EASTERN OYSTER, *Crassostrea virginica*: PRODUCTIVITY AND PHYSIOLOGY UNDER VARYING TEMPERATURE AND SALINITY CONDITIONS

BY

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ABSTRACT

In the early 1990s, the introduction of suspended culture methods and the development of a market for cocktail oysters (65–75 mm) propelled the oyster aquaculture industry in New Brunswick forward, now producing over 140,000,000 oysters (337,000 floating bags) on 2,649 hectares of private shellfish leases. Recently, the industry has been threatened by an oyster disease outbreak, known as MSX in the Bras D'Or Lakes region (Nova Scotia). The possibility of the MSX parasite spreading into New Brunswick waters, as well as an increasing number of aquatic invasive species in the area, is a real concern for the industry and has prompted an investigation on possible mitigation strategies.

The benefits of incorporating an upriver site into the production cycle of oyster aquaculture operations were evaluated. Seed (~ 26 mm shell height) and adult (~ 66 mm shell height) oysters were taken from downriver (salinity ~ 20–30‰) aquaculture sites and transferred upriver (salinity ~ 5–20‰) and monitored for shell and meat growth (May-October). At the same time, the impact of the relay fishery (harvest of oyster in closed marginally contaminated areas for relay to tenures in open areas) on oyster productivity was evaluated; wild upriver oysters were transferred downriver and monitored. Productivity data revealed that seed transferred upriver grew and survived as well as seed that remained downriver, while the mortality rates of adult oysters were lower at the upriver site. Meat content was unaffected in adult oysters transferred upriver. However, adult oysters transferred upriver had a gain in shell height (least squares means

± standard error) over the oysters that remained downriver (2.7 ± 0.5 mm vs. 1.8 ± 0.5 mm) and, in shell width (Median [95% CI]; 2.8 mm [1.9, 3.6] vs. 1.0 mm [0.3, 1.2]). Final organic meat content were approximately 35% less in adult oysters transferred downriver compared to those that remained upriver, confirming anecdotal reports from fishers of the relay fishery. Stress response of adult oysters was also assessed (80 and 150 days post transfer). Stress response measured revealed that regardless of their origin (upriver, downriver), oysters maintained upriver showed high levels of lysosomal destabilization and tissue atrophy. By contrast, the transfer downriver had the opposite effect, i.e. low lysosomal destabilization and low tubule atrophy. Results suggest that there exists no association between productivity parameters and the level of stress response measured by the biomarkers.

The field investigation prompted further exploration on the impact of hemolymph fluid osmolality on neutral red retention assay (NRA) outcome. The prescribed saline solution for the assay assumes animals are isosmotic to surrounding water; however oysters sampled were hyperosmotic. Hemolymph osmolality was manipulated under laboratory conditions by subjecting adult oysters to temperature (0, 5, 10, 15, 20ºC) and salinity (6, 27‰) regimes. At 10–20ºC, oysters remained isosmotic to ambient waters, while hyperosmotic at 0–5ºC. When mimicking spring freshets (salinity 6‰), hyperosmotic condition was observed in all temperature groups, and was inversely proportional to temperature. Monitoring of valve activity confirmed a restricted exchange at 6‰ salinity (e.g. spring freshet). With these laboratory results, use of filtered

hemolymph fluid (NRA_{MOD}) is recommended instead of the prescribed standard solution (standard NRA) adjusted to ambient seawater.

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Dedicated to Robert, Mathieu and Caroline,

the stars that brighten my life each and every day.

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LIST OF ABBREVIATIONS

ΔLDI	Δ lysosomal destabilization index
AIS	aquatic invasive species
AO	ambient osmolality
AVC	Atlantic Veterinary College
C	condition index
CI	confidence interval
DFO	Department of Fisheries and Oceans
DH	destabilized hemocyte
DMSO	dimethyl sulphoxide
DNR	downriver
HO	hemolymph osmolality
LSM	least squares means
LDI	lysosomal destabilization index
MSX	*Haplosporidium nelsoni*
M	million
M	mortality
Md	median
NB	New Brunswick
NBDAAF	New Brunswick Department of Aquaculture, Agriculture and Fisheries

NS	Nova Scotia
NRA	neutral red retention assay
NRA_{MOD}	modified neutral red retention assay
PE	Prince Edward Island
S	shell surface area
SE	standard error
SH	shell height
SL	surface area of the lumen
SLT	surface area of the whole tubule, including the lumen
SW	shell width
TAI	tubule atrophy index
UPR	upriver
W	whole wet weight

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Chapter 1 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Eastern oysters, *Crassostrea virginica,* occupy ~8,000 km of coastline from the Gulf of St. Lawrence, Canada (48ºN) to Argentina (50ºS), including the Gulf of Mexico, Caribbean and Brazilian coasts (Gosling 2003a). Habitat loss, mismanagement of the fisheries, diseases and water degradation has led to massive declines in wild stocks since the late 1800s, prompting the industry to explore more sustainable methods such as farming to increase production (Dewey *et al.* 2011; Landry *et al.* 2001; MacKenzie 1996). As with the development of any aquaculture sector, site availability, efficient production cycle and high quality seed became the primary area of concern for ensuring sustainability and economic viability of the industry.

1.2 Oyster production

Oyster farming presumably began in 1600s in Hiroshima Bay (Japan) using stones and bamboo for wild seed collection (Fujiya 1970). Through the years, massive importations were made of the Pacific oyster, *Crassostrea gigas,* from Japan to Canada, US, Australia and France. By 2005, *C. gigas* comprised 97% of the world oyster production (http://en.aquaculture.ifremer.fr/, last accessed November 20, 2014). Unlike *C. gigas*, introductions made of the *C. virginica* mostly failed (Carlton

& Mann 1996). As a result, despite its vast natural geographical distribution, farmed *C. virginica* productions originate primarily from operations located in North America (FAO 2004).

In 2012, the United States accounted for 99,047 Mt ($74M US) of the farmed *C. virginica* production, while in Canada, the production level was 4,026 Mt ($13M US) (FAO 2014). Most of the oyster production (64%) in Canada comes from *C. gigas*. It is only farmed in British Columbia (7,200 Mt) ($10.2M CAN) (www.dfo-mpo.gc.ca/aquaculture, last accessed November 18, 2014). Prince Edward Island (PE) and New Brunswick (NB) account for 90% of *C. virginica* landings (wild and cultured) in Canada. Following a dramatic decline (>90%) in oyster production in Eastern Canada due to Malpèque disease, harvesters and government officials responsible for management of public beds in NB and PE enhanced oyster production by adopting desilting (i.e. removing silt from shell beds) and seeding practices (McKenzie 1996). As a result of those efforts, today in PE the wild oyster fishery accounts for 80% of the landings with only 20% from aquaculture. In NB, a slower recovery from the Malpèque disease outbreak (Logie *et al.* 1961), coupled with degrading ecological health of public beds (Landry *et al*. 2001) has prompted a stronger interest in farming of oysters. Private and public investments in recent years have contributed to increased farmed production, representing now ≥50% of the landings in that province.

1.3 Oyster culture in New Brunswick

The oyster industry is concentrated in the bays and estuaries of the Gulf of St. Lawrence as far south as the Nova Scotia (NS) border and north to Caraquet Bay, NB. The development of oyster aquaculture began in the last century with impressive progress made over the past two decades. In the earliest stages of this developmental phase, bottom culture was the main technique used, but the recent introduction of suspended (or floating) culture, in addition to the development of a market for cocktail oysters (65-76 mm), contributed to an unprecedented growth of the industry. In 2005, standing stock (whole wet weight) of cultured oysters in the province was evaluated at 1,249 mT (100M units) over 544 hectares, with an estimated 42% of the tonnage at commercial size (76 mm) (Comeau *et al.* 2006). By 2012, oyster inventory was estimated at more than 140M units cultivated in floating bags (337,000) on 2,649 hectares of private shellfish leases (NBDAAF 2012; Marie-Josée Maillet, NBDAAF, pers. comm.). Sales were estimated at 15M units ($5.2M), with a farm gate value of between $0.28/unit (cocktail) and $0.38/unit. It is estimated that over 90% of the oysters sold today are cocktail oysters (Maurice Daigle, La Maison Beausoleil Inc., pers. comm.). Due to the growing demand for cultured product, it is expected that the oyster industry in NB will continue to grow; however, given the limited number of traditional culture sites (i.e. lease areas) and the dependence of the industry on wild seed collection, there is uncertainty as to whether the industry can sustain this level of growth. The industry is currently facing a shortage of available lease acreage and there are many unknowns as to the quality and health of the seed source, particularly

worrisome in light of the recent MSX outbreak in NS (McGladdery & Zurbrigg 2006).

