality. In addition, a study was conducted by Butler (1949) on a population of Eastern oyster from Chesapeake Bay that had been exposed to extensive flooding of freshwater in the summer and spring of 1945—1946. This flooding caused the salinity range to be 10—15‰ with time periods of 0‰. During this flood up to 70% of oysters died, and survivors were in poor condition. These oysters would differ from the study by Butler (1949), as these oysters have been exposed to suboptimal

environmental condition for their entire lifecycle, indicating they have adapted to it. However, it might account for why oysters were overall just in fair conditions in the ideal compared to the aquaculture oysters in excellent condition, but since the baseline CI, before the experiment, was not accounted for this it can only be speculated.

2.6. Conclusions

This experiment had several hypotheses. The first hypothesis was pertaining to variation between individuals. It was hypothesized that variation in AE would occur between individuals, as a result of genotype. This hypothesis is consistent with the results of this experiment, in that there was a lot of variation observed between individual oysters. However, it cannot be determined, at this point, whether this variation is related to genotype, as further analysis would need to be conducted (See chapter 3 and 4). Hypothesis (2) was related to the effects of the thermal stress challenge on AE. It was hypothesized that the thermal stress challenge would significantly reduce AE in oysters. That being said, there would be some oysters that could acclimate to the stress better than others due to genetic differences. The results of this experiment were not congruent with this hypothesis. It was found that there was no significant difference between AE in ideal condition group compared to the thermal stress challenge group. However, there was great variation of absorption efficiencies found in the thermal stress challenge, indicating that some oysters were able to acclimate better than other oysters. Again, as discussed earlier, there is no evidence to support that oysters that acclimated well to higher water temperatures exhibited a genotypic superiority without further genetic study (See chapter 3 and 4). Hypothesis (3) was that there would be significant differences between wild and aquaculture oysters relating to AE, with the wild population having a lower AE then aquaculture oysters in optimal conditions, but wild oysters having higher AE in the thermal stress challenge. The results of this experiment, did not satisfy this hypothesis. Although aquaculture oysters did exhibit a higher AE in the ideal condition group, and the wild group had an overall higher AE in the thermal stress challenge, the results were not considered statistically significant.

Finally, as there was wide variation between individuals in this study, further analysis should be conducted to determine if the variability between oysters in this experiment was

related to genotypes that are advantageous or disadvantageous to AE in the Eastern oyster. If this is true, making a link between phenotype and genetics would easily allow the detection of markers associated with oysters that have a high AE. Having validated markers for food absorption would be beneficial in an oyster selective breeding program.

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CHAPTER 3: SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) IN THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) CORRELATED TO ABSORPTION EFFICIENCY USING RAD-SEQUENCING AND *DE NOVO* ANALYSIS

3.1. Introduction

In the Atlantic Canadian aquaculture sector, the Eastern oyster (*Crassostrea virginica*) is considered to be an important species of bivalve mollusk, and market demand currently exceeds production. This market demand indicates, that the oyster aquaculture industry will continue to grow in upcoming years (Chopin 2015). However, the industry is being challenged due to many factors, including environmental fluctuations through climate change (Shackell & Loder 2012), spat supply variation (Doiron 2008), and disease outbreak (Ford 1996; Ford & Tripp 1996; McGladdery & Stephenson 2005; Bower 2014). Through climate change, ocean temperatures and acidity is increasing, while salinity and dissolved oxygen are decreasing. Deviations in water currents and nutrient concentration will also occur as a result of climate change (Shackell & Loder 2012). Currently, wild recruitment is still the main source of spat supply for the region (Doiron 2008). This method of spat recruitment is highly variable from year to year; however, with acidity increasing and water currents changing (Shackell & Loder 2012), this method of collection could become unreliable. Furthermore, oyster pathogens that have never been present in the Maritimes, pose emerging threats to oysters in the Maritimes Provinces due to their ability to survive in these changing conditions (Ford & Tripp 1996; McGladdery & Stephenson 2005; Bower 2014).

All of these factors threaten the long-term sustainability of the industry, which has led to initiatives for a hatchery-based industry being developed in the Maritime Provinces (Aquaculture Association of Nova Scotia 2009, 2012). A hatchery-based industry, would not only allow oyster growers to have consistent spat supply from year to year, but also allows for a selective breeding program to be implemented for important traits such as resiliency to disease and climate changes. This would allow oyster growers to increase production, and keep up with current demands. However, one important factor to consider

before increasing production of oyster aquaculture, is the wild population. Currently, wild populations are recovering from the outbreak of Malpeque disease that devastated the Maritime Provinces in the early 1900's (Needler & Logie 1947; Logie 1958; McGladdery & Bower 1999; McGladdery & Stephenson 2005; Bastien-Daigle *et al.* 2007). Overall, increased biomass of oysters in a particular location, fuels concern of the ecological footprint of oyster culture through potential of exceeding the carrying capacity of an ecosystem (Bastien-Daigle *et al.* 2007). There is caution in the sector, to not exceed the carrying capacity of an ecosystem by growing too many oysters in a particular location (Guyondet *et al.* 2005; 2013).

In this context, a potential solution for this, is having oysters that exhibit a higher growth rate and feed efficiency ratio. Feed efficiency ratio can be defined as the weight unit biomass increase over weight unit feed consumed (Thodesen *et al.* 2001). A major component of feed efficiency ratio is absorption efficiency. Absorption efficiency can be defined as the efficiency at which the ingested food is absorbed (Bayne & Newell 1983). Oysters that exhibit a higher absorption efficiency would theoretically, use food resources more efficiently. Absorption efficiency can be affected by many factors including diet features and environmental conditions (Bayne & Newell 1983). In addition, there is a potential genetic component related to absorption efficiency (Prudence *et al.* 2006; Huvet *et al.* 2008), which means it can be used as a trait to be selected for in a hatchery-based breeding program.

In the previous chapter (2), absorption efficiency was measured with the addition of a thermal challenge. The thermal challenge was used to identify oysters that exhibit resiliency and were able to absorb feed efficiently even under suboptimal conditions. The oysters used in this experiment came from two locations from Bouctouche, NB. The first group, came from an aquaculture site with optimal growing conditions, and the second, came from a wild site considered to have suboptimal growing conditions. Both groups of oysters would be considered from the same population, as wild recruitment is still the main method of spat collection. However, both population are under different selection pressures. Aquaculture oysters are being inadvertently selective bred for growth, by culling oysters based on growth performance (Doiron 2008; Rubio 2010), while the wild oysters

are being subjected to natural selection, as they live in suboptimal conditions and only oysters that can adapt would be able to thrive. The results of this experiment, indicated that there was no significant differences in terms of absorption efficiency between wild and aquaculture oysters, and that the oysters were able to absorb feed similarly, even in temperatures considered to be stressful. However, there was a great deal of variability in absorption efficiency found within each group of oysters. Genetic variation for growth has been found in wild populations of other species such as rainbow trout (*Oncorhynchus mykiss*) (Aulstad *et al.* 1972). Since absorption efficiency is a component of growth, this variability seems to be fitting for the wild population of oysters. Interestingly, it appears that the aquaculture oysters, despite being manually graded on growth performance, are exhibiting absorption efficiency traits similar to the wild population. This finding provides evidence that domestication has not occurred. The results of this experiment, allow for sequencing of individuals with high and low absorption efficiency to determine genetic markers that are associated with absorption efficiency.

Feed efficiency ratio has been successfully improved in other aquaculture species such as coho salmon (*Oncorhynchus kisutch*), Japanese flounder (*Paralichthys olivaceus*), rainbow trout, and Atlantic salmon (*Salmo salar*) (Thodesen *et al.* 1999; Ogata *et al.* 2002; Silverstein *et al.* 2005; Neely *et al.* 2008). Feed efficiency ratio is of great importance to the finfish aquaculture industry, including Atlantic salmon, as feed is a high cost of production, and relies on fishmeal and oil that has been criticized as being unsustainable (Ytrestøyl *et al.* 2015). Therefore, having salmon that can effectively utilize food resources, including fishmeal and fish oil replacements, while growing fast has been an important goal of the industry. Selective breeding programs for finfish for traits such as growth first began in 1967 (Aulstad *et al.* 1972). It was later discovered that feed efficiency could be inadvertently improved by selecting for growth, which reduced the amount of energy and protein needed for maintenance cost (Gjedrem 2010). Selective breeding leading to domestication of Atlantic salmon has improved feed efficiency ratio substantially from 0.93 for wild salmon to 1.16 for aquaculture salmon (Thodesen *et al.* 1999).

The Eastern oyster has also been selective bred for growth rate in locations such as the Eastern US (Allen *et al.* 1993). However, unlike salmon/finfish aquaculture, feed

efficiency ratio has not been considered, as feed isn't a major cost. That being said, as concerns have arisen about exceeding the carrying capacity of an ecosystem (Bastien-Daigle *et al.* 2007; Guyondet *et al.* 2005; 2013), it is important that it is considered in future selective breeding programs. In addition, in finfish production, selection for growth has inadvertently improved feed efficiency ratio (Gjedrem 2010; Thodesen *et al.* 1999). In contrast, research has been conducted on the Pacific oyster (*Crassostrea* gigas) and Sydney rock oysters (*Saccostrea glomerata*) to compare physiological parameters that improved growth rate in wild and selective bred oysters. From these studies, it was discovered that low metabolic rate and higher filtration rate were attributed to higher growth, but absorption efficiency was not significantly different between the two groups (Bayne *et al.* 1999; Bayne *et al.* 2000; Tamayo *et al.* 2014). Therefore, this indicates that absorption efficiency is a trait that must be selected for, in addition to faster growth.

In the past, selective breeding programs for the Eastern oyster have mostly relied on family-based breeding schemes (Allen *et al.* 1993). However, the use of MAS allows for genetic markers to be discovered, for traits that are not visually observed through phenotype (Guo *et al.* 2008). Understanding the genetic basis behind different phenotypes has been an important field of research in recent years. Genome-wide association (GWA) studies have established that there are specific variations occurring, usually, in non-coding regions of the genome involved in gene regulation (Nica & Dermitzakis 2013). Quantitative trait loci (QTLs) studies in recent years has increased our understanding on how specific variances within a genome can quantitatively change the expressed phenotype (Nica & Dermitzakis 2013). Single nucleotide polymorphisms (SNPs) are an example of QTLs that have a high potential in discovering how these variations in a genome are involved in the expression of phenotype (Yue 2013).

It has been difficult to conduct genetic studies on non-model organisms, such as the Eastern oyster, as they were expensive and time consuming due to the lack of available genomic resources (Ekblom & Galindo 2011). However, recently there have been great advances in next generation sequencing (NGS) technology that allow for a large amount of genomic information to be acquired rapidly from any species without a prior knowledge of its genome; and at much lower cost. Restriction Site Associated DNA sequencing (RAD-seq)

(Baird *et al.* 2008) technology makes use of NGS Illumina sequencing platform and can be used to identify SNPs across an entire genome (Davey & Blaxter 2011). Additionally, it is a cost-effective alternative to traditional genome wide sequencing, for non-model species where thousands of SNPs can be discovered that are correlated to a specific phenotype (Davey & Blaxter 2011). This method relies on the use of restriction enzymes to cut at specific location along the genome which is then sequenced; resulting in significant sequencing depth at these locations (Davey & Blaxter 2011). Software such as Stacks, developed by Catchen *et al.* (2011), has the ability to analyze the products of RAD-seq *de novo* (without the use of a known reference genome) and discover SNPs that are correlated with an expressed phenotype.