1.4 Traditional culture sites

When oyster culture began in the mid-19th century in Atlantic Canada, oyster seeds were transferred from natural reefs to bottom areas for grow out (Lavoie 1995). The bottom is typically comprised of loose shells on sandy or rocky substrate. Clusters of growing oysters cemented to existing shells are commonly found on these natural beds. Although bottom culture is still practiced today, it is limited for the most part to the public fishery (or public beds). Suspended and off-bottom culture methods are now commonly used in the area. Regardless of the technique, suspended, off- or on-bottom, culture sites are usually protected from strong currents or wave action by a natural barrier (e.g. island or dune), supplied with nutrient-rich food and have a water depth of less than 5 m (1 m tides) (Lavoie 1995). These areas generally have ideal growing conditions, where the salinity fluctuates between 20–30 gL^{-1} and temperature peak at 25–26º C. In these same estuaries, however, cultured oysters can experience a wide range of salinities (10–30 gL^{-1}) and temperatures (-1.5–26º C) (Comeau *et al.* 2012). The occurrence of predators and fouling organisms, like mud and green crabs, starfish and barnacles are widespread. The presence of these predaceous and fouling organisms is less problematic however for off-bottom or suspended culture techniques than for bottom-culture.

1.4.1 Current husbandry practices

The oyster industry relies heavily on wild collected seed from a number of high seed producing areas in the bays and estuaries of NB and PE (Drinnan & Stallworthy 1961; Méthé & Léger 1994). Gametogenesis begins the previous fall with full gonad development completed by the following summer. Once water temperature exceeds 20ºC (~July), spawning is triggered either as a single or extended event, through the release of eggs and spermatozoids in the water column (Loosanoff & Davis 1952). Fertilization is external in the water column. Within the following 24 hr, the larvae form a shell, and develop swimming appendages and feeding organs. Oyster larvae actively swim vertically in the water column and passively drift with tidal currents for ~ 2 wk, depending on environmental factors such as temperature, salinity and food (Gosling 2003b). During this period they feed off phytoplankton and get preyed upon by the plankton community. Once the free-swimming larvae reach ~300 µm shell length, a foot will develop and they will descent in the water column and permanently attach to calcareous substances, such as shells, stones by secreting a cement-like substance. Settlement generally occurs over a 1–2 week period in July (Mayrand *et al.* 2013; Méthé & Léger 1994). During this time growers have set out artificial collectors suitable for recruitment, such as Chinese hat, plastic drain tiles or pipes covered with a cement mixture (lime, cement, sand), on a floating long (60 m) line system (Doiron 2008; Ferguson 1984). For enhancement purposes, desilting of shells on public fishery grounds has been carried out by fishers. Once settled, oyster seed will remain permanently attached. No further work is required for seed settled on bottom grounds. However, oyster seeds that have settled on artificial

collectors need to be removed and separated through a mechanical or manual threshing process (Doiron 2008). This is generally carried out in early September. The seed (5–15 mm SH) are subsequently placed in 2–4 mm plastic mesh bags at a predetermined volume (or count), approx. 2–4 L bag-1 (500–1,000 units bag^{-1}). Most growers place their bags in larger cages holding 5–6 bags $cage^{-1}$ or attach two cylindrical buoys to either side of the bag; both systems are kept in suspension on a long line (60 m) system (Doiron 2008). Some growers will attach bags to rebar tables that are normally placed in the intertidal area, also known as off-bottom culture. In late November, cages/tables (or floating bags) are sunk a minimum of 1m below the water surface (or directly placed on the bottom), to avoid being dragged due to ice buildup during the winter months. The density of oysters in each bag is increased prior to winter. High density is not an issue as oysters do not filter during this period of low water temperatures (Comeau 2014). Within two weeks after ice has left the area or has melted, lines are re-suspended in the water column, mortalities are sorted out, and densities returned to optimal levels. In the year following spat fall, the number of oysters is reduced to 500 units bag-1; in the third year, 200–250 units bag-1 (Comeau *et al.* 2006). Oysters usually reach cocktail size (65 mm) by the end of their third year; commercial size (76 mm) in their fourth or fifth year of production. Two or three times a year in the spring/summer period, oyster growers sort their oysters mechanically or manually according to size and thereby reduce densities in the bags (Mallet *et al.* 2013). During the season, growers must also deal with fouling on culture gear (e.g. barnacles, mussels, algae, oyster seed, etc.) through air drying or

other manual/mechanical intervention (Mayrand *et al.* 2013; Mallet *et al.* 2009). Oyster mortality in cultured (<5% $year^{-1}$) and wild stocks is primarily related to predaceous or mechanical activities (Doiron 2008; Harding 2003; Mayrand *et al.* 2013) in Atlantic Canada, except in the few (2) reported instances disease outbreaks. In the 1930s, oyster populations in NB were nearly extirpated (>90% mortality) by Malpèque disease (Frazer 1938; Needler & Logie 1947). To replenish decimated stocks, twenty years later oysters were brought over to NB from PE (Logie *et al.* 1961). Oysters are now tolerant to Malpèque disease, except for certain areas within the Bras d'Or Lakes (Nova Scotia) region (Li *et al.* 1975). A more recent (Fall 2002) disease outbreak in the Bras d'Or lakes caused by the *Haplosporidian nelsoni* parasite (aka MSX) resulted in 80% mortality of cultured and wild adult stocks in the St. Patrick's Channel region of the lakes (Stephenson *et al.* 2003).

1.5 Disease expression under different environmental conditions

For a commercially important bivalve such as oysters, understanding the environmental factors affecting health and productivity, particularly in the presence of pathogens, is important for an efficient management of cultured stocks. Although pathogens are ultimately the cause of infectious disease, their presence does not always translate into disease expression. The interaction between environmental factors (i.e. temperature, salinity), the pathogen (e.g. *H. nelsoni*) and the susceptibility of the host (i.e. oyster) can cause disease. The environmental conditions often dictate

the pathogen-host interaction because they influence the physiological condition of both the host and the pathogens.

1.5.1 Estuarine environment

The estuarine environment has been linked to a variety of effects on aquatic organisms. Changes in physicochemical regime, a regular occurrence in estuaries, have a direct and indirect effect on sessile filter feeders living therein. Fluctuations in freshwater flow from major rivers, including drought events, have a pronounced impact on oyster productivity and disease dynamics by modifying food availability, salinity regimes, water temperature, and turbidity levels (La Peyre *et al.* 2009). Oyster mortalities have been observed in connection with episodic drops in salinity in many estuaries (Banks 2011; Eberline 2012). At the same time, low salinity environment strengthens oyster tolerance to diseases (Fisher & Newell 1986a). Strong currents, tidal mixing and coastal upwelling from wave action within an estuary can also influence productivity (Pierce & Conover 1954) and disease expression in oysters.

1.5.2 Seasonal variations

Major seasonal cellular and metabolic changes weaken the host immune system making oysters more susceptible to pathogen infection during warmer months than at other times of the year (Cheney *et al.* 2000; Chu & La Peyre 1989; Li *et al.* 2010; Patrick *et al.* 2006; Paynter & Burreson 1991). The high energetic cost of spawning has been linked to massive summer mortality in *C. gigas* in Marennes-Orléan, France (Patrick *et al.* 2006). During the post-spawning period oysters are in a

weak physiological state and more susceptible to environmental stress (Cho *et al.* 2005; Li *et al.* 2010). In fact, they are less capable of developing a strong defense due to reduced heat shock protein synthesis, phagocytosis, lysozymes and antimicrobial activity (Chu & La Peyre 1989; Li *et al.* 2007). Decreased protein and carbohydrate levels at time of spawning have also been linked to reduced hemocyte mobility (Fisher & Newell 1986b).

Host susceptibility is affected by seasonal changes in hemocyte defense mechanisms, including quantity and quality (Fisher *et al.* 1989). For instance, McCormick-Ray & Howard (1991) showed that granulocyte presence decreased from 60% (Jan-March) to less than 32% in May, moreover their mobility decreased from 60% to 50% over the same time period. Although the role of different types of hemocytes is not known, granulocytes are metabolically more active than other cell types (Huffman & Tripp 1982). And, mobility is essential for phagocytosis, encapsulation, inflammation (aka tissue infiltration or wound repair), hence defense activities of the cell (Fisher 1988). Interestingly, maximum phagocytic activity has been found at water temperatures of 10–37ºC, with inhibition occurring below 8ºC (Fisher & Tamplin 1988; Alvarez *et al.* 1989). In addition, Alvarez *et al.* (1989) determined that phagocytosis can occur under anaerobic conditions. In periods of low salinity (e.g. spring freshet), hemocyte spreading and mobility is also reduced, possibly resulting from an osmotic imbalance (Fisher & Newell 1986a; Fisher & Tamplin 1988). Finally, in the post-hibernation period (water temperature ~5 ºC),

oysters are often covered in silt, resulting in a stress response with reduced or delayed valve activity (Comeau *et al.* 2011).

1.5.3 Disease expression

The ability of a pathogen to infect and establish itself in a host is highly dependent on its surrounding environment (Bushek *et al.* 2012; La Peyre *et al.* 2009). It is well documented that oysters experience a higher incidence of diseases in high salinity environments (Ewart & Ford 1993; Paynter & Burreson 1991). *H. nelsoni* and *Perkinsus marinus* are highly virulent in waters with high salinity (Ford 1985; Ford & Tripp 1996). High water temperature has also been responsible for P. marinus infection/mortality (Ford 1996; Ford & Smolowitz 2007) and high Vibrio spp. counts (Baross & Liston, 1970) in oysters. Similarly, juvenile oyster disease (JOD) which primarily affects young oysters (15–25 mm in shell height) was also observed at culture sites where salinities were above 18 ‰ (Boettcher *et al.* 2006). Elevated infections of *P. marinus* have also been associated with low dissolved oxygen (Chu & Hale 1994). The physiological state of the animals also influences the outcome of host-pathogen interactions. When the host is exposed to physiological stress, its susceptibility to pathogens may increase. The virulence of a parasite can be highly variable in relation to the physiological fitness of the host (Burreson *et al.* 2000).

1.6 Stress responses in bivalves

First introduced by Selye (1936), the “general adaptation syndrome” in what became known as the “stress concept” was defined as a “generalized effort by an

animal to adapt to a new condition". Selye (1976) further clarified the term as "the nonspecific response of the body to any demand". It is based on a chronological development of a stress response: alarm, resistance, exhaustion. As a result of exposure to a stress factor, an organism may improve its well-being by adapting to environmental change, to grow and reproduce more efficiently. However, exposure to a stress-related factor may render them less fit, less able to adapt to the change, disturbing their normal functions and ultimately reducing their chance for survival. The reaction of bivalves to a stress factor defined as a stress response can be positive or negative (Bayne *et al.* 1985). For instance, alterations in the environment, deleterious compounds, and reproductive efforts can all induce a stress response in marine bivalves that may affect their normal biological and physiological state rendering them more susceptible to pathogens. When the physiological response of an oyster to environmental stress suppresses the immune functions, it can have serious implications in their susceptibility to disease agents (Hégaret *et al.* 2004). Exposure of oysters to environmental stress enhances pre-existing infection and the prevalence of infections (Fisher 1988; Fisher *et al.* 1989; Lacoste *et al.* 2002; Soudant *et al.* 2013).

1.6.1 Physiological/behavioral adaptation to environmental stressors

Many species endure a wide range of environmental conditions from the upper estuary to the marine environment that induces a certain level of stress response (Bayne 1985). The greatest effects are created by temperature and salinity fluctuations (Shumway 1996). To acclimate to seasonal changes in water

temperature, oysters (poikilotherms) alter their feeding and respiration rates and valve activity while maintaining a stable scope for growth.

Through a process known as homeoviscous adaptation, fatty-acid composition of cell membrane lipids varies to ensure membrane fluidity and efficient cellular functions at all temperatures (Sinensky 1974). To acclimate to fluctuating salinities, oysters can exert osmotic control at the whole animal level, as well as the cellular level (Deaton 2009). Under deleterious conditions (i.e. high freshwater flow) they adopt an avoidance (behavioral) strategy to control the concentration of their body fluids and, hence avoiding stressful conditions (Loosanoff 1958). The behavioral response (isolation reflex) is a short-term solution providing the animal time to acclimate physiologically to change (Pierce 1971).

1.6.2 Cellular volume homeostasis

1.6.2.1 Homeoviscous adaptation

Many organisms, including poikilotherms such as oysters vary the fatty-acid composition of their cell membrane lipids with thermal change—a process called *homeoviscous adaptation* (Sinensky 1974; Hazel 1995). Physical properties of membrane lipids are affected by temperature, which in turn alters membrane fluidity. For example, as water temperature increases more saturated fatty acids are synthesized into phospholipids to ensure constant fluidity of the cell membrane for efficient molecule transport and biochemical reactions (Hazel & Williams 1990; Sinensky 1974), while a decrease in temperature necessitates a desaturation of fatty acid. To compensate for a temperature change, oysters also remodel membrane lipids

with a higher unsaturation index at low temperatures. In oysters, however, this change in fatty acid composition occurs over a week (warm acclimation) to several weeks (cold acclimation) (Pernet *et al.* 2007).

1.6.2.2 Osmoregulation

As osmoconformers, oysters are capable of thriving at varying salinities (Galtsoff 1964; Shumway 1996). According to Shumway (1977a), during periods of decreases (increases) in ambient salinity, most bivalves experience passive inflow (ouflow) of water with limited solute loss and incomplete cell volume regulation. This is partially attributed to their capacity of coping with osmotic stress. In the presence of salinity change, water movement occurs initially (temporally) at the animal level with a limited increase/decrease in tissue water content (Shumway 1977b), then at the cellular level through cell swelling/shrinkage (Strange 2004). Since cell membranes are freely permeable to water, a transmembrane osmotic gradient will cause cells to swell (or shrink) due to the immediate water flow into (or out) of the cell. However, like most bivalves, oysters lack a sophisticated osmo-regulating mechanism (Shumway 1996). They do not regulate, but rather dilute (concentrate) extracellular osmolality by rapidly swelling (shrinking) in response to the osmotic influx (out flux) of water produced by hypo (hyper) osmotic stress (Pierce 1982). This influx/out flux of water places osmotic stress on the cells. Cell volume change occurs for the most part through extracellular anisosmotic (alterations in extracellular osmolality) and intracellular isosmotic (alteration in intracellular solute content) (Burton 1983; Deaton 2009). Oysters are poor regulators of their

extracellular fluid (hemolymph), being poikilosomotic, or osmoconformers whose extracellular fluid remains isosmotic with the fluid in their mantle cavity (Loosanoff 1953; Shumway 1996).

Osmotic cell volume is maintained through an effective intracellular isosmotic regulation process, primarily by influx and efflux of osmotically active solutes across cell membranes: inorganic ions such as sodium and (primarily) chloride ions and small organic molecules (organic osmolytes). This occurs within seconds to minutes after cell volume perturbation. In a hypersaline environment, sodium and chloride ions rapidly diffuse into the cells, while in a hyposaline environment these ions are actively removed from the cells. However, cell volume is not regulated if the cost of metabolic impairment due to volume change does not outweigh that of cell volume regulation (Strange *et al*. 1996). For instance, under normal conditions (active), oysters are slightly hyperosmotic (or isosmotic) to their surrounding environment. They tend to reflect the environmental variation by osmotic fluctuations in cell volume (Davenport 1979). This is done by adjusting osmotic pressure or by adjusting intracellular contents of osmotically active solutes, through full (or partial) restoration of cells to their original volume (Pierce 1982). Although initially inorganic ions are used for rapid cell swelling (shrinkage), cells will replace these by organic osmolytes when exposed to hypertonic (hypo) conditions for prolonged periods of time. Organic solutes have no deleterious effects on cellular structure and function, even when large shifts in the concentration occur, unlike inorganic ions (Strange 2004). These

osmolytes (i.e. taurine, alanine, glycine and proline) are found in high concentrations in the cytosol (Heavers & Hammen 1985; Pierce *et al.* 1992; Pierce 1982).

Osmotic equilibrium with the environment differs in oysters inhabiting different aquatic biotopes due primarily to population polymorphism (Pierce *et al.* 1992). For example, oysters from Chesapeake Bay have narrower salinity tolerances than their congenic along the Atlantic coast (Pierce *et al.* 1992), although the authors suggest that this reduced tolerance may be linked to the presence of parasites, *P. marinus* and *H. nelsoni*.