3.1. Objectives and Hypothesis

It has been identified by the oyster aquaculture industry, that having oysters which efficiently utilize phytoplankton resources even under suboptimal conditions, is an important trait for long-term viability of the industry. Since, absorption efficiency has been found to have a genetic component (Prudence *et al.* 2006), it can likely be improved in oysters through selection of this trait in oysters in a hatchery marker-assisted selective (MAS) breeding program. Therefore, the main objective of this experiment is, to identify markers in the Eastern oyster's genome correlated to high absorption efficiency using RAD-seq technology. The main hypothesis of this experiment is that oysters contain SNPs in their genome which are correlated with either high or low absorption efficiency phenotypes

3.2. Materials and Methods

3.2.1. Absorption efficiency trial

All data and oysters in this experiment, came from the absorption efficiency trial that was described in chapter 2. As stated in chapter 2, oysters in this experiment came from Bouctouche River, NB. 120 oysters from two locations were collected for this study, half from site (A) (sampled from an aquaculture site) and half from site (W) (sampled from a wild oyster bed). A week long thermal stress challenge was conducted on the oysters before

absorption was measured. Oysters in the treatment (stress) group received a temperature of 30ºC and the control (optimal) group received a temperature of 20ºC. After the thermal stress challenge, an absorption efficiency trial occurred, where individual absorption efficiencies were calculated using the methods adapted from Conover 1966 (See chapter 2).

3.2.2. DNA extractions, library synthesis, and RAD-seq

After the absorption efficiency trial, tissue samples from the mantle were collected from each oyster. DNA was extracted from tissue samples using the Qiagen DNeasy blood and tissue kit (Qiagen, Hilden Germany), following the manufacturer's protocols. In preparation for RAD-seq, DNA extractions were sent to Université Laval, Quebec, for cDNA library construction. Libraries were constructed on a total of 120 oysters used in the absorption efficiency trial. DNA was digested using the restriction enzymes PstI and MspI (4 bp recognition sites). Individual barcodes were attached to the PstI overhang to identify each individual, and the MspI extension was used ensure proper fragment sizes for sequencing. Libraries were sent to Genome Quebec, Montreal, QC, and were run on two lanes of an Illumina HiSeq2500, with paired end 100 nucleotide sequencing.

3.2.3. Stacks software

Bioinformatic analysis was carried out using the Stacks software (Version 1.18) (Catchen *et al.* 2011; 2013). The web-based Galaxy platform GenOuest (Rennes, France) was used to run the Stacks software. Several pipelines were run including *process RAD-tags, de novo map* and *populations.* Using the *process Rad-tag*s pipeline, samples were demultiplexed into individual RAD-tag files. Additionally, RAD-tags were cleaned, sorted and given a quality scoring. All poor quality reads were discarded based on the Phred scoring system.

The next pipeline used was *de novo map,* to align RAD-tags files into stacks of matching alleles and then merge stacks together to form a locus. *De novo map* pipeline was run using default parameters. A population map can be used in this pipeline to define two population for comparison, in this case, the populations chosen were related to individuals with high or low absorption efficiency. The population map was constructed using the 15 least

efficient oysters as population (1) and from the 15 highest efficient oysters as population (2). In the population map, only oysters that had reads over a million were used to ensure high quality analysis. In addition, treatment group and source of oysters from the absorption efficiency trial, were not considered during the construction of the population map, since they were not found to be significant**.**

The last pipeline used was, *populations*, which is a tool that performs core population statistics and then filters based on set parameters. The *populations* pipeline was run using the map created in *de novo map.* In this pipeline, a minimum stack depth of 10 was retained, to ensure that stack depth had high enough coverage to be accurately called a SNP. In addition, SNPs were only retained if the genotype was present in 70% of the individuals used in the analysis. To minimize likelihood of false positives and experimental-wise error rate, a minimum minor allele frequency of 0.05 and Bonferroni window at a significance level of α=0.05 was used.

3.2.4. Further filtering

Results of the *population* pipeline gave a set of SNPs correlated to high or low absorption efficiency and their core population statistics. Fishers exact test (FET) p-value was selected as an important parameter that describes whether the F_{ST} value, which is a measure population diversity, is statistically significant. Microsoft Excel (2013) was used to filter data and determine SNPs that met the required FET P-value at a significance level of α=0.05 and minimum threshold F_{st} ratio of >0.05 with the most optimal SNP F_{ST} being >0.10.

3.2.5. Annotation

SNPs were annotated by using RAD-seq sequences outputted from the *population* pipeline, for each given SNP. These sequences were annotated initially, using a Blastn (National Center of Biotechnology Information (NCBI), Bethesda MD) search against the NCBI nr nucleotide database. Additionally, RAD-seq sequences were aligned using a Blastn (NCBI, Bethesda MD) search against an unpublished laboratory Eastern oyster RNA-seq transcriptome contig database from the Dalhousie Aquaculture Genomics Laboratory. The

aligned RAD-seq sequences, were then annotated using a Blastx (NCBI, Bethesda MD) search against the NCBI protein database.

3.2.6. Determination of SNPs correlated phenotypes based on odds ratio

SNPs were determined to be correlated to either a high or low absorption efficiency phenotype by use of odds ratio (OR), calculated in the *populations* pipeline in Stacks. In this case, the significant SNPs chosen were related to the odds of having a genotype related to the phenotype of population (1) as opposed to population (2) (odds ratio = odds population (2)/ (odds population (1))**.** Therefore if the odds ratio for an individual was a positive number then the odds were in favour of that individual exhibiting the phenotype of population (1) and if it is less than zero then it has a higher odds to exhibit a phenotype related to population (2). In this study, odds ratios that were less than zero were converted to an equivalent negative integer for clarity purposes. As a result, the more negative the value the higher the odds/chance of being correlated to oysters exhibiting a lower absorption efficiency.

3.3. Results

3.3.1. Population map developed for *de novo map* pipeline

In population (1), 11 wild and 4 aquaculture oysters were used in the population map all containing a value of 0% absorption efficiency. In population (2), 8 wild oysters and 7 aquaculture were used in the population map, with the absorption efficiencies ranging from 59—94%.

3.3.2. Filtration steps and best candidates SNPs

Table 2: SNP counts related to filtering steps used in analysis to determine best candidates SNPs correlated to absorption efficiency in the Eastern oyster

Filtering steps	SNP count
Stacks: populations filtering steps	42089
FET p-value	929

Filtering steps	SNP count
F_{ST} ratio	108
Markers used in selective breeding program (F_{ST} ratio>0.10)	17

Table 1. shows a description of total SNP counts at each filtering step. The *populations* pipeline in Stacks resulted in 42,089 SNPs that contained genotypes that were present in 70% of the individuals used in the analysis, and had a minimum minor allele frequency of 0.05 and a significant Bonferroni window. After filtering by FET p-value, 929 SNPs were significant. Finally, after filtering by F_{ST} ratio at the >0.05 threshold, 108 SNPs were considered to be significant. 17 best candidate SNPs were selected by further filtering by $F_{ST} > 0.10$.

3.3.3. Annotating best candidate SNPs

Table 3: Results of Blastn search against the NCBI nr nucleotide database against RAD-seq loci identified with each best candidate SNP discovered. SNP loci have been annotated with GO ID functions, average E-value and similarity, and sequence length shown

Locus ID	SNP ID & (Position in Locus)	Annotation	GO ID functions	Avg E-value (Similarity)	Length aa
Locus_14235	**1** (89)	N/A	N/A	N/A	N/A
Locus_15610	**2** (40)	*Crassostrea gigas*: interleukin-17 receptor D-like (LOC105340255) mRNA 96	Integral component of membrane: non-cytoplasmic; transmembrane; cytoplasmic	3.43E-15 (83.0%)	96
Locus_15729	**3** (88)	N/A	N/A	N/A	N/A
Locus_16396	**4** (70)	N/A	N/A	N/A	N/A
Locus_17969	**5** (72)	N/A	N/A	N/A	N/A
Locus_18367	**6** (16)	N/A	N/A	N/A	N/A
Locus_19241	**7** (22) **8** (85)	N/A	N/A	N/A	N/A
Locus_29276	**9** (27)	N/A	N/A	N/A	N/A

Locus ID	SNP ID & (Position in Locus)	Annotation	GO ID functions	Avg E-value (Similarity)	Length aa
	10 (56)				
Locus_35511	**11** (8)	*Crassostrea gigas*: thioredoxin domain containing 11-like (LOC105347231) mRNA 96 N/A	cell redox, homeostasis N/A	3.86E-11 (84.0%) N/A	96 N/A
Locus_40270	**12** (15)	N/A	N/A	N/A	N/A
Locus_4298	**13** (60)	N/A	N/A	N/A	N/A
Locus_44164	**14** (45)	N/A	N/A	N/A	N/A
Locus_6771	**15** (50) **16** (86)	*Haplochromis burtoni*: ELKS Rab6-interacting, CAST family member 1-like (LOC102303751) transcript variant mRNA 96	Part of the family of RIMS-binding: Neurotransmitter regulation	4.93E-14 (78.7%)	96
Locus_9767	**17** (11)	N/A	N/A	N/A	N/A

The results of the Blastn search on RAD-seq sequences provided by Stacks software is summarized in Table 3. It was found that three loci contained more than one SNPs at different position within the sequence; SNPs 7 and 8 were from Locus_19241; SNPs 9 and 10 were from Locus_29276; and SNPs 15 and 16 were from Locus_6771. Only four SNP loci matches in the NCBI database were found. SNP 2 and 11, were matched back to a sequence related to the Pacific oyster (*Crassostrea gigas*), an interleukin-17 receptor and thioredoxin domain containing 11-like, respectively. SNP 15 and 16, which were found in the same loci, was annotated as ELKS Rab6-interacting, CAST family member 1-like, which was linked to the fish species *Haplochromis burtoni.* Although few RAD-seq sequences were matched in the Blastn search, it is notable that all matched sequences were considered to be protein coding.

Table 4: Results of Blastx search against the NCBI protein database using matched RAD-seq loci with Eastern oyster RNA-seq transcriptome database contigs (unpublished data). SNP loci have been annotated with #GO ID functions, average E-value and similarity

Locus ID	SNP ID & (Position in Loci)	Annotation	GO ID functions	Avg E-value (Similarity)	Length aa
Locus_14235	**1** (89)	Sterile alpha and TIR motif	protein binding through signal transduction	0.00E+00 (76.0%)	4526
Locus_15610	**2** (40)	Interleukin-17 receptor	Integral component of membrane: non-cytoplasmic; transmembrane; cytoplasmic	0.00E+00 (63.8%)	3510
Locus_15729	**3** (88)	N/A	N/A	N/A	N/A
Locus_16396	**4** (70)	uncharacterized protein (LOC105332190)	Unknown	0.00E+00 (70.95%)	3850
Locus_17969	**5** (72)	Phosphotidylinositol phosphatase	zinc ion binding; nucleic acid binding	9.80E-152 (67.3%)	1990
Locus_18367	**6** (86)	N/A	N/A	N/A	N/A
Locus_19241	**7** (22) **8** (85)	N/A	N/A	N/A	N/A
Locus_29276	**9** (13) **10** (51)	N/A	N/A	N/A	N/A
Locus_35511	**11** (45)	Thioredoxin domain	cell redox, homeostasis	0.00E+00 (59.0%)	4022

Locus ID	SNP ID & (Position in Loci)	Annotation	GO ID functions	Avg E-value (Similarity)	Length aa
Locus_40270	**12** (29)	colorectal mutant cancer isoform	Negative regulation of canonical Wnt signaling pathway; Protein binding in cytoplasm	2.30E-168 46(68.65%)	2364
Locus_4298	**13** (60)	uncharacterized protein (LOC105332626)	Protein binding through signal transduction Regulation of apoptotic process	0.00E+00 (62%)	2045
Locus_44164	**14** (45)	N/A	N/A	N/A	N/A
Locus_6771	**15** (50)	"ERC" partial	Presynaptic active zone Coil	0.00E+00 (67.9%)	5145
	16 (86)				
Locus_9767	**17** (72)	Uncharacterized protein (LOC105341785)	Unknown: protein coding	8.97E-12(65.0%)	350

Results of the Blastx search against the NCBI protein database, using matched RAD-seq sequences with Eastern oyster RNA-seq transcriptome database contigs (unpublished data), is summarized in the Table 4. Of the 17 best candidate SNPs, 10 were matched back to protein coding sequences in the NCBI database. SNPs 4, 13 and 17 were annotated as uncharacterized proteins. SNPs 2 and 11 were annotated as an Interleukin-17 receptor and Thioredoxin domain, respectively, and resulted in the same annotation in both the blastn and blastx search. SNPs 15 and 16, found within the same loci, were annotated as an "ERC" partial protein. SNPs 1, 5, and 12 were annotated to be genes relating to sterile alpha and TIR motif, Phosphotidylinositol phosphatase, and a “colorectal” mutant cancer isoform, respectively. SNPs 3, 7, 8, 9, and 14 loci were not successfully annotated is this study. Broadly speaking, these genes related to proteins with annotated functions relating to

cellular binding (via protein, ion, or ion binding), compositional functions via membranes, cell redox homeostasis, and presynaptic functions in the active zone.