1.6.2.3 Gaping behavior (valvometry)

Bivalves have the ability to impede exchange with their surrounding environment by controlling fluid concentration with valve closure (i.e. isolation reflex). Through a highly effective hermetization of their mantle cavity, oysters escape adverse environmental changes and, hence, protect their hemolymph cells from the influence of extreme conditions (Berger & Kharazova 1997: Shumway 1977b).

Water temperature also affects valve activity in oysters (Galtsoff 1926). In acute cold conditions, oysters close their valves and remain isolated from the surrounding environment for months at a time (Comeau *et al.* 2012). With the capacity to withstand an accumulation of acidic byproducts from anaerobic metabolism and prolonged asphyxia in cold temperatures, oysters and other bivalves can survive months with their valves closed (or partially closed).

Unfavorable salinity concentrations also initiate the behavioral response, isolation reflex, allowing bivalves to isolate their body fluids from the unfavorable environment (Shumway 1977b). By utilizing an isolation behavior, they can overcome extremely diluted or concentrated seawater and avoid osmotic shock (Davenport 1979). In diluted seawater, for example, oysters can block the water-salt exchange with the environment, thereby maintaining the mantle cavity fluids at a higher osmolality than at ambient salinity. Unlike in a cold environment, these oysters have to eventually interact with their environment; such a behavior is therefore only suitable in areas where salinity varies over periods of hours and not weeks or months.

1.6.3 Whole animal responses

Whole animal responses, such as growth and survival rates are often used to examine acute and chronic stress in shellfish. These parameters provide long-term outcomes, which actually originate from subcellular and cellular disturbances (i.e. cascading effect) (Moore *et al.* 2006). Studies at the animal level also include clearance rates and behavioral changes, including valve closure (McFarland *et al.* 2013). More difficult to measure than growth and survival, but useful in that they provide real-time data sets—easier for the interpretation of the effects, for making timely decision in the application of mitigation measures.

1.6.4 Cellular and subcellular responses

Measuring cellular and subcellular responses in an animal provides a rapid and highly sensitive method in determining a stressful event before pathology, at the

tissue level or a whole animal response, is observed. Hemocyte activity (phagocytosis, aggregation or infiltration into infected or wounded tissues) may be altered in response to stress, providing a cellular response that is a useful indicator of stressful conditions (Fisher 1988). Lysosomes are subcellular organelles that have also been widely used to measure stress-induced responses in both invertebrates and vertebrates. Studies of digestive and hemolymphatic cells of bivalves have demonstrated that lysosomal membranes are disrupted or enlarged as a result of exposure to stress factors (e.g. contaminants, environmental conditions) (Moore 1985).

1.6.4.1 Lysosomal membrane stability

As subcellular organelles, lysosomes are involved in a magnitude of activities including cell membrane repair, digestion, apoptosis, protein turnover and immune response. They are capable of degrading intracellular and extracellular macromolecules through to use of acid hydrolases (e.g. lysosomal enzymes). But, they also sequester (accumulate) a range of bacteria, viruses, toxic compound etc. that wreak havoc to the stability of their membrane. Membrane integrity (permeability) appears to be a generic common target for stressors (Moore *et al*. 2008). The effect of abiotic stress factors (seasonal, environmental, and mechanical) on the lysosomal membrane stability is well documented for many bivalve species (Moore 1976; Ringwood *et al.* 2002; Tremblay & Pellerin-Massicotte 1997). For instance, seasonal (Ringwood *et al,* 2002), thermal (Moore 1976) and tidal (Tremblay & Pellerin-Massicotte, 1997) effects influence lysosomal membrane stability.

Lysosomal integrity has been identified as an indicator of bivalve fitness (Moore *et al.* 2006). Somatic tissue growth is strongly correlated with lysosome membrane stability in blue mussels (Krishnakumar *et al.* 1994). Moore *et al*. (2006) presents a conceptual model based on empirical data showing linkages between lysosomal stability (based on biomarker responses) and physiological health (fitness) of an organism. The model presents linkages between lysosomal dysfunction with cell, tissues and whole animal responses. Moore *et al.* (2006) presents the model as a practical approach to the development of a toolbox to assess eco-system health.

1.7 Biomarkers

The National Academy of Sciences (USA) defined biomarkers as '*indicators [of] signaling events in biological systems or samples in the presence of contaminants'* (NRC 1987). Biomarkers are primarily used for studying biological effects of contaminants; however, the need to determine seasonal variations and basal levels of biomarkers from natural stressors has prompted investigators to use biomarkers for other stress-related factors (Bodin *et al.* 2004). As a result, today biomarkers are used to determine the magnitude of host responses from many stress factors in addition to contaminants, other stressors include environmental (biotic or abiotic related), mechanical (i.e. aquaculture-related), etc. (Harding 2003; Ringwood *et al.*1998; 1999a,b; 2002).

Before a biomarker can be used, changes over time (increasing, decreasing, sigmoidal, bell-shape) and confounding influences (i.e. temperature, nutritional

status, post spawning period) must be well understood. Proper interpretation of the response requires a full understanding of these factors (Bodin *et al*. 2004; Ringwood *et al*. 2002). The response measured must not be linked to the effect of extraneous factors. Cause and effect relationships must be well-established between the exposure and the biological responses. Few biomarkers exist for which this relationship has been unambiguously determined. For example, it is difficult to determine whether the response measured is an indicator of impairment or part of the homeostatic response by the organism who is successfully dealing with the stress factor.

Host response can be measured at the molecular (DNA, protein synthesis), cellular (lysosomal destabilization), tissue (hemocyte infiltration, digestive tubule atrophy) and whole body (survival, reproductive effort) levels (Sarkar *et al.*2006; Viarengo *et al.* 2007). The use of biomarkers must allow for early detection of internal changes induced by exterior stresses at the cellular (or molecular) level before its observable at the tissue and whole animal level, potentially compromising oyster productivity, overall health and hence, of ecological relevance. Biomarkers can also be general (lysosomal membrane destabilization) or specific (vitellogen hormone); and can have relatively low (digestive tubule atrophy) to high (cortisol) sensitivity (Marin & Matozzo 2004; Viarengo *et al.* 2007).

1.7.1 General (nonspecific) biomarkers of immune response

Ideally, a biomarker is expected to be able to detect early immune responses; validated in and confirmed by the lifecycle endpoints (productivity). The degree to which stress indicators (referred to as biomarkers) reflect these outcomes judges its

effectiveness (Moore *et al.* 2006). At the molecular level, the comet assay (single cell gel electrophoresis), which detects cleaved DNA fragments, has been widely used as a biomarkers of genetic damage (Frenzilli *et al*. 2009; Kumaravel *et al*. 2009). Heat shock proteins are also expressed in presence of stress, providing chaperone functions to stabilize proteins (folding and refolding) (Ueda *et al*. 2009). At the cellular level, general non-specific biomarkers of stress that are widely accepted include 1) lysosomal membrane stability measured using a histochemical method (release of acid hydrolases) or neutral red retention assay (release of red dye); 2) oxidative stress (ROS) measured by using the lipofuscin accumulation assay, an end-product (lipofuscins) of membrane lipid peroxidation, also strongly correlated to lysosomal damage, or by observing antioxidative enzymatic activities, such as levels of catalase and GSH transferase activities (GST); 3) neutral lipid assay is also used to measure lipid content build up in lysosomes (increase in cytosolic lipids content or decrease in fatty acid processing) from autophagic uptake in exposed animals (Viarengo *et al.* 2007). Biomarkers which determine lysosome membrane stability in bivalves are extremely useful in providing an early warning signal of cellular of cellular dysfunction (Moore 1982, 1985). They will detect the earliest possible change of primary intracellular disturbances, before these disturbances quickly spread to lifecycle endpoints (growth and survival) and become quickly irreversible (Moore 1985).

1.7.2 Neutral red retention assay

It is well documented that lysosomal membrane stability, as measured with the neutral red retention assay (NRA) can be used for monitoring the effects of anthropogenic and environmental stress factors in bivalves (Lowe & Pipe 1994; Moore 1976; Moore *et al.* 2006; Ringwood *et al.* 1998). The lysosomal membrane stability in NRA is measured by observing the efflux of the contents of lysosomes, such as neutral red dye, into the cytosol. The neutral red dye is a weak base that permeates the lysosomal membrane and becomes trapped inside until structural changes to the lysosome membrane are induced and 'dye leakage' is observed through light microscopy. The neutral red retention assay determines leakage of the lysosomal contents into the cytosol, indicative of damage to the lysosome membrane. The additional challenge of neutral red dye to an already impaired membrane makes NRA a good indicator of stress (Babich & Borenfreund 1990).

1.7.3 Ecological assessments using NRA

The NRA has been successfully applied to measure cellular responses in marine animals following environmentally induced stress or injury (Bayne *et al.* 1981; Hauton *et al.* 1998, 2001; Ringwood *et al.* 1998, 2002). In fact, stress responses from salinity fluctuations, temperature change, and air exposure have been correlated with overall stress in bivalves using NRA (Camus *et al.* 2000; Hauton *et al.* 1998; Mayrand *et al.* 2013; Song *et al.* 2007b). Recently, the NRA was used to investigate hemocyte response to climate change (e.g. low pH) in *C. gallina* and *M. galloprovincialis*, (Matozzo *et al.* 2012). More relevant to investigators of aquatic toxicology, NRA was one of the core benchmark biomarkers selected within the EU

BEEP (Biological effects of environmental pollution) project and MEDPOL (Mediterranean pollution) monitoring and research program to measure lysosomal reactions of aquatic organisms to pollutants of ecological importance (MEDPOL 2007; Viarengo *et al.* 2007).

1.7.4 Advantages and disadvantages of the NRA

The NRA has several advantages over other indicators of stress (Domouhtsidou & Dimitriadis 2001; Izagirre & Marigomez 2009; Lucas & Beninger 1985; Nicholson 2001), it is simple, highly sensitive and non-invasive utilizing live cells (hemolymph or digestive gland) to accurately measure lysosomal membrane stability (Lowe *et al.* 1995). Lysosomes play a central role in an organism's innate defense system by destroying foreign invaders such as parasites, bacteria and viruses and are involved in numerous cellular functions including nutrition, tissue repair, and turnover of membranes, organelles, and proteins. They also sequester pollutants, making them a valuable biomarker of environmental and/or chemical exposure (Moore *et al.* 2008).

Nevertheless, seasonal changes in physiological state, such as gametogenic cycle and nutritional status interfere with lysosomal stability (Izagirre *et al.* 2008; Ringwood *et al.* 2004; Ringwood *et al.* 2002; Song *et al.* 2007a). Environmental variables (i.e. salinity, temperature, algae) also affect NRA response (Dimitriadis *et al.* 2012; Hauton *et al.*1998; Keppler *et al.* 2005; Nicholson 2001; Song *et al.* 2007b). As a result, the interpretation of NRA results can be problematic, particularly in field-

collected animals because of the high variability in bivalve physiology and environmental conditions (Ringwood *et al.*1999a).

1.8 Objectives

The **overall goal** of this thesis is to discuss mitigation strategies that the oyster aquaculture industry can potentially incorporate into the production cycle of their field operations in relation to environmental conditions. The long term sustainability of an oyster operation depends on its ability to mitigate the impact of mortality cause by physiological stress, disease infections and predation on cultured stocks. A proactive approach, that has proven to be both economically and biologically feasible for oyster operations elsewhere in North America, is the transfer of oysters upriver into lower salinity water. As part of my thesis, the benefits of incorporating an upriver site in the production cycle of an operation are investigated.

The **primary aim of the field** study was to evaluate the health and productivity of oysters cultivated in upriver (i.e. close to the river source) and downriver (i.e. close to the ocean) areas. With oyster culture in Atlantic Canada being carried out primarily in the downriver areas in the shallow bays and estuaries where salinity varies between ~18–30 gL^{-1}, incorporating an upriver site in the production cycle of an oyster operation could provide greater flexibility in presence of pathogens, pests and predators for continued growth of the industry. To evaluate the benefits of incorporating an upriver site in the production cycle of an aquaculture operation, growth, survival and physiological health (including assessment of the

stress response) of cultured oysters were measured over a growing season (May to October). Wild adult oysters, which had never been exposed to environmental conditions downriver, were also incorporated in the experimental design to determine if upriver environment had any sustained effect on growth and survival in the transfer habitat and whether oysters are stressed by being relayed along a river system. The **specific objectives** were:

1) to assess oyster performance in both downriver and upriver environments;

2) to assess how oysters that have periodically encountered large seasonal fluctuations in biotic and abiotic factors at the upriver area perform downriver.

The **primary aim of the laboratory study** was to determine the potential of the prescribed saline solution (Schlieper 1972) to affect lysosomal destabilization in the standard NRA. Results from the field study (above) raised doubts regarding the robustness of the standard NRA when used to measure stress responses in oysters (*Crassostrea viriginica*) in cold waters. The saline solution used in the application of the NRA assumes osmotic balance is maintained with the surrounding waters. However, at low water temperature (i.e. October) oysters, as well as other bivalves, limit exchange with the surrounding seawater through valves closure (Comeau *et al.* 2008; Loosanoff 1958). Although oysters are osmoconformers, the salinity of their internal tissue may differ from their aqueous environment when their valves are closed. This osmotic differential has the potential to affect the results of the

immunoassay. In order for the assay to be of maximum use, the stress response measured must be related to a stressful event (or stressor) and not related to osmotic stress imposed on hemolymph cells from a pressure gradient. The potential for artifacts when using the current NRA procedure in oysters exposed to 1) low water temperatures, and 2) in combination with reduced salinity to mimic a spring freshet (or autumn flood), was verified as well as an attempt to improve this method (referred to as NRA_{MOD}). The NRA_{MOD} consists of using a novel adjustment to the existing technique (NRA), whereby haemolytic fluid is used instead of the prescribed saline solution. The **specific goal** was to improve the NRA, making it more adaptable for use in bivalves living in cold water. I hypothesized that using hemolymph fluid of the individual that is perfectly adjusted to the osmolality of the animal tested, instead of the prescribed saline, would improve the accuracy of the assay and result in an easier-to-use and more efficient assay.

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Chapter 2 SURVIVAL AND GROWTH PERFORMANCE OF *Crassostrea virginica* ALONG AN ESTUARINE GRADIENT

Méthé D, Comeau LA, Stryhn T, Guyondet T, Burka JF, Landry T & Davidson J (2014) Survival and growth performance of *Crassostrea virginica* along an estuarine gradient. Aquaculture International. doi 10.1007/s10499–014–9866–3.

2.1 Abstract

In eastern Canada, an increasing number of pests, predators, and pathogens in downriver areas (close to the ocean) have led the aquaculture industry to consider growing oysters in upriver areas (close to the river source). In this study, oyster growth performance was compared between downriver and upriver environments by means of stock transfer experiments within the Richibucto estuary. In May 2009, seed oysters (~ 26 mm shell height) originating from two downriver sites (salinity ~ 20–30 ‰) were transferred upriver (salinity ~ 5–20 ‰). Follow up measurements in October 2009 revealed that the seed transferred upriver grew and survived as well as seed that remained downriver, while the mortality rates of adult oysters (shell height ~ 66 mm) were lower at the upriver site. Meat content was unaffected in adult oysters transferred upriver. However, there were indications that the upriver environment promoted shell growth (mm) in adult oysters. Oysters transferred upriver had a gain in shell height (least squares means ± standard error) over the oysters that remained

downriver (2.7 ± 0.5 mm vs. 1.8 ± 0.5 mm) and, in shell width (Median [95% CI]; 2.8 mm [1.9, 3.6] vs. 1.0 mm [0.3, 1.2]). Therefore, the holding of adult oysters upriver during the spring-summer period confers productivity advantages, and may protect from diseases and predation. By contrast, productivity losses were recorded when relocating adult oysters originating from the upriver environment. More specifically, final organic meat content were approximately 35% less in adult oysters transferred downriver compared to those that remained upriver (0.48 ± 0.04 g vs. 0.74 ± 0.04 g). Results suggest that transfers along the river impacts physiological processes such as gametogenesis and shell formation in adult oysters.

2.2 Keywords

Oyster; salinity; shellfish productivity; upriver; relaying

2.3 Introduction

Oyster culture in Atlantic Canada is generally carried out in the downriver (close to the ocean) area, in the shallow bays and estuaries where salinity varies between ~18–30 ‰. It is well documented that maximum growth and survival for eastern oysters (*Crassostrea virginica*) is in the vicinity of 14–28 ‰ (Medcof & Needler 1941; Loosanoff 1953; Chanley 1958; Shumway, 1996). However, the downriver area also provides a suitable environment for disease vectors causing high mortality in wild and cultured stocks. For example, a 58 % mortality rate in oyster spat reared downriver (12–18 ‰) compared to 0 % upriver (9–14 ‰) was attributed

to *Perkinsus marinus* infections, causative agent of Dermo disease (Albright *et al.* 2007; Bushek *et al.* 2012). Similarly, juvenile oyster disease (JOD) which primarily affects young oysters (15–25 mm in shell height) was also observed at culture sites where salinities were above 18 ‰ (Boettcher *et al.* 2006).