3.3.4. Determination of SNPs correlated phenotypes based on odds ratio

Table 5: Significant SNPs correlated to population (1) correlated to low absorption efficiency oysters and (2) correlated to high absorption efficiency oysters. Odd ratios were expressed in terms of positive and negative for clarity. Negative values were determined by the following formula (-1/odd ratio)

Locus ID	SNP ID & (Position in Loci)	OR	Correlated Population
Locus_14235	1 **(89)**	**9.3**	**2**
Locus_15610	2 **(40)**	**5.1**	**2**
Locus_15729	3 **(88)**	**17.5**	**2**
Locus_16396	4 **(70)**	**10.8**	**2**
Locus_17969	5 **(72)**	**-10.3**	**1**
Locus_18367	6 **(86)**	**7.5**	**2**
Locus_19241	7 **(22)**	**8.3**	**2**
	8 **(85)**	**8.3**	**2**
Locus_29276	9 **(13)**	**13.2**	**2**
	10 **(51)**	**13.2**	**2**
Locus_35511	11 **(45)**	**-6.9**	**1**
Locus_40270	12 **(29)**	**-12.0**	**1**
Locus_4298	13 **(60)**	**9.2**	**2**
Locus_44164	14 **(45)**	**7**	**2**
Locus_6771	15 **(50)**	**-12.4**	**1**
	16 **(86)**	**-14.5**	**1**
Locus_9767	17 **(72)**	**-9.2**	**1**

Odd Ratio was used to decipher if correlated to population (1) or (2) for each of the 17 SNPs discovered in relation to absorption efficiency. These population, as discussed previously, were created based on whether the oysters exhibit high or low absorption

efficiency. Population (1) is correlated to oysters exhibiting low absorption efficiency and population (2) to oysters that exhibited high absorption efficiency. Table 5. describes the SNPs that were discovered to be significant in terms of absorption efficiency, their odd ratio and the correlated population. It was determined that SNPs 5, 11, 12, 15 and 16 were discovered to be correlated to population (1) (low absorption efficiency), and SNPs 1, 2, 3, 4, 6, 7, 8, 9, 10,13 and 14 were discovered to be correlated to population (2) (high absorption efficiency).

3.4. Discussion

In total, 17 significant SNPs were discovered that were correlated to either high or low absorption efficiency. The results of the initial blast search on the RAD-Seq sequences using the NCBI database only resulted in the annotation of three SNPs. However, RAD-seq sequences are only short-read sequences and therefore it is to be expected. The second blast search, which were first aligned to contigs, yielded better results, with 10 of 17 SNPs being annotated to proteins. This is a very important result, as it means that the SNPs discovered could have a role in phenotypic response. This phenotypic response change can occur through changes in interactions with cell mechanisms involved in mRNA synthesis, maturation, transport, translation or degradation (Shen *et al.* 1999). In addition, SNPs related to a genes are less likely to be altered during the process of crossing over and therefore could be heritable (Salem 2012).

When comparing the annotation of the two searches, it was found that SNPs 2 and 9 were annotated similarly with both being interleukin-17 receptor (D-like) and thioredoxin domain proteins, respectively. In addition, SNP 15 and 16 loci in the first search was annotated as an ELKS Rab6-interacting, CAST family member 1-like, while in the second search it was annotated as an "ERC" partial using the NCBI database. However, ERC genes encodes for ELKS Rab6-interacting proteins (Wang *et al* 2002; Deguchi-Tawarada *et al.* 2004). Overall, it was found that the protein encoding genes could be categorized into four broad categories based on their annotated putative function: cellular binding (via protein, ion, or ion binding), compositional function via membranes, cell redox homeostasis, presynaptic functions in the active zone or unknown functions.

Some annotated SNP loci sequences indicated a relation to the immune system in mollusks. SNP 1 locus was annotated to be potentially within a sterile alpha and TIR motif. This type of enzyme is found to have a role in innate immunity. Toll/interleukin-1 receptor (TIR) domains are usually found in the cytoplasm and are attached to membrane-bound receptor proteins such as sterile alpha motifs (Gerdol *et al.* 2017). They are usually involved in fundamental signal transduction relating to the immune system through toll-like receptors (TLRs). Sterile alpha and TIR motifs have been identified in several mollusks such as the Eastern oyster and blue mussels to have a role in innate immune system (Zhang *et al.* 2014; Philipp *et al.* 2012). Additionally, SNP 2 locus was found to be annotated potentially within an interleukin-17- receptor (D-like). The interleukin-17 family in general are an important part of the immune system and can aid in inflammatory responses (Huang *et al.* 2015; Iwakura *et al.* 2011). The interleukin-17 receptor family are a single-pass transmembrane receptor that aid in transporting interleukin-17 homodimer or heterodimers across the membrane. Many types of interleukin domains have not been found in mollusks (Takeuchi *et al.* 2012). However, a study done by Roberts *et al.* (2008) studied the accumulation of the interleukin-17 receptor in the Pacific oyster (*Crassostrea gigas*) and found that it was upregulated rapidly during an injections of bacteria, indicating that it might be an early response to pathogens to stimulate the production of immune genes (Roberts *et al.* 2008). In addition, the first blast search conducted identified it as an interleukin-17-D-like receptor. This is an interesting finding, as the interleukin D-receptor structure and function is not fully understood (Huang *et al.* 2015; Iwakura *et al.* 2011).

Additionally, SNP 11 loci was found to be within a potential thioredoxin domain, which is a protein that is involved in a variety of different biological functions including redox signaling (Patwari *et al.* 2006). Their activity occurs through cysteine thiols at its active site that become oxidized in reduction processes, and have been found to have a potential role in several biological function including, disease protection, stress induced apoptosis, glucose metabolism, homeostasis and cell differentiation (Patwari *et al.* 2006). In molluscs such as the Pacific oyster, thioredoxin domains have been found to be upregulated in exposure to *Vibrio splendidus* (Lorgeril *et al.* 2011) and also high acidic environments (Timmins *et al.* 2014). In addition, It has also been found in the Manila clam (*Ruditapes philippinarum*), to be an antioxidant enzyme against bacterial outbreaks in mitochondria

(Umasuthan *et al.* 2012). Furthermore, a study by Jimenez-Hidalgo *et al.* (2014) found for the first time, a thioredoxin domain in the intestines of Roundworm (*Caenorhabditis elegans*). This is an interesting finding as it has never been found in eukaryotic intestines before this study. This study found that the presence of thioredoxin-3 in the intestines had a potential role in protection against pathogens. A direct relationship was not found between thioredoxin-3 proteins in the intestine of roundworm and digestion. However, a subtle phenotype was observed in individuals containing mutant thioredoxin-3 proteins and were found to be smaller with a shorter defecation cycle (Jimenez-Hidalga *et al.* 2014). The exact thioredoxin protein that SNP 11 locus is related to, is not known and therefore how it could relate to absorption efficiency is unknown. However, the SNP had an OR of -6.9, which indicates that it was correlated to low absorption efficiency individuals. This indicates potentially, despite literature showing links in mollusks species to pathogen defence and environmental stress, there could be a link in digestion as well, but would need to be further studied.

It is difficult to speculate how these annotated immune proteins could have a role in absorption efficiency in the Eastern oyster, as their roles have not been studied yet. That being said, it has been found that proteins related to the immune system do have a role in feeding behaviour. For example, lectins are a type of carbohydrate binding protein that are found in the mucosal layer of vertebrates and invertebrates, which are known to have function in the immune system by the detection of foreign materials (Cambi & Figdor 2003). However, interestingly, they have also been found in the Eastern oyster to have a key role in the feeding organs, in accepting microalgae for digestion or rejecting microalgae in the form of pseudofeces (Pales Espinosa *et al.* 2009). Additionally, lectin-like receptors have been found to sometimes have a synergetic or antagonistic effect on TLR signalling but mechanisms between the two are unknown (Cambi & Figdor 2003). Therefore, the fact that lectins appear to sometimes interact with other immune related proteins, could indicate that other immune related proteins could also have a role in feeding behaviour or absorption efficiency, but further investigation would be needed to confirm this.

Other SNPs appeared to have functions independent of the immune system such as SNP 5, which was annotated within a potential phosphotidylinositol phosphatases. These complex enzymes have an important role in regulation of insulin signaling, and glucose metabolism in humans (Dyson *et al.* 2012). This class of enzymes were first discovered as being a lipid phosphatase with the ability to degrade phosphoinositides or inositol phosphates by removal of the phosphate group, which is important for many metabolic pathways (Dyson *et al.* 2012). In addition, gene knockout studies on mice and mutations on phosphotidylinositol phosphatases, have found that they play a critical role phosphoinositide-related metabolism (Dyson *et al.* 2012). However, the function of phosphotidylinositol phosphatases in mollusks is unknown and further analysis would be need to be conducted to further understand its potential role in absorption efficiency.

Locus_6771 that had two SNPs within SNPs 15 and 16, which was annotated potentially to be within an ELKS Rab6-interacting, CAST family member 1-like /"ERC" partial. This type of protein are found to be components of active zone vesicles. The active zone is involved with release of neurotransmitters in humans (Hida & Ohtsuka 2010). Very little information is known about how this protein functions in invertebrates. One study on roundworm *(Caenorhabditis elegans*), tried to better understanding of ELKS protein through creating roundworms that are lacking ELKS protein and found they did not have any physiological or behavioural impact (Deken *et al.* 2005). It therefore is hard to determine if this SNP could have a potential role in absorption efficiency, as no information is known about this protein in mollusks.

SNP 4, 13, and 17 were annotated as being possibly uncharacterized proteins found in the Pacific oyster. Because these proteins, are not fully understood, or characterized, it is difficult to speculate their involvement in absorption efficiency. Additionally, SNP 12 was annotated to be a colorectal cancer mutagen isoform. Although a colorectal mutant cancer protein isoform has been found in other species of oysters such as the Pacific oyster (Accession # XM_011449171.2), it is difficult to interpret how this protein function could impact the physiology of oysters, as it hasn't been extensively studied.

The present study is one of the first studies to look for genetic markers in the Eastern oyster related to absorption efficiency. Little information is known about physiological components of oysters that pertain to absorption efficiency. Amylase and other digestive enzymes can improve digestive function and increase absorption efficiency (Prudence *et al.* 2006); however SNPs relating directly to digestive enzymes such as amylase, were not found in this study. Instead, the SNPs in this study were annotated to proteins found to be related to the immune system, cell signalling via neurotransmitters, metabolic processes, and a few uncharacterized. Therefore, the direct link between these SNPs and absorption efficiency is not to be known.

In this experiment OR was used to determine the odds of a SNP being related to a particular phenotype. Odds ratio is a measurement of strength of association and is used in GWA studies to determine the odds of an individual having a specific genotype (Clarke *et al.* 2011). In addition, OR can be additive between SNPs and therefore the total OR can be determined over a genome, to determine whether an individual is likely to exhibit a specific phenotype, therefore, the use of odds ratio could be applicable tool in MAS breeding programs in the future. The discovery of these SNPs associated with absorption efficiency, could be useful in a hatchery based industry where MAS could be used. Identification of these 17 SNPs in a broodstock, could be used as a quantitative approach to determine the likelihood of a particular oyster exhibiting a high or low absorption efficiency. It therefore is recommended that further validation of these markers be conducted, by applying these markers to screen broodstock for spawning, to generate an F1 generation that could then be analyzed to determine the effectiveness of these markers as a QTL for food absorption.

3.5. Conclusion

The main goal of this experiment was to identify markers in the Eastern oyster's genome that were correlated to high absorption efficiency in both optimal and suboptimal condition using RAD-seq technology. The hypothesis of this experiment was confirmed with 17 significant SNPs discovered correlated to absorption efficiency. These SNPs were discovered through the use of RAD-seq and the Stacks software. The loci containing SNPs were annotated, and were matched with protein coding sequences, indicating that the SNPs

could potentially have a phenotypic response. The function of these genes in the cells, were broadly categorized as cellular binding (via protein, ion, or ion binding), compositional function via membranes, cell redox homeostasis, and presynaptic functions in the active zone. Further study, indicated in mollusks they were mainly related to immune system, cell signalling via neurotransmitters and metabolic processes. Additionally, three SNPs were annotated within uncharacterized proteins and five SNPs could not be annotated. Little information is known about the pathways involved in absorption efficiency; therefore, it cannot be determined at this time how these SNPs alter the protein pathway, resulting in either increased or decreased absorption efficiency without further study. To further validate these SNPs as effective markers for absorption efficiency, they will be applied to a broodstock and the F1 generation phenotype and genotype will be evaluated.

3.6. References

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CHAPTER 4: SELECTIVELY BREEDING OYSTERS FOR BETTER ABSORPTION EFFICIENCY USING SNPS THAT ARE CORRELATED TO ABSORPTION EFFICIENCY IN THE EASTERN OYSTER (CRASSOSTREA VIRGINICA)

4.1. Introduction

In Atlantic Canada, the Eastern oyster (*Crassostrea virginica*) is an important species of bivalve mollusk, and market demand currently exceeds production (Chopin 2015). However, the long term viability of the industry is being challenged through climate change (Shackell & Loder 2012), spat supply variation (Doiron 2008), and disease outbreak (Ford 1996; Ford & Tripp 1996; McGladdery & Stephenson 2005; Bower 2014). Climate change in Atlantic Canada, is causing major modifications to the environment, and as a result, ocean temperatures and acidity are increasing, while salinity and dissolved oxygen are decreasing. Water currents and nutrient concentration are also expected to be modified as a result of climate change (Shackell & Loder 2012). Through these modification to the ocean environment, wild recruitment as the main source of spat for the oyster sector could be challenged. Additionally, pathogens of the Eastern oyster, historically only found in southern regions are now considered to be a threat to the industry in northern regions (Ford 1996; Ford & Tripp 1996; McGladdery & Stephenson 2005; Bower 2014).

These challenges to the oyster aquaculture industry has led to the discussion of a hatchery-based industry to be developed in the Maritime Provinces (Aquaculture Association of Nova Scotia 2009, 2012). Additionally, many other locations worldwide are also investing in hatchery-based science to deal with many of these same challenges (Allen *et al.* 1993; Flimlim 2012; Center for Shellfish Research 2008; Dumbauld *et al.* 2011). Developing a hatchery-based industry will allow oyster growers to have access to consistent spat supply from year to year, and the ability to keep up with market demand. It would also allow for selective breeding programs to be implemented, so that oyster growers have better control over the traits their spat carries.

Compared to the Eastern US, the use of hatchery-based selective breeding programs for the Eastern oyster has had limited success in Atlantic Canada. The development of a hatchery industry for the Eastern oyster has been ongoing in the Eastern US since the

outbreak of MSX (*Haplosporidium nelsoni*) in the 1950's, which caused selection to occur for oysters that exhibited disease tolerance (Haskin and Ford 1979; Guo *et al.* 2008*b*). Since this time, the US, has extensively developed their hatchery-based industry. However, they rely heavily on family-based breeding systems that took many years to establish, and were initially met with inbreeding issues (Allen *et al.* 1993; Guo *et al.* 2008*b*). Despite being the same genetic species, the selective breeding program in the US, can't be applied to the Eastern oyster stocks in the Atlantic region, as oysters in Atlantic Canada have been found to be a genetically different population of oysters (Reeb & Avise 1990; Karl & Avise 1992; Cunningham & Collins 1994; Hoover & Gaffney 2005; Varney et al 2009; 2016). In addition, Canadian Food Inspection Agency (CFIA) will not permit the movement of oyster spat from oysters in the Eastern US to Atlantic Canada, due to the presence of several diseases that are not currently present in Atlantic Canada. These diseases are OIE reportable diseases and CFIA will not risk introduction to Canadian waters by oyster transfers from the US.

An effective and efficient method of establishing a hatchery-based selective breeding program for oysters in Atlantic Canada is through marker-assisted selection (MAS). Marker-assisted selection has been established in parallel with inventions of sequencing technology, such as next generation sequencing (NGS) (Yue 2014). These technologies have allowed specific variances in the genome to be detected that are correlated to specific traits (Nica & Dermitzakis 2013). The use of MAS breeding programs are especially useful when the trait cannot be observed easily phenotypically (Guo *et al.* 2008a). Currently, MAS is being slowly implemented in to aquaculture breeding schemes. For example, in Atlantic salmon (*Salmo salar*), acute infectious pancreatic necrosis (IPN) is a major issue in salmon farming. However, three markers have been found to be related to resistance, and these markers have been implemented into MAS breeding programs (Moen *et al.* 2009; Yue 2014). In contrast, although quantitative trait loci and linkage mapping has been extensively studied in certain mollusks such as the Pacific oyster (*Crassostrea gigas*), (Li & Guo 2004; Hubert & Hedgecock 2004; Hedgecock *et al.* 2007; Sauvage *et al.* 2010; Guo *et al.* 2012; Zhong *et al.* 2014; Hedgecock *et al.* 2015; Wang et al 2016), it is only in the beginning stages of MAS being implemented into selective breeding programs (Zhong *et al.* 2017). The use of MAS is much more challenging in non-model species, where a

reference genome isn't available. However, with the development of NGS technologies, such as RAD-seq (Baird *et al.* 2008), and bioinformatic platforms, such as Stacks (Catchen *et al.* 2011; Catchen *et al.* 2013), the discovery of markers in non-model organism, including single nucleotide polymorphism (SNPs), is highly plausible. These platforms allow for segments of DNA to be screened for markers that are correlated to a particular trait or population and then applied in MAS programs.

In the Atlantic Canadian Eastern oyster aquaculture industry, several traits have been identified as important to implement in a selective breeding program, including disease resistance, stress resiliency, growth and feed efficiency. Having oysters that exhibit resiliency in changing conditions will allow the oyster aquaculture industry to be successful long-term, while facing changes relating to climate change and disease. Additionally, as discussed previously, the current demand for oysters exceeds production (Chopin 2015), which indicates oyster aquaculture will likely increase in upcoming years. While this is happening, wild populations are slowly recovering from the outbreak of Malpeque disease in the early 1900's (Needler & Logie 1947; Logie 1958; McGladdery & Bower 1999; McGladdery & Stephenson 2005; Bastien-Daigle *et al.* 2007). Consequently, concerns of the ecological footprint of oyster culture has emerged through exceeding the carrying capacity of an ecosystem (Bastien-Daigle *et al.* 2007). This is especially important, as food resources in oyster aquaculture, come from natural phytoplankton resources. Therefore, having oysters that exhibit fast growth and feed efficiency through selective breeding has been identified as important traits for a selective breeding programs in Atlantic Canada

Feed efficiency ratio can be defined as the weight unit biomass increase over weight unit feed consumed (Thodesen *et al.* 2001). A major component of feed efficiency ratio is absorption efficiency. Absorption efficiency can be defined as the efficiency at which the ingested food is absorbed (Bayne & Newell 1983). In other aquaculture species, such as Atlantic salmon (*Salmo salar*) growth has been selected for, which has subsequently improved feed efficiency ratio as well (Gjedrem 2010). Growth has also been selectively bred for in species such as the Sydney rock oyster (*Saccostrea glomerata*), and the Pacific oyster. However, in contrast to Atlantic salmon, studies have looked at the physiological differences between oysters that have been selected for fast growth compared to non-

selective bred oysters, and found that metabolic rate was lower and filtration rate was higher in oysters that exhibit fast growth, but absorption efficiency was similar in both (Bayne *et al.* 1999; Bayne *et al.* 2000; Tamayo *et al.* 2014). This indicates, in order for food utilization to be improved in addition to growth, it needs to be selected for in addition to growth.

4.2. Objective and Hypothesis

In chapter 3, SNPs correlated to high and low absorption efficiency were discovered in the Eastern oyster genome. Additionally, in a related study SNPs have also been discovered correlated to high and low metabolic rate in study conducted in parralel to this study. The main objective of this experiment is to validate these SNPs by use of MAS. This experiment aims to find the newly discovered SNPs in the genome of potential broodstock collected at a separate time than those used in SNP discovery, and then spawn and evaluate growth and survivability of the F1 generation. This experiment has two main hypotheses. The first is that these SNPs will be discovered in the genome of a new set of oysters being used for broodstock selection. Additionally, that there will be several combination of SNPs found correlated to different phenotypes. This is related to the fact that, it is likely that genetic diversity will be high in the selected broodstock, since the Eastern oyster has not been domesticated. Furthermore, there is a distinct possibility that some oysters will not contain any of the SNPs. The second hypothesis is that due to MAS, the F1 generation will exhibit significant differences in growth between groups. It is important to note, that metabolic rate SNPs that were discovered in a related study will also be applied in this experiment and will be discussed for clarity purposes.

4.3. Materials and Methods

4.3.1. Broodstock populations

Oysters were collected in November 2015, from two locations in Bouctouche, NB. The first population, was from an aquaculture site, using suspended culture techniques that represented optimal conditions, with a salinity range from 20–28‰ and temperatures seasonally ranging from 0–26ºC (N46'30"35.35; W64'40"31.33). The second location, was

a wild oyster population which represented suboptimal growing conditions with a muddy bottom, inefficient access to food, oxygen, predation and fluctuating salinity. At this location, salinity ranged from 0—20‰ and temperature ranged from 0—24ºC (N 46'27"20.9; W64'44"42.8). A total of 120 oysters were collected (60 from each population) to be used in selection of broodstock (~76mm shell height (SH)). Oysters from the aquaculture farm were retrieved directly from culture bags and wild oysters were collected from the muddy bottom using an oyster drag. At time of collection water temperature at the first location (Bouctouche Harbour) was 3.5ºC and salinity was 25.1‰. The second location (Bouctouche River) temperature and salinity at time of collection was 5.0ºC and 14‰, respectively.

Oysters were transferred to the Dalhousie Aquatron, Halifax, NS within 24 hours of collection. The oysters were held in four 150L tanks with a flow rate of 1.25L/min tanks at 4ºC and 22‰, to mimic natural conditions. Feeding ration was at 10^6cells/L five days a week, using a blend of *Isochrysis galbana* (T.ISO)*, Chaetoceros mulleri, Chaetoceros calitrans* and *Monochrysis lutheri.* Oyster mortality was checked daily.