As oysters experience a higher incidence of disease downriver, incorporating an upriver (close to the river source) site in the production cycle of an oyster operation could provide benefits to the industry (Paynter & Burreson 1991; Ewart & Ford 1993; McGladdery & Zurbrigg 2006; Ford & Smolowitz 2007). Given that parasite infection is positively correlated with salinity, an area that experiences low salinity events for part of the seasonal cycle may protect oyster stocks and hence increase overall productivity of the farms (Ford & Haskin 1988; Ragone & Burreson 1993; Elston & Ford 2011). For example, in the 1950s, the oyster industry in Delaware Bay benefited from using the low-salinity environment of the upper Bay to protect seed stocks from an endoparasitic pathogen, *Haplosporidium nelsoni*, responsible for MSX disease (Elston & Ford 2011). In recent years, severe mortality (~ 90%) within the Bras d'Or Lakes in Cape Breton, NS (15–20 ‰) was also caused by an outbreak of MSX disease (McGladdery & Zurbrigg 2006). Within the Gulf of St. Lawrence (Fig 1), oysters are considered resistant to Malpèque disease (Mary Stephenson, Fisheries and Oceans, pers. comm. November 12, 2014). Previous studies indicated a clear spatial gradient of reduced threats with reduced salinity (Paynter & Burreson 1991; Chu *et al.* 1993; Bushek *et al.* 2012). Also, population differentiation in sessile organisms may exist along a salinity gradient, thus providing

a fitness advantage for marine invertebrates exposed to heterogeneity environments often present upriver (Sanford & Kelly 2011). As a result oyster growers in Atlantic Canada are increasingly considering the use of the upriver area. Moreover, predaceous and competing species, such as oyster drills, starfish, boring sponges and mussels are less abundant in the upriver area (Wallace 1966).

In Atlantic Canada, incorporating an upriver site in the early stage of the production cycle would entail unidirectional transfers of seed from the downriver areas. Growers depend on natural spat collected from the downriver areas to replenish their stocks (Méthé & Léger 1994). Since oyster larvae (2 week planktonic stages) travel predominantly downstream (North et al 2010), spat presumably originate from parents located all along the riverbed of the estuary. As a result, spat collected downriver should be well adapted to upriver and downriver conditions. Eierman & Hare (2013) showed that larval pools from downriver areas (salinity 30 ‰) had similar survival rates across different salinities (10 and 30 ‰) in a laboratory setting. Conceivably, unidirectional transfers of seed stock upriver may prove to be an acceptable practice for the industry to reduce exposure to pathogens, bacteria and predaceous activities in young stocks.

Information regarding the tolerance of adult oysters to transfers along the salinity gradient of an estuary is more limited than for seed oysters. As sessile organisms adult oysters spend a lifetime adjusting to local conditions. For example, Loosanoff (1953) showed that *C. virginica* oysters from a low salinity environment have a greater capacity to close and open their shells and resume pumping activities

at lower salinities than those living further downstream in the estuaries. Having rarely been exposed to episodic drops in salinity in the downriver area, oysters transferred to the upriver site may not have the capacity to resume pumping activities at these lower salinities (Loosanoff 1953). A prolonged period (March-November) of low salinity (<10 ‰) in upriver areas of the Chesapeake Bay has resulted in heavy mortalities of adult oysters (Engle 1946).

While oysters in eastern Canada have rarely been transferred to the upriver area, it should be mentioned that some growers are presently transferring or relaying wild oysters from the upriver area to their aquaculture site located downriver. Regulatory agencies support this relay fishery, whereby oyster growers are permitted to harvest wild market-size oysters upriver, where they are freely available but nevertheless susceptible to bacterial (*E. coli*) contamination from sewage effluent, a human health concern. Their transfer to aquaculture sites downriver allows a prescribed depuration to take place. It also contributes to increasing the salt content of the meats (improved taste). There is nevertheless anecdotal evidence of high mortalities in the transferred stocks, presumably related to transfer shock (Paul Cormier [NB Aquaculture, Fisheries and Agriculture], personal communication, July 23, 2014). Investigating the impact of this relay fishery on oyster productivity is necessary to guide industry.

In this study, the first objective was to evaluate the benefits of incorporating an upriver site in the production cycle of an oyster operation. Oysters were taken from downriver aquaculture sites and were transferred upriver, where they were

monitored for shell and meat growth during a five month period. The second objective was to evaluate the impact of the relay fishery on oyster productivity. Wild upriver oysters were transferred downriver and monitored over the same time period.

2.4 Material and Methods

2.4.1 Study area and experimental design

The experiment was conducted at two sites (upriver, downriver) located 20 km apart in the Richibucto River in eastern New Brunswick, Canada (Fig. 2.1). The underlying bedrock of the Richibouctou watershed consists of soft sandstone and siltstone and is located within vast bogs (Turcotte-Lanteigne & Ferguson 2008). On May 25, 2009, seed oysters (d1, d2) (same genetic stock) originating from two downriver areas of similar environmental conditions were transferred to the upriver site. The d1 oysters originated from a wild collection within the same estuary (N 46° 40' 54.434"; W 64° 51' 57.529"). The d2 oysters were also a wild collection, but from an adjacent estuary (Bouctouche Bay) (N 46° 31' 26.928"; W 64° 43' 56.911"). The initial shell heights (mean ± SE) were similar at the two sources (d1 24.0 ± 0.3 mm; d2 28.0 ± 0.3 mm), although significantly different. At both study sites (~3m depth) oysters were held in cages (60 cm × 120 cm × 10 cm) constructed of plastic-coated wire mesh and divided into 32 compartments, each holding an individual oyster (Fig. 2.2). Cages were suspended in the upper water column near the surface. A total of 64 d1 and 64 d2 oysters were equally distributed amongst 4 cages at both sites. Cages were deployed for 155 days, from May 25 to October 28, 2009.

Additionally, ~ 4 year old oysters (UPR, DNR) originating from upriver and downriver areas were reciprocally transferred. The UPR oysters were hand-collected from a wild population adjacent to the upriver site (N 46° 35' 11.227"; W 64° 58' 51.376"). The DNR oysters (wild seed from Bouctouche, NB) were obtained from a Richibucto Harbor aquaculture site downriver (N 46° 41' 42.231"; W 64° 49' 50.243"). The initial shell heights (mean ± SE) were similar at the two sources (UPR 62.5 ± 0.8 mm; DNR 69.9 ± 0.4 mm), although significantly different. Oysters were held in cages, as described above (Fig. 2.2). A total of 64 UPR and 64 DNR oysters were equally distributed amongst 4 cages at each site. Cages were deployed for 155 days, from May 25 to October 28, 2009.

2.4.2 Environmental parameters

At each site, salinity was determined from electrical conductivity measurements taken every 3 h using *in situ* YSI 6560 probes (YSI, Yellow Springs, Ohio). Rate of flow (or discharge) data was obtained from Environment Canada's (EC) Water Survey of Canada (1964–2010; Station no. 01BS001). To compute the water discharge (m^3s^{-1}), EC established a water level-discharge relationship from multiple measurements (channel depth, width, flow velocity) taken at different water levels in the river. Water temperature was measured every 3 h for the duration of the study using *in situ* Minilog-TR probes (AMIRIX Systems Inc., Halifax, NS). Relative fluorescence was measured using *in situ* YSI 6025 chlorophyll sensors. Relative fluorescence units (RFU) were corrected for instrument drift, and converted into a 0–1 scale.

2.4.3 Productivity parameters

Shell height (SH [anterior-posterior]) and width (SW [dorsoventral]), whole wet weight (W) and approximate shell surface area (S) of each oyster was measured prior to deployment (May 25) and when retrieved (October 28). SH and SW were measured to the nearest 0.01 mm using an electronic caliper. W was measured to the nearest 0.01 g. The S of each shell was determined to the nearest 0.01 cm^2 using University of Texas Health Science Center of San Antonio (UTHSCSA) Image Tool Software. The overall growth (SH, SW, W, S) of individual oysters was calculated as the final minus the initial measurement, an approach that is consistent with the linear growth reported for the size range under investigation (Doiron & Mallet 2010). Mortality (M) was assessed by counting live/dead oysters in each cage at the end of the study.