4.3.2. Metabolic SNPs

SNPs were discovered relating to high and low metabolic rate, in a related study. A metabolic rate trial was conducted by measuring oxygen consumption of oysters at maximum feeding rate and after starvation, to identify individuals with high and low metabolic rate. Oysters in the metabolic rate trial came from the same population of oysters in Bouctouche River, NB as the oysters in the absorption efficiency trial and the present study. SNPs were discovered using RAD-seq and Stacks software, in parallel to the SNPs discovered correlated to absorption efficiency. For purpose of clarity, metabolic rate SNPs will be discussed in terms of broodstock selection spawning and the F1 generation.

4.3.3. Identification and haemolymph sampling

For the purpose of broodstock selection and cataloguing purposes, each oyster was measured, identified as wild or aquaculture, and given a unique number ranging from 1—120. Haemolymph samples were collected from each live oyster by drilling through the

shell using a dremel tool with a 1/16th inch drill bit. Before drilling, the approximate location of the adductor was estimated on the posterior end, and drilling occurred directly below the estimated location of the adductor muscle, on the dorsal side of the oyster. While drilling through the oyster, the drill bit was placed on a slight angle so that drilling occurred slightly towards the anterior direction of the oyster. A 1/2inch No. 20 needle was inserted into the drilled hole under the adductor muscle, into a haemolymph sinus, in close proximity to the pericardial sac, and 100µL of haemolymph was extracted from each oyster. Drilled holes were left to heal naturally.

4.3.4. DNA extractions RAD-sequencing and analysis

DNA extractions were conducted on all haemolymph samples using the protocol by Clark (2015), which was adapted from the Ivanova *et al.* (2006) 96 well plate extraction protocol. After this, DNA samples were sent to Université Laval, Quebec for cDNA library construction. Libraries were constructed on a total of 120 oysters. DNA was digested using the restriction enzymes PstI and MspI (4 bp recognition sites). Individual barcodes were attached to the PstI overhang to identify each individual, and the MspI extension was used ensure proper fragment sizes for sequencing. Libraries were sent to Genome Quebec, Montreal, QC, and were run on two lanes of an Illumina HiSeq2500, with paired-end 100 nucleotide sequencing.

4.3.5. Broodstock selection

RAD-seq results were analyzed using the software Stacks version 1.18 developed by Catchen *et al.* (2011; 2013). The web-based Galaxy platform GenOuest (Rennes, France) was used to run Stacks software. Using the *process RAD-tag*s pipeline, samples were demultiplexed, into individual RAD-tag files. Additionally, RAD-tags were cleaned, sorted and given a quality scoring and poor quality reads were discarded. The *de novo map* pipeline was run using default parameters with only one population being inputted, so that locus would be assembled but no comparison could occur, since only one population was inputted. The *populations* pipeline was then run using default settings and the results from *de novo map.* After this, a National Center of Biotechnology Blastn (NCBI, Bethesda MD) search was conducted using SNP Loci sequencing described in chapter 3, with all Loci

assembled from the broodstock RAD-sequencing results. This blastn was conducted using the Megablast setting at an E-value threshold of 10^{-4}. Matched Loci were then assigned back to the individual oyster and odds ratio (OR) was used to determine the strength of the SNP and if it was relating to high or low absorption efficiency phenotype. For each individual oyster that contained one or more SNPs, ORs were cumulatively assessed to determine the likelihood of exhibiting a high or low absorption efficiency. As discussed in chapter 3, a negative OR, was correlated to oysters that exhibited a low absorption efficiency and positive an OR was correlated to high absorption efficiency individuals. For metabolic rate, negative OR was correlated to individuals with low metabolic rate, and positive OR to high metabolic rate. The cumulative OR was then used to develop oyster spawning groups, based on threshold OR ranges and likelihood of exhibiting a particular phenotype. Only oysters that contained SNPs and OR within threshold ranges were used as broodstock spawning groups.

4.3.6. Conditioning

Broodstock were kept in four flow-through 150 L tanks, with a flow rate of 1.25L/min. Oysters were batch fed twice daily at 100cell/µL using a combination of species of fresh algae including *Isochrysis galbana* (T.ISO)*, Chaetoceros mulleri, Tetraselmis chui,* and *Monochrysis lutherii.* At the beginning of the conditioning period, water temperature was approximately 4ºC. Water temperature was increased by 1ºC/day until water temperature reached 18ºC. At this point, water temperature was held at 18ºC for an additional three weeks to allow time for gametogenesis to occur.

4.3.7. Spawning

Spawning occurred on May 16, 2017 using a combination of natural spawning and strip spawning. Strip spawning was used as the secondary method in this experiment, and was only used if spawning could not be induced through thermal stimuli. Broodstock were placed into shallow troughs, based on their spawning group. Induction involved placing the oyster into a water bath at 16ºC for approximately 40min and then moving them into a warm water bath at 28ºC for an additional 40min. All oysters that were induced to spawn were placed in individual water baths at the beginning of their spawning event so that

gametes could be isolated. Water baths were alternated between the warm (28ºC) and cold bath (16ºC) for approximately 3 hours, and all oysters that did not spawn were stripped. Strip spawning occurred by shucking open the oyster and making small cuts using a scalpel blade and washing gametes into individual beakers. All individuals spawned were identified as male or female. Eggs and sperm were pooled separately, and based on broodstock group. The eggs were washed and hydrated and approximately 30mL of sperm was added to the corresponding group of eggs. Each group was monitored for approximately 60-90 min to ensure that fertilization and first divisions were occurring and finally were added to a larvae rearing tank.

4.3.8. Larvae rearing

Larval rearing was conducted in a static rectangular, flat bottom, polyethylene tanks with a volume of 946L (Figure 2). Approximately 48h post-fertilization, larval tanks were drained using a 55µm sieve over a 20µm sieve to catch larvae, and remove debris. Tanks were cleaned using Virkon™ (Virkon™ Disinfectant Technologies, Suffolk UK) and rinsed with filtered seawater. Larvae counts and shell height (SH) (umbo to outside to outside edge) were estimated, and then larvae were immediately returned to their respective tanks. Tank cleanings, larval counts and size estimations occurred every 48h during this time. Water temperature was acclimated to room temperature (~19ºC) and salinity was maintained at 22‰. Larvae were kept in this static system until development of an eyespot occurred. During larvae rearing they were fed a combination of algae species including *M. lutherii, I. galbana* (T.ISO)*, C. mulleri, Nannochloropsis sp.,* and *Tetraselmis sp.*.

4.3.9. Spat rearing

After development of an eye spot, larvae were transferred to a flow-through downwelling system for settlement. The downwelling system consisted of 19L bucket with a 150µ fine nylon mesh bottom. A layer of shells between 250—500µm was used as cultch. Water flow was configured with a spray bar at the bottom of the bucket that maintained a 1.5inch layer of water that was constantly moving though the 150µm fine mesh. In addition, the downwelling system was covered using black tarp to encourage settlement. Feed was constantly dispersed using a drip system adapted from a chicken feeder. Feed was dripped

slowly into the buckets (Figure 2). Water was maintained at temperature of 23.5ºC and salinity of 22‰. Once settlement occurred, spat was transferred to an upwelling system. This system consisted of a similar system as the downweller, except that water was moving up through the 150µm fine mesh screen and over the top of the bucket. The system was maintained on a recirculating system, but changed to a flow-through for one hour daily to flush out waste and debris. Water temperature was increased to 25ºC and salinity maintained at 22‰. Oysters were fed the same mixture of algae species mixture as during larvae rearing and grown to 1.5mm SH.

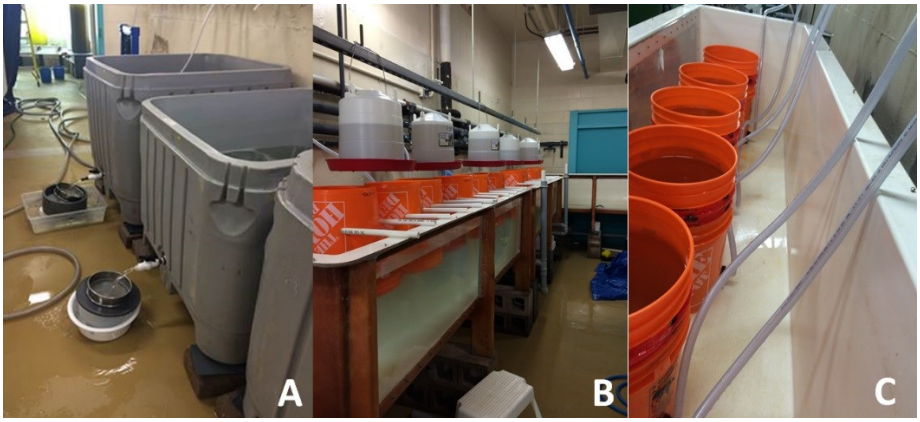

Figure 3: Tanks used for larvae and spat rearing. (A) Larvae rearing tanks used from post-fertilized egg stage to eyed stage. *Note: picture was taken during larval sieving process for tank cleaning purposes. (B) Downwelling system with overhanging algae drip system used during eyed larvae to settlement stage. (C) Upwelling system used for spat stage.

4.3.10. Grow-out system

Once oysters reached a minimum size of 1.5mm SH, they were transferred to an aquaculture site in Bouctouche Harbour, NB. The aquaculture site used suspended OysterGro (Bouctouche Bay Industries Ltd., Bouctouche, NB) cages each containing 6 Vexar bags. Spat were transferred to 1mm mesh bags, with approximately ~1,100 individuals per bag. Oysters will be grown in these bags until spring 2017 or until they reach approximately 30mm SH. Cages were suspended in the water column to provide optimal growing conditions during summer and fall period and sunk below ice during the winter period which is an industry standard.

4.3.11. Evaluating F1 generation.

Spat growth, survival and genotype will be assessed again in spring 2017. Genotype will be assessed by subsampling each group, extracting DNA and conducting RAD-seq analysis. SNPs corresponding to traits for high or low food absorption efficiency (determined in chapter 2) will be investigated in the F1 generations genome, to determine if SNPs were heritable and if the F1 generation containing SNPs showed superiority in terms of growth, survival. The results from the spring 2017 will be available in a study outside of this thesis.

4.4. Results

4.4.1. Broodstock selection

Table 6: Significant absorption efficiency SNP loci information and the prevalence found during broodstock selection. This table indicates each SNP found, the loci it was found within and corresponding OR. The number of individual oysters found to contain each SNP locus is indicated with the corresponding % of individuals with the SNP. The SNP variant(s) is also indicated with the related % frequency.

Locus ID	SNP ID & position	OR	# of oysters found to have loci	% of oysters with SNP in loci	SNP Allele(s) prevalence
Locus_14235	**1** (89)	9.3	37/120	2.7%	AG (100%)
Locus_15610	**2** (40)	5.1	33/120	39.3%	TT (100%)
Locus_15729	**3** (88)	17.5	38/120	10.5%	TT (100%)
Locus_16396	**4** (70)	10.8	42/120	30.9%	AG (61%) /AA (39%)
Locus_17969	**5** (72)	-10.3	74/120	1.3%	TC (100%)
Locus_18367	**6** (16)	7.5	34/120	52.9%	AA (100%)
Locus_19241	**7** (22)	8.3	33/120	39.3%	TC (62%) /TT (38%)
	8 (85)	8.3	33/120	12.1%	AG (100%)
Locus_29276	**9** (13)	13.2	39/120	23.0%	GG (100%)

Locus ID	SNP ID & position	OR	# of oysters found to have loci	% of oysters with SNP in loci	SNP Allele(s) prevalence
	10 (51)	13.2	39/120	N/A	N/A
Locus_35511	**11** (8)	-7.0	37/120	16.2%	AG (100%)
Locus_40270	**12** (15)	-12.0	42/120	9.5%	AA (100%)
Locus_4298	**13** (60)	9.2	15/120	13.3%	TT (100%)
Locus_44164	**14** (45)	7.0	0/120	N/A	N/A
Locus_6771	**15** (50)	-12.4	56/120	35.7%	TC (60%) /CC (40%)
	16 (86)	-14.5	56/120	28.5%	AG (68%) /GG (32%)
Locus_9767	**17** (11)	-9.2	0/120	N/A	N/A

Table 6. shows all significant loci discovered in chapter 3, that contained SNP's correlated to absorption efficiency. After the NCBI Blastn search, 74 oysters out of the 120 oysters sequenced were found to have the loci detected in chapter 3 related to absorption efficiency and metabolic rate. In addition, in the 74 oysters, not all loci were in every individual and therefore each individual had different combination of loci present. All loci discovered in chapter 3 were found, except for Locus_9767 and Locus_44164. Furthermore, of the loci found in the broodstock, only a fraction of the individuals actually contained the SNP correlated to absorption efficiency (Table 6). SNPs variants were found to be heterozygous, homozygous or both, and is summarized in table 6.

Table 7: Broodstock selection groups developed by cumulative OR for SNPs correlated to absorption efficiency and metabolic rate (A=aquaculture, W=wild). Note: Positive OR is correlated to high absorption efficiency and negative OR to low absorption efficiency

Group # /likely phenotype based on correlated SNPs	Oyster ID	Cumulative OR for absorption efficiency	Cumulative OR for metabolic rate
1: High absorption efficiency and high metabolic rate	W53	34.1	14.9
	W45	28.4	14.9
	W13	28.3	14.9
	W42	23.4	19.1

Group # /likely phenotype based on correlated SNPs	Oyster ID	Cumulative OR for absorption efficiency	Cumulative OR for metabolic rate
	W11	23.4	23.7
	W37	21.5	4.8
	W52	20.5	23.7
	A66	18.3	4.8
	W5	17.5	8.7
2: High absorption efficiency and high metabolic rate	A20	13.2	4.8
	A17	13.2	8.7
	W6	13.2	13.5
	W59	13.2	23.7
	A63	10.4	32.1
	A12	8.3	10.1
	A68	8.3	18.8
	W7	7.5	0.8
	W51	7.5	4.8
	W4	7.0	4.8
3: Efficient: High absorption efficiency with no information on metabolic rate	A4	30.7	N/A
	A59	20.9	N/A
	A22	16.6	N/A
	W55	15.9	N/A
	A58	10.8	N/A
	A19	7.5	N/A
	A27	7.5	N/A
	A8	7.5	N/A
	W24	7.5	N/A
	W48	7.5	N/A
4: Non-efficient: Low absorption efficiency and high metabolic rate	W58	-7.0	14.4
	W50	-7.0	18.4
	W2	-10.9	37.5
	A3	-13.6	13.5
	W26	-14.2	8.7
	A67	-19.4	4.8
	W22	-21.7	8.7
	W30	-21.7	18.4
	A13	-24.4	4.8
	A43	-26.9	3.6
	W19	-26.9	18.4

Oyster groups were selected based on OR for both absorption efficiency and metabolic rate, as shown in Table 7. Odds ratio was used in the selection process, as it gave a quantitative measurement that could be used in an additive way to determine the likelihood for an organism to exhibit high or low absorption efficiency. When the cumulative OR of

all SNPs present for an individual is correlated to a positive value, it's more likely to exhibit a phenotype related to high absorption, and negative values to low absorption. Although metabolic rate SNPs discovery is not included in this thesis, for clarity of understanding groupings, metabolic rate will be discussed. The ideal phenotype for efficiency and growth is high absorption efficiency and low metabolic rate, however SNPs correlated to low metabolic were not found in the broodstock. Therefore, two groupings (1 and 2) containing SNPs with high absorption and high metabolic were used for spawning, to better understand how the two set of SNPs would affect phenotype. However, when developing grouping 1 and 2, only high absorption efficiency cumulative OR's were considered in the grouping, with the minimum thresholds being 17.5 and 7.0, respectively. For group 3, all oysters that contained a minimum cumulative OR of 7.5 for absorption efficiency and no SNP information relating to metabolic rate was used. Finally, group 4, was made up of oysters containing SNPs relating to low absorption efficiency and high metabolic rate were used, with a cumulative ORs of less than -7.0 for absorption efficiency and greater than 3.6 for metabolic rate. Furthermore, only 40 oysters fell within the thresholds used in developing groups and individuals, and their cumulative ORs are shown in table 7. The SNP's found in each broodstock group are described in table 8. There was a different set and prevalence of SNPs found in each oyster.

There were several SNPs found within each broodstock spawning group. Table 8 shows the SNPs prevalent, their OR and how many individuals contained each SNP. One oyster in group 1, and two oysters in group 2, contained a SNP correlated with low absorption efficiency. Additionally, the SNPs that were most prevalent were SNPs 3 4 and 7 in group 1; SNPs 6, 7 and 9 in group 2; SNP 6 in group 3; and SNPs 15 and 16 in group 4.

Table 8: Description and prevalence of SNPs found in each broodstock group

Broodstock Group	SNP	OR	Annotation	# of oysters with SNPs in the group
1: High absorption efficiency and	1	9.3	Sterile alpha and TIR motif	1/9

Broodstock Group	SNP	OR	Annotation	# of oysters with SNPs in the group
high metabolic rate	2	5.1	Interleukin-17 receptor	2/9
	3	17.5	N/A	4/9
	4	10.8	uncharacterized protein (LOC105332190)	5/9
	6	7.5	N/A	3/9
	7	8.3	N/A	4/9
	8	8.3	N/A	1/9
	9	13.2	N/A	1/9
	13	9.2	uncharacterized protein (LOC105332626)	1/9
	16	-14.5	"ERC" partial	1/9
2: High absorption efficiency and high metabolic rate	4	10.8	uncharacterized protein (LOC105332190)	1/10
	6	7.5	N/A	3/10
	7	8.3	N/A	3/10
	9	13.2	N/A	5/10
	12	-12	colorectal mutant cancer isoform	2/10

Broodstock Group	SNP	OR	Annotation	# of oysters with SNPs in the group
	13	9.2	uncharacterized protein (LOC105332626)	1/10
3: Efficient: High absorption efficiency with no information on metabolic rate	2	5.1	Interleukin-17 receptor	2/10
	3	17.5	N/A	1/10
	4	10.8	uncharacterized protein (LOC105332190)	2/10
	6	7.5	N/A	5/10
	7	8.3	N/A	2/10
4: Non-efficient: Low absorption efficiency and high metabolic rate	2	5.1	Interleukin-17 receptor	4/11
	4	10.8	uncharacterized protein (LOC105332190)	1/11
	6	7.5	N/A	1/11
	9	13.2	N/A	1/11
	11	-7.0	Thioredoxin domain	3/11
	12	-12	colorectal mutant cancer isoform	1/11
	15	-12.4	"ERC" partial	9/11
	16	-14.5	"ERC" partial	7/11

4.4.2. Spawning

Natural spawning was only induced using temperature stimuli for four individuals (A4, A22, A27, and A58) in group 3, others were stripped spawned. In all other groups' natural induction method was not successful; therefore strip spawning was carried out.

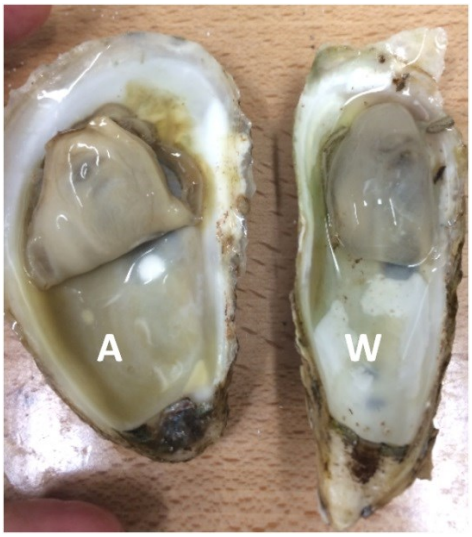

Figure 4: Visual comparison in gamete development of oysters (A = aquaculture; W= wild). Gametes are indicated by yellow-beige coloured mass diffused over the digestive gland

4.4.3. Larvae rearing, spat and grow out

An approximate count of oysters at each landmark day during development is indicated in Table 9. At post-fertilization stage, egg counts ranged from 320,000—1,229,666. During spawning, gametes of wild oysters appeared (based upon visual observation) less developed compared to aquaculture oysters (Figure 4). Additionally, it was found that groups 1 and 2, which contained more wild oysters, had a lower post-fertilization egg count compared to groups 3 and 4 (Table 7). It took approximately 22 days to reach the eyed stage and at this time larval SH ranged from 180–260µm. Overall counts at the spat stage ranged from 23—12,480 individuals. Since group 1 only had 23 oysters, this group was removed from the study; remaining groups will be genotyped at a later date. At the field grow out site, spat SH ranged from 1.5–7mm at 91–92 days.

Table 9: Approximate counts at different landmarks in larval development and spat.

Spawning group	Post-fertilization	Eyed larval stage	Spat
1	320,000	<2500	23
2	673,333	345,000	4748
3	1,100,000	360,000	12,480
4	1,229,666	173,333	8,252

4.5. Discussion

4.5.1. Marker-assisted selection and SNPs

Marker-assisted selection is a powerful tool that is being used to select for traits of interest in farmed organisms. In comparison to terrestrial farmed organisms, it has taken a long time for MAS to be established in aquaculture farmed species (Goddard *et al.* 2010). One of the first aquaculture species to be used in MAS breeding programs was Atlantic salmon, for IPN resistance. The QTL associated with IPN resistance was first confirmed by Moen *et al.* (2009), by using a genome scan of 10 different families (full-sib) challenged by IPN (Moen *et al.* 2009). However, for many non-model aquaculture species, the use of genome scans is not a feasible method for discovery of QTLs. Recent advancement in NGS technology, such as RAD-seq (Baird *et al.* 2008), has opened up the possibility of discovering and genotyping a large amount of genetic markers in a cost and time efficient way. In fact, the power of RAD-seq has been demonstrated by the study conducted by Houston *et al.* (2012), which found SNPs related to the quantitative trait loci QTL associated with IPN resistance in farmed salmon, and confirms the usefulness of RAD-seq as an approach to MAS.

Marker-assisted selection is in the initial phases of being used in shellfish species. For example, the Pacific oyster has been extensively studied to identify QTLs and genetic linkage maps (Li & Guo 2004; Hubert & Hedgecock 2004; Hedgecock *et al.* 2007; Sauvage *et al.* 2010; Guo *et al.* 2012; Zhong *et al.* 2014; Hedgecock *et al.* 2015; Wang et al 2016); however, it is only in beginning phases of application in a MAS breeding programs (Zhong *et al.* 2017). RAD-seq has also been used in the Pearl oyster (*Pincata fucata*), to discover QTLs associated to growth related traits. In this study a total of 39 QTL-peak loci for growth related traits were discovered and linkage groups were developed. However, like

the Pacific oyster, it has still not been applied in a MAS breeding program (Li & He 2014). In this study, RAD-seq was used to identify significant SNPs associated with high and low absorption efficiency and metabolic rate in the Eastern oyster. Significant SNPs were then identified in a different broodstock pool and were spawned to determine the F1 generations growth, survivability and genotype. Although family-based selective breeding of the Eastern oyster has been occurring since the 1950's (Allen *et al.* 1993; Guo 2008b), direct selection for growth related traits such as metabolic rate and absorption efficiency has never been conducted. This is also the first known, study to use MAS in the Eastern oyster. Although the effectiveness of these SNPs is still not fully understood (since the F1 generation has yet to be assessed in terms of growth and survivability) this study still gives insight that SNPs identified through RAD-seq can be successfully applied in a broodstock for MAS.