Sixteen oysters per cage were selected (2 middle rows) for the condition index (C). The C was calculated using the organic tissue weight (dried tissue weight minus ash weight) and its relationship to dry shell weight as follows (Landry et al. 2001):

$$C\ \% = (organic\ tissue\ weight\ /shell\ dry\ weight) * 100$$

The dried tissue weight was obtained by oven-drying the meat for at least 24 h at 70 ºC and weighing resultant mass. The ash weight was obtained by heating the oyster meat at 500 ºC for 6–7 h, weighing the resulting ash. The dried shell weight was measured following air-drying the shell at room temperature for 24 h. The dry shell weight, dry tissue, and ash weights were measured to the nearest 0.0001 g.

2.4.4 Dynamic Energy Budget model simulations

In order to test the influence on oyster (adult) growth of the different temperature regimes at the two sites, a numerical ecophysiological model was set up using the Dynamic Energy Budget (DEB) theory (Kooijman 2000). The model describes how an individual oyster utilizes the energy it acquires from food ingestion and assimilation to fulfill the requirements of the growth/maturation and maintenance of its somatic and reproductive tissues. Given that environmental conditions (water temperature and food concentrations, here chlorophyll *a*, was used as a proxy to assess food biomass for bivalves) are known for the period studied, the model can reproduce the individual growth over that time period. Model equations are detailed in Pouvreau *et al.* (2006) and the set of parameters specific to *Crassostrea virginica* was retrieved from Filgueira *et al.* (2014).

At each site, growth observations were pooled for both stocks and two models were built, one for each site. Each model was based on the environmental conditions of the respective site and was validated against the pooled growth observations by tuning the only site-specific parameter, i.e. the half-saturation coefficient that scales the food ingestion to available food at each site.

Finally, to test if water temperature alone could explain growth differences between the two sites, each model was run one more time using the temperature forcing of the opposite site, such that for each site, oyster growth could be predicted based on the water temperature of the other site. The growth (in terms of shell height)

at the end of the experiment was then compared to the “non-inverted” temperature scenario.

2.4.5 Statistical data analysis

Separate statistical analyses were carried out for different outcomes: SH, SW, W, S, C and M.

A linear mixed model with random cage effects was used due to the hierarchical (nested) structure of the experimental design (Dohoo *et al.* 2009). The statistical design had a split-plot character because two oyster sources were reared within the same cages which in turn were located at two sites. Thus, the cages corresponded to wholeplots and individual oysters corresponded to subplots; furthermore, site and source were the wholeplot and subplots factors, respectively. The linear mixed models had site, source and its interaction, as well as overall cage rows and columns as fixed effects. In addition to the random cage effects, cage-specific row and column effects were also analyzed.

The assumptions of normality and homoscedasticity of linear mixed models were evaluated by residual plots based on the standardized results. The deviation of residuals from normality was assessed using the Anderson-Darling normality test. Whenever model assumptions could not be met, an optimal power transformation was obtained by Box-Cox analysis (Dohoo *et al.* 2009). A value of 10 was added to the SW data (market-sized oysters) prior to log-transformation, based on trial and error and inspection of residuals.

In the presence of a significant interaction between the various factors, pairwise comparison tests with Bonferroni correction were performed to determine significance between groups. All estimates were reported as least squares mean (LSM) ± standard error (SE); in some instances following back-transformation the back-transformed value was interpreted as the median, with the standard error computed using the delta method (Weisberg 2005). In the log back-transformation, the confidence intervals are presented, instead of standard errors.

A few extreme outliers were removed after they were found statistically significant by the outlier detection test (based on deletion residuals): W (3 obs.) and C (1 obs.) for the adult oysters (UPR, DNR); surface area (1 obs.) for the seed oysters (d1, d2). In addition, a number of missing values were due to mortality (32) and missing (18) oysters in the field.

A binary logistic regression analysis of the M data (dead/alive) was carried out with random cage effects because the experimental design was of a hierarchical (nested) structure, as described above.

For all analyses, the significance level was set at $P \leq 0.05$. The analyses were carried out using Minitab software (Version 15.1.30) (Minitab Inc., State College, PA), STATA software (Version 13.0) (StataCorp LP, College Station, TX) and SAS software (Version 9.2) (SAS Institute Inc., Cary, NC).

2.5 Results

2.5.1 Environment

While relative chlorophyll fluorescence at both sites were the same (upriver vs downriver, respectively) (0.064 ± 0.008 vs 0.080 ± 0.011, P = 0.25), the upriver site was characterized by higher temperature (18.1 ± 0.3 °C vs 16.7 ± 0.3 °C, P < 0.001) and lower salinity (14.6 ± 0.3 ‰ vs 25.5 ± 0.2 ‰, P < 0.001). At both sites, the tidal influence based on a harmonic analysis explains ~13–14% of the observed salinity variation (Pawlowicz *et* al. 2002; Foreman 1977). Although the influence from the diurnal (and semi-diurnal) constituents is weak, the lunar cycle still has an influence on the observed salinity, particularly at the upriver site. The tide explains the observed periodic lows (Δ 5 ‰), occurring at the beginning of July, Aug, September and even October (albeit less obvious) (Fig. 2.3). The largest diurnal salinity variations due to tides were observed at the upriver site with amplitude ranging from 0.3‰ to 1.2‰ during neap and spring tides respectively. At the upriver site, salinity reached < 10 ‰ in July and October during ebb tide, coinciding with heavy rains (precipitation) based on freshwater discharge data from Environment Canada. The lowest observed salinity (3–4 ‰) occurred in mid-October during a lunar event and when freshwater discharge at a peak (~ 100 $m^3 s^{-1}$ for 4 consecutive days). Monthly discharge averages for June–September (~11 $m^3 s^{-1}$) were comparable to the historical (1964–2010) monthly averages (10.5 $m^3 s^{-1}$) (Environment Canada 1964–2010, http://www.wsc.ec.gc.ca).

2.5.2 Mortality rates

The binary logistic regression analysis indicated a significant difference in mortality between seed sources (P = 0.05), but not between sites (P = 0.22). The d2

oyster seeds had a lower risk of dying (odds-ratio = 0.39) than d1 seeds, regardless of site. The mortality rates in adult oysters were significantly lower (P = 0.03; odds-ratio = 0.11) at the upriver site, regardless of source. Live and dead oyster counts are summarized in Table 2.1 (seed) and Table 2.2 (adults). Mortality rates were below 15% (seed) and 8% (adults).

2.5.3 Oyster productivity

Oyster seed (d1, d2) transferred upriver grew as well as seed that remained downriver. No significant difference in growth as measured by shell height, width, surface area, condition index, or wet weight was detected between rearing sites, regardless of source (Table 2.3). Nor were there any difference between rearing sites for final dry shell and organic meat weights.

In contrast, the upriver environment positively affected the shell growth of adult oysters (Table 2.4). For example, in terms of shell height, DNR oysters that were transferred upriver grew by 2.7 ± 0.5 mm, whereas those that remained downriver grew by 1.8 ± 0.5 mm. However, there was no effect on wet weight (P = 0.54) and condition index (P = 0.15), and DNR oysters had similar final organic meat weights, 0.71 ± 0.04g upriver and 0.62 ± 0.04 downriver. For UPR stocks, the transfer of adults to the downriver area seemed unfavorable representing a difference of ~ 2.2 mm in shell height and ~ 2.1 mm in shell width compared to their counterpart that remained upriver. Moreover, a significant reduction in meat content (organic weight) was observed at the downriver site. UPR oysters had a final meat (organic) weight of 0.74 ± 0.04 g upriver, compared to only 0.48 ± 0.04 g, downriver.

The DEB model simulations indicated that transferring oysters from one site to the temperature regime of the other site does not induce any growth difference (less than 0.08% difference in individual growth [in terms of shell height] in the different temperature regimes for both transfer experiments, i.e. upriver to downriver temperature [0.25 mm] and downriver to upriver temperature [0.50 mm]). According to these model results, the difference in water temperature between the two sites is not a valid explanation for the observed difference in adult oyster growth.