In this experiment, only 74 individuals contained SNP loci; however, not every individual contained the same loci, and of the loci present only, a small percentage of individuals tested positive for the SNP (Table 6). RAD-seq is a method of sequencing that relies on randomly shearing of genome with use of restriction enzymes (Davey & Blaxter 2011). This means that it is not guaranteed that the same loci will be sequenced each time. During SNP discovery, the SNP locus was only retained if it was present in 70% of the individuals, to ensure that the loci was well represented. That being said, most loci were not found in 70% of individuals in this experiment, but this is most likely due to the random shearing in the RAD-seq technique.

The cellular mechanisms involved in absorption efficiency, digestion, and growth are not fully understood in bivalve mollusks. Only a few functional genes have been found to be related to digestion and growth in bivalves. For example, the amylase gene has been found to be linked to absorption and growth (Prudence et al 2006; Huvet *et al.* 2008), and the insulin-related peptide has been found to be related to metabolic rate, growth and reproduction in the Pacific oyster (Hamano & Awaji 2002; Hamano *et al.* 2005; Cong *et al.* 2013). Additionally, the myostatin gene is related to growth in Farrer's scallop (*Chlamys farreri*) (Wang *et al.* 2010) and bay scallop (*Argopecten irradians*) (Guo *et al.* 2011). As discussed in chapter 3, 17 significant SNPs were discovered and correlated to absorption

efficiency. The SNPs were annotated to potential proteins related to the immune system, cell signalling via neurotransmitters, metabolic processes, and a few that were uncharacterized or failed to be annotated at all. These SNP's were then applied to a broodstock generation. Interestingly, the SNPs found most commonly in the broodstock relating to high absorption efficiency were either uncharacterized proteins or failed to be annotated in the NCBI database. In contrast, the SNPs found most commonly related to individuals with low absorption efficiency were two SNPs found within the loci annotated potentially as an ERC partial sequence, involved in cell signalling via neurotransmitters, which has not been extensively studied in invertebrates such as the Eastern oyster. It therefore has become apparent that since so few genes have been found to be related to growth and digestion, and so many SNP loci that were significantly correlated to absorption efficiency have not been characterized, or functions in invertebrate species are not known, that further research in this field is needed in order to better understand function role in absorption efficiency, digestion and growth.

4.4.1. Limitations in experiment

There were a few limitations in this experiment. For instance, gametes of wild oysters were less developed (based on visual inspection) compared to the gametes of aquaculture oysters. Both groups were considered to be the same population and received the same treatment, and therefore it was expected that both groups should have performed similarly in terms of gametogenesis. That being said, the two groups of oysters came from location that were very different conditions in Bouctouche, NB. The aquaculture site used suspended culture techniques with salinity and temperature ranging from 20—28‰ and 0—26ºC, depending on season and tide. The wild oysters were located upriver the salinity fluctuated greatly from 0—20‰, depending on the tide. This site had suboptimal conditions for oysters, as it contained a muddy bottom, where oysters were partially buried, less access to oxygen and feed (phytoplankton).

There is no way of knowing exactly what caused this inconsistency in reproductive output. One hypothesis, was that the wild oysters could have been in a lower condition at the time of transfer and/or the transfer was more stressful. Oysters in the wild population at the time

of transfer, were at 14‰, but were immediately transferred to 22‰ at the aquatron. Therefore, the rapid change of salinity could have been a source of stress. Since the oysters from upriver were exposed to fluctuating salinities (0—20‰), they should be more likely to cope with rapid changes in salinity. However, since 22‰ is out of their normal range, it might have been more difficult to adapt and spawn. A study by Hosoi *et al.* (2003) looked at the effect of salinity change on osmolytes on the Pacific oyster. This study looked at the ability for an oyster to osmo-regulate within 72 hours after an abrupt salinity change from 15‰ to 30‰, and found that the oysters weren't able to return to original state after the 72 hours (Hosoi *et al.* 2003), indicating that the event could be stressful. However, the study by Hosoi *et al.* (2003) did not look at long-term effects of increased salinity, and therefore it is unknown whether this could be a long-term event. In addition, 22‰ is within the optimal range (10—27‰) for the Eastern oyster (Galtsoff 1964), indicating that it should not have been a long-term stress event.

Another hypothesis that could have resulted in observed differences in reproductive development, was that there could have been a genetic trade-off between adaption to suboptimal conditions and reproductive effort. This link has been found before, in the Pacific oyster relating to summer mortality. Summer mortality in the Pacific oyster is linked to an interactional effect of problems in physiology and metabolic rate, resulting from a great amount of effort being put into gametogenesis during high water temperature and eutrophication (suboptimal conditions) (Koganezawa 1974). It also has been found that resiliency to summer mortality is a highly heritable factor in the Pacific oyster, and the oysters that are resilient, put less effort into reproduction then oysters susceptible to summer mortality (Huvet *et al.* 2010; Samain *et al.* 2007). This is an interesting parallel to the present study, as the wild oysters from suboptimal conditions, appeared to have less reproductive development after being conditioned, compared to aquaculture oysters. Another study, by Butler (1949) found that oysters in lower salinity were overall behind in development by two months compared to oysters reared in high salinities. This could indicate that although the oysters were at a higher salinity during conditioning, they might have been unequal conditioning start points. However, further analysis would be needed to understand these differences.

Another limitation for this experiment, was mortality and lower initial stocking densities. In most hatcheries, initial stocking densities are usually between 15,000—20,000/L (Helm *et al.* 2004); however, in this experiment stocking densities were much lower. During larvae development in hatcheries and in the wild, mortality is often high during D-stage larvae and during metamorphosis. It is estimated that mortality between the D-stage and metamorphosis is between 30—50% (Helm *et al.* 2004). Mortality in this experiment was a factor in all groups, but especially for group 1. In addition, Group 1 also had a lower stocking density to begin with (~320,000). Interestingly, all but one oyster in that group were wild oysters. As discussed previously, gametogenesis appeared much lower in wild oyster group and therefore could have contributed to the lower stocking density. In addition, gamete viability is an important factor in larvae survival and is highly dependent on the oogenesis process (Helm *et al.* 2004). Therefore, if the wild oysters were not fully ripe for spawning, this could have been a reason for the lower initial counts and mortality, for group 1. In addition, it took ~22 days for larvae to develop an eye-spot, however in normal hatchery environments it takes 15—20 days. The reason for this remains unknown, however it is speculated that it could have resulted from cooler room temperature (19ºC), as optimal water temperature for larval rearing is around 28ºC and temperature is known to be a major factor in development (Helm *et al.* 2004). Since the larvae stage at this time of development normally has high mortality levels, a longer developmental period could have resulted in higher mortality (Helm *et al.* 2004).

4.4.2. Experimental hypothesis

There were two main hypotheses in this experiment. The first was that SNPs would be discovered in a broodstock and that there would be several genotypes found using MAS that would be correlated to phenotypes relating to growth, such as absorption efficiency and metabolic rate. This hypothesis was found to be correct, as the SNPs were discovered and there were many combinations of SNPs exhibited in the broodstock that could be correlated back using OR to different phenotypes of absorption efficiency and metabolic rate. Different levels of OR were also obtained, which indicated some oysters were related more or less to a specific phenotype. In addition, some oysters that were used in this study

were not found to have any of the discovered SNPs, and therefore were not used in the selection process to breed an F1 generation.

The second hypothesis that, through MAS for high or low absorption efficiency and metabolic rate, the F1 generation will exhibit significant differences in relation to growth. Currently, this hypothesis cannot be confirmed, as the F1 generation are still growing to a size at which they can be assessed, based on growth and survivability and genotype. It is predicted that group 1 and 2, will have a higher absorption efficiency and higher metabolic cost. Oysters with higher metabolic rates/costs have been associated with slower growing individuals (Bayne & Newell 1983; Bayne *et al.* 1999; Bayne et al 2000; Tamayo *et al.* 2014). However, having oysters that utilize food resources efficiently, would also mean they would have higher access to energy from these food resources (Bayne & Newell 1983). Therefore, it might be expected that selecting for higher metabolic cost and higher absorption efficiency could cancel each other out. Additionally, since group 3 has been correlated to high absorption efficiency but no information is known about metabolic rate, it is unknown how this group of oysters will respond in terms of growth. Group 4 was correlated with SNPs relating to low absorption efficiency and high metabolic rate. Therefore, it is predicted that these will most likely be low performing individuals in terms of growth. This prediction is related to the presumption that have high metabolic costs and utilize food resources inefficiently, will need more food to access the energy it needs to grow and therefore it is predicted that this group will be the low performing individuals (Bayne & Newell 1983; Bayne *et al.* 1999; Bayne et al 2000; Tamayo *et al.* 2014).

4.5. Conclusion

In this experiment, DNA of oysters from Bouctouche, NB were screened for SNPs correlated to absorption efficiency and metabolic rate. These SNPs were successfully found in oysters used for broodstock selection. Oysters that were identified to have the SNPs present were then placed in spawning groups based on their cumulative OR and were spawned. The F1 generation were grown in hatchery conditions until they had settled and reached a minimum of 1.5mm (SH). They were then transferred to a grow-out system where they will be grown to 30mm and then assessed based on growth, survivability and

genotype (spring of 2017). Interestingly, of the SNPs correlated to high absorption efficiency, the ones that were found most often, either failed to be annotated or were classified as uncharacterized proteins. The SNPs found most often, correlated to low absorption efficiency were annotated as ERC partials, which is related as cell signalling via neurotransmitters. Finally, this experiment is the first study to knowledge, to use SNPs discovered using RAD-seq *de novo* analysis in a MAS breeding program for the Eastern oyster. Although the effectiveness of these SNPs is still not yet fully understood, as the F1 generation has yet to be assessed in terms of growth and survivability, this study provides insight in that SNPs identified through RAD-seq can be successfully applied in a broodstock and used in MAS.

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CHAPTER 5: CONLUSIONS

5.1. Comparison of absorption efficiency between wild and culture oysters, *Crassostrea virginica*, (Bouctouche, New Brunswick) during a thermal challenge

5.1.1. Summary and conclusion

Absorption efficiency (AE) is defined as the efficiency at which ingested food is absorbed (Bayne & Newell 1983) and is a key component in growth. Oysters that exhibit a higher AE, would theoretically not only use food resources more efficiently, but have the capability to extract more energy from less feed, which could be converted to growth. As climate change occurs, the ocean is being disrupted, which is changing the physical conditions of the ocean (Shackell & Loder 2012). These changes in the ocean are potential stressors to the Eastern oyster (*Crassostrea virginica*). Environmental factors in the water column including temperature, feed concentration, presence of organic matter etc. have been known to affect AE in bivalve mollusks (Thompson and Bayne 1974; Bayne 1976; Widdows 1978; Widdows *et al.* 1979 Elvin & Gonor 1979; Griffiths 1980). Therefore, having oysters that exhibit a high AE (i.e.use food resouces efficiently), even under suboptimal conditions, could be a beneficial economic trait for the oyster aquaculture industry, while mitigating ecological footprint of oyster culture. The main objective of this study, was to measure AE in two groups of oysters from a Bouctouche River, NB, under ideal and suboptimal conditions. The first population of oysters was from an aquaculture site considered to be in optimal conditions, and the second was a wild population of oysters considered to be in suboptimal condition. Oysters in the treatment (suboptimal) group received a temperature of 30ºC and the control (optimal) group received a temperature of 20ºC. The study intended to better understand if there were differences in AE between wild and aquaculture, between individuals, and between stress and ideal conditions.

The results of this experiment indicated that overall there were no statistically significant differences between wild and aquaculture oysters in terms of AE, and that oysters from both groups were able to utilize food similarly, even under suboptimal conditions. This indicates that the two groups of oysters, despite coming from different environmental conditions, were able to absorb feed similarly, in terms of efficiency, and one group did

not show superiority over the other. However, oysters in this experiment appeared to have a lower AE than those in the literature with an overall mean of 35.64±2.6%. In other studies on mollusks being fed algae. Several factors could have contributed to this including a higher feed concentration (100cells/µL) during batch feedings (Bayne 1976; Winter 1978), indigestibility of feed, and, potentially, diatoms species which are harder to digest and absorb nutrients (Thompson and Bayne 1974; Widdows 1978; Griffiths 1980; Romberger & Epifanio 1981; Kuenster 1988; Ren *et al.* 2006). In addition, there were major fluctuations in AE between individuals with an overall range of 0-94.84%. This variability could have resulted from genetic differences between individuals. Genetic variation for growth has been found in wild populations of other species such as rainbow trout (*Oncorhynchus mykiss*) (Aulstad *et al.* 1972) and is often seen in controlled hatchery conditions at the larval stage (Gosling 2015). Since AE is a component of growth, it could account for some of the variability. Information obtained in this experiment has the potential to be used to discover markers associated with oysters with either high or low AE.

5.1.2. Future directions

It could be beneficial in future studies investigating AE, to use a continuous feeding regime, ie lower food concentration. Using species in which digestibility is known, could also be beneficial so that there is a baseline knowledge of the AE level, which would aid in determining an efficient oyster from a non-efficient. Moreover, it would also be benificial to investigate common species of algae found in Atlantic Canada, at different seasons to determine the effect it could have on AE. Additionally, since a hatchery industry is currently being developed, it could be useful in understanding what species of phytoplankton are most digestible/efficiently absorbed at the larval stage.

In this experiment, a thermal stress challenge was used to determine how it would affect AE. However, there was no significant difference between ideal conditions versus the suboptimal group, indicating the oysters were able to adapt to the change in temperature. In future studies, it would be interesting to determine the critical point at which oyster's AE is being effected by thermal stress. Furthermore, climate change is causing changes in

temperature but also salinity, acidity, feed availability, water currents etc. (Shackell & Loder 2012). Therefore, it could be useful to determine how other ocean dynamics being effected by climate change alter AE in oysters.

Other future directions stemming from this study, could be looking at more than one factor effecting growth, such as AE, metabolic rate and filtration in terms of growth. It could be useful to study all three factors, to better understand differences between aquaculture oysters versus wild, thermal stress versus ideal conditions, and individual differences between oysters for all three factors in the Eastern oyster.

5.2. Single nucleotide polymorphisms (SNPs) in the Eastern oyster (*Crassostrea virginica*) correlated to absorption efficiency using RAD-sequencing and *de novo* analysis

5.2.1. Summary and conclusions

As discussed previously, it is understood that there is a genetic relationship related to AE in mollusks (Prudence *et al.* 2006; Huvet *et al.* 2008). In chapter 2, an AE trial was conducted with an added stress thermal challenge, on two groups of oysters (wild and aquaculture oysters) from Bouctouche, NB. The main objective of this experiment, was to discover single nucleotide polymorphisms (SNPs) in the Eastern oyster genome related to high or low AE using Restriction site Association DNA sequencing (RAD-seq) technology. After the AE trial, oysters were euthanized, and a small tissue sample was collected from each oyster. DNA was then extracted from each sample, and sent to undergo RAD-seq analysis. The software Stacks (Catchen *et al.* 2011; 2013) was used to analyze the RAD-seq data.

This experiment resulted in 17 significant SNPs being discovered relating to AE in the Eastern oyster. It was found that 10 of the SNPs discovered were matched with protein coding sequences, indicating a higher likelihood of having a phenotypic effect. However, very little information is known about the function of the genes containing these SNPs in molluscs. Based on their annotation, the functions of these proteins were related to the immune system, cell signalling via neurotransmitters, metabolic processes, or were uncharacterized. It is unknown how these proteins and SNPs result in a phenotypic

response relating to AE, as very little information is known about the cellular pathways involved in digestion in mollusks. The SNPs discovered in this experiment, have the potential to be useful in future marker-assisted hatchery-based selective breeding programs

5.2.2. Future directions

As discussed, very little information is known about the pathways and cellular mechanism involved in AE in mollusks, and therefore this should be investigated further. The SNPs discovered in this experiment have a great potential to be successful candidates in marker-assisted selection (MAS) breeding program, however, further analysis of these SNPs is needed. It was determined that 10 out of the 17 SNPs were matched to protein coding sequences, which indicates that they may result in a phenotypic response. Phenotypic response could be altered through changing interactions that are involved with mRNA synthesis, maturation, transport, translation or degradation (Shen et al 1999). However, how these SNPs result in a phenotypic response and what other effects it could be having on the physiology is unknown, and therefore could be investigated in the future. Furthermore, six SNPs were not matched to protein coding sequences, and therefore understanding how these SNPs result in a phenotypic response could also be a potential future investigation. Additionally, it would be useful to align SNP loci in a genetic linkage map, to better understand inheritance patterns. Furthermore, if these SNPs are validated as a useful tools in determining individual exhibiting high or low AE, it would be very useful to develop a diagnostic test that could detect these SNPs without use of time consuming processes, such as sequencing technology and bioinformatics analysis. This could allow growers to have better access to the tools necessary to implement a MAS hatchery-based breeding programs.

5.3. Single nucleotide polymorphisms (SNPs) in the Eastern oyster (*Crassostrea virginica*) correlated to absorption efficiency using RAD-sequencing and *de novo* analysis

5.3.1. Summary and conclusions

Previously, an AE trial was conducted and 17 significant SNPs were discovered. In addition, in a related study SNPs associated to high or low metabolic rate were discovered.

This experiment used these SNPs in a MAS breeding program, by scanning DNA from a broodstock group of oysters for the SNPs correlated to AE and metabolic rate, using RAD-seq and National Center of Biotechnology Information (NCBI) blast tools. A total of 120 oysters were selected from two population in Bouctouche, NB, 60 wild oysters and 60 aquaculture oyster. Haemolymph samples were collected from each oyster, in a non-lethal matter. DNA was extracted from the haemolymph samples and were sent away for RAD-seq. The RAD-seq data from the broodstock was analysed and the target SNPs were discovered in the broodstock. The broodstock that were found to contain SNPs were categorized based on cumulative odds ratio. Group 1 contained SNPs correlated to high AE and metabolic rate; group 2, with SNPs correlated to high AE and metabolic rate; group 3, was based on all oysters that contained a minimum cumulative odds ratio of 7.5 for AE, but had no SNP information relating to metabolic rate; and group 4, was all oysters containing SNPs relating to low AE and high metabolic rate. After groups were developed, spawning was induced using a combination of temperature stimuli and stripping. The F1 generation was grown in a hatchery condition until they reached a minimum of 1.5mm. At this time, they were sent to an oyster farm in Bouctouche, NB, to be further grown in a suspended culture system until they reach 30mm. In spring 2017, or when oysters reach 30mm, growth, survival and genotype will be assessed. Since the F1 generation have not yet been accessed in terms of growth, it cannot be determined if these SNPs resulted in significant differences between groups in terms of growth.

5.3.2. Future Directions

In this experiment there two major limiting factors. The first was related to gamete development in the wild oysters. Wild oyster gametes were much less developed compared to aquaculture oysters. Understanding, why this discrepancy occurred might be of interest in future study, to determine if it could have been an adaptive mechanism for the wild population, or if it was related to stress and/or condition of wild oysters. The second limitation was low initial egg counts and mortality, especially in group 1. As gametogenesis in broodstock is an important aspect in larvae viability it could have been a major cause of mortality. In addition, if the gametes of the broodstock in the wild were not developed, it could have led to a lower initial egg-count. Moreover, temperature was at ~19 ºC, which is

lower than the optimal temperature for Eastern oyster larvae (Helm *et al.* 2004). Since temperature is a factor in development and major mortality occurs during this time, it could have also increased prevalence of mortality. Therefore, it may be of importance to conduct the same experiment again but correcting these limiting factors.

This experiment had the hypothesis that, due to MAS, the F1 generation will exhibit significant differences in growth between groups. If this hypothesis is proven true, then it would be very important to continue the process of MAS into the F2 generation to ensure viability of the SNPs in upcoming generations. Additionally, it would of interest to better understand the physiological component of these SNPs to ensure that there isn't a trade-off in other important physiological functions. Finally, it would also be of great important to create a genetic linkage map of the parental generation, and the F1 generation to better understand patterns of inheritance.

5.4. Additional comments

This study demonstrated that SNPs can be discovered that are correlated to specific traits, such as ones that are not grossly phenotypical like AE. In the future, as a hatchery-based selective breeding continues to develop, other traits can also be selected for in MAS programs. In addition, there are other physiological factors that have been linked with faster growth rate including filtration rate that SNPs could be identified for. It could be of interest to discover one set of SNPs/markers that are correlated to high AE, low metabolic rate and higher feeding rate/filtration rate, as all three have been linked to growth (Bayne & Newell 1983; Bayne *et al.* 1999; Bayne et al 2000; Tamayo *et al.* 2014). These general growth SNPs could then be used with the SNPs discovered in this study to give further support, as general growth markers.

A hatchery-based selective breeding program could be a potential solution to many of the issues that the oyster aquaculture industry is facing, currently, and in the future. However, potential concerns emerges to the environment by introduction of selectively bred oysters into grow-out sites. Eastern oysters sexually mature at a young age (6—10 weeks post-settlement) (Galtsoff 1964), but take three to five years to reach market size, (Bastien-Daigle *et al.* 2007; Doiron 2008), and therefore the aquaculture production cycle gives

ample time for breeding to occur with wild stocks. Consequently, concerns emerge over if the introduction of selectively bred oysters into an ecosystem could potentially change the population genetics of an ecosystem, and introduce deleterious alleles into the population (Camara & Vadopalas 2009). Currently in Atlantic Canada, this risk is considered low (Bastien-Daigle *et al.* 2007) but as the hatchery-based selective breeding program continues to develop, the risk arises. In order to mitigate this risk, either restriction of breeding between wild and aquaculture oysters needs to occur by implementing a triploidy or tetraploidy program in aquaculture bred oysters, or addition research needs to be conducted to better understand the implications resulting from wild and aquaculture oysters breeding.

5.5. Conclusion

The main goal of this projects was to discover genetic markers that were correlated to high AE which is a factor that effects growth. This study started out by measuring AE in two population (wild and aquaculture) of oysters from Bouctouche River, NB with an added thermal stress challenge to ensure oysters were able to be efficient even under suboptimal conditions. After this, RAD-seq analysis was conducted on tissue samples from the AE trial. Bioinformatic analysis was conducted and 17 significant SNPs were discovered in terms of either high or low AE phenotypes. The 17 significant SNPs discovered, were then identified in a new set of broodstock and spawned. The F1 generation were reared in hatchery conditions until they reached a suitable size, and were moved to a grow-out system in Bouctouche, NB. The F1 generation will in assessed at 30mm in terms of growth and survival in the spring of 2017.

There are many factors leading to the development of hatchery-based oyster aquaculture industry including, variability in annual spat collection (Doiron 2008), climate change (Shackell & Loder 2012), and the increasing demand for oysters (Chopin 2015). By implementing a selective breeding program within a hatchery-based industry, oyster growers not only have consistent spat supply, but also have control over the traits that their spat carries. It was determined that having oysters with higher growth rate while using food resources as efficiently as possible was pertinent for the oyster aquaculture industry. This

study was not only the first study to discover SNPs relating to AE in the Eastern oyster, but the first study to use MAS in the Eastern oyster, by use of RAD-seq and *de novo* analysis. The long-term results of this experiment are not fully known yet, but the potential for these methods to be applied in future studies are very probable, especially for other traits where the phenotype is not visually observed. It is expected that a hatchery-based selective breeding program will continue to be developed in the region, and MAS has the ability to be deliver results in a quick, and cost efficient manner, compared to traditional family-based selective breeding.

5.6.References

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