2.6 Discussion

2.6.1 Mortality

Despite spending 5 months (May-October 2009) exposed to the ecological conditions found at the upriver site, seed and adult oysters, including those originating from downriver, exhibited mortality rates comparable to those reported by industry (Doiron 2008). Ongoing wild oyster health monitoring in the vicinity of the study sites has not detected any diseases of concern (Mary Stephenson, Fisheries and Oceans, pers. comm. November 12, 2014). The average mortality rate was below 15% (seed) and 8% (adults). More importantly, in adult oysters the mortality rate upriver was significantly lower than the mortality rate downriver. This result suggests that in Richibucto Harbor both seed and mature crop may be transferred upriver from late May until October with no apparent concern for survival. This conclusion is further supported by numerous reports showing the species' tolerance to low salinities. Loosanoff (1953) and Wells (1961) reported 7.5 and 7.0 ‰, respectively,

as the species' lower salinity threshold for survival. However, the duration of low salinity exposure is also an important factor affecting survival. For instance, a six-month (April-October) exposure to salinities in the range of 3–9 ‰ ultimately resulted in massive (50–95%) mortality in Chesapeake Bay (Engle 1946). In our study, oysters were exposed to salinities below 10 ‰ for only four consecutive days in July and nine consecutive days in October. Moreover, low temperatures, particularly in October (<10°C), presumably lowered the oysters' metabolic activity (Loosanoff 1953; Wells 1961; Pollack *et al.* 2011). Consequently, if the oysters were closed and operating anaerobically during the freshets, the accumulation of toxic metabolic waste within the tissues would have been minimized.

It is noteworthy that the freshets recorded upriver were caused by the combined effects of ebb tides and freshwater discharges. In keeping with this information, oysters in eastern Canada may have greater difficulty tolerating spring freshets than autumnal freshets. In spring, the melting of snow adds substantial quantities of freshwater into the estuarine systems. In the Richibucto River, for instance, the monthly freshwater discharge averages 84 m^3s^{-1} in April compared to 17 m^3s^{-1} in October (Environment Canada 1964–2010, http://www.wsc.ec.gc.ca). These differential discharges are consistent with the Bataller *et al.* (1999) report, which indicated that the salinity in the upriver area of the Richibucto was approximately 10 ‰ lower in spring than autumn. Moreover, in spring, the freshwater discharge starts in late March and extends over 1–2 months, therefore coinciding with the spring awakening of oysters (Comeau *et al.* 2012; Comeau 2014) and presumably the onset

of aerobic metabolism/oxygen intake. Exactly how spring freshets impact the survival of oysters in eastern Canada remains to be investigated. In the interim, since fresh-water kills are well documented (Andrews *et al.*1959; Fisher *et al.*1989; La Peyre *et al.* 2009) and disease outbreaks generally occur at high water temperature (Shumway 1996), it may be advisable to avoid transferring oysters upriver until after spring freshets.

Based on climate change predictions, precipitations in New Brunswick will increase by 10% in 2050 and 15% in 2080 (Vasseur & Catto 2008), however it will be scattered uniformly throughout the year, still within acceptable salinity conditions for optimal growth and survival. Therefore oysters reared upriver from May through October should not experience a substantial change in salinity levels.

2.6.2 Growth performance

Oyster shell growth observed at the upriver site lends further support to incorporating an upriver site in the production cycle. Seed oysters transferred upriver grew as well as those that remained downriver, both in terms of shell length and meat content. These results are consistent with Shaw (1966), whereby seed oysters (25 mm shell height) from a low salinity area (8–16 ‰) of Chesapeake Bay (Maryland), transferred to both low (8–16 ‰) and high (17–35 ‰) salinity areas showed no significant differences in growth rates after two seasonal cycles.

For mature stocks, the transfer to the upriver area was associated with a significant but modest enhanced growth, corresponding to a gain of ~ 0.9 mm in shell height and ~ 1.8 mm in shell width. The exact reason for this outcome is unknown.

Although shell erosion is common in exposed sites (Mallet *et al.* 2009, 2013), we found no indications of damaged new shell growth downriver. High level of calcium carbonate in the river system is unlikely given the presence of bogs and the bedrock composition within the watershed (Turcotte-Lanteigne & Ferguson 2008). It is well known that oysters grow faster in warmer waters (Ingle & Dawson 1952; Shumway 1996). However, according to our DEB model, the 1.4°C increment in water temperature (~ 217 degree days) at the upriver site cannot explain the difference in shell growth among groups of adult oysters. As well, food quantity cannot explain the difference in shell growth. Presumably any influence of food (or wave/wind exposure) on shell growth (erosion) would have been detected in seed oysters, which was not the case. Carrying out a larger study that has more sites would be required to determine which site level predictors are associated with these outcomes. On the other hand, it may be useful to determine whether the higher shell growth upriver was linked to gametogenesis, trading off storage (lipids utilized in gametogenesis) for shell growth after the transfer to the upriver site. Such tradeoffs have been observed in other species; e.g., freshwater snails will delay reproductive effort in the presence of predators (water bugs), in order to form larger shells (Hoverman *et al.* 2005; Hoverman & Relyea 2007). Butler (1949) had previously reported gametogenesis inhibition in *C. virginica* at salinities less than 6 ‰. Loosanoff (1953) also demonstrated that oysters spawned lightly at 7.5 ‰. In our study, salinity at the upriver site fluctuated between 8–20 ‰ prior to the expected spawning period in July (Mayrand *et al.* 2013), while salinity was higher and more stable downriver (25–30

‰). The higher survival rates observed in adult oysters at the upriver site relative to the downriver site could be linked to low spawning effort. Therefore it is possible that transfers along the river impacted physiological processes in adult oysters, such as gametogenesis and shell formation.

2.6.3 Wild upriver oysters

In our study, wild oysters originating from upriver had 31–44% better shell growth than cultured oysters originating from downriver, regardless of their study site position along the river. The underlying cause for this result is unknown. The genetic pool would not have been reduced as cultured stocks originated from wild spat fall collection. The observed differences may have been a function of compensatory growth from a lifetime of living on upriver beds. Compensatory growth is a period of rapid growth relative to age in animals, such as after a period of food deprivation, and usually manifests itself as short-term accelerated growth (Eldridge & Eversole 1982; Kraeuter *et al.* 2007). In our study, simply raising upriver oysters off-bottom would have improved water flow and increased food availability. A sessile lifestyle in a heterogeneous environment (i.e. upriver area) may have also led to the development of traits that improve their relative fitness later in life (Ernande *et al.* 2004; Sanford & Kelly 2011). Through the production of variable phenotypes, from a single plastic genotype, oysters have the flexibility (morphological, physiological, and /or behavioral) to respond to changes in environmental conditions (Berger & Kharazova 1997; Hamdoun *et al.* 2003; Drent *et al.* 2004).

Interestingly, considerable tissue (organic) loss was observed in wild oysters transferred downriver compared to those that remained upriver (0.48 g vs 0.74 g), supporting anecdotal evidence from relay fisheries. Since tissue measurements made no distinction between somatic and gonadic tissues, it is difficult to discern what actually occurred. For instance, wild oysters from upriver, having not developed and grown in downriver conditions, may have been faced with an 'unplanned' spawning event after the transfer downriver. Since the upriver site is not conducive to gametogenic development which is normally initiated in the fall, it could be argued that the observed tissue loss was the result of increased energy demands (i.e. vitellogenesis) after the transfer downriver (Honkoop 2003; Bayne 2004). Honkoop (2003) showed in Sydney rock oyster (*Saccostrea glomerata*) that the cost of reproductive effort (production, maintenance) was 84% greater than those of somatic tissues.

2.7 Conclusion

After five months at the upriver site, aquaculture-derived oysters survived at levels comparable to that of the industry. Moreover, seed and mature oysters originating from downriver grew as well upriver as those that remained downriver, suggesting that transferring them upriver for part of their seasonal growth cycle (summer-autumn period) may serve in providing protection from diseases and predators lurking in the downriver area. The transfer to the upriver area seemed even more favorable for mature stocks, representing a gain of ~ 0.9 mm in shell height and

~ 1.8 mm in shell width compared to their counterpart that remained downriver. Based on these findings, it may be advantageous for oyster growers to transfer oysters (seed and mature crop) upriver for part of the seasonal cycle (May to October), as it may serve to augment shell growth while maintaining meat content, while providing protection from diseases and predation.

Mature oysters collected upriver had 31–44% better shell growth than aquaculture oysters collected downriver, regardless of their study site position along the river. Nevertheless, it seems that ecological conditions downriver led to an expensive energetic cost in upriver-originated oysters transferred downriver as considerable meat content loss was observed (0.48 ± 0.04 g vs. 0.74 ± 0.04 g). In light of these findings, in Richibucto collecting wild adult oysters from upriver in May and relaying them to an aquaculture site downriver is not recommended. These results also suggest that downriver oysters may exhibit meat loss once returned to the downriver environment.

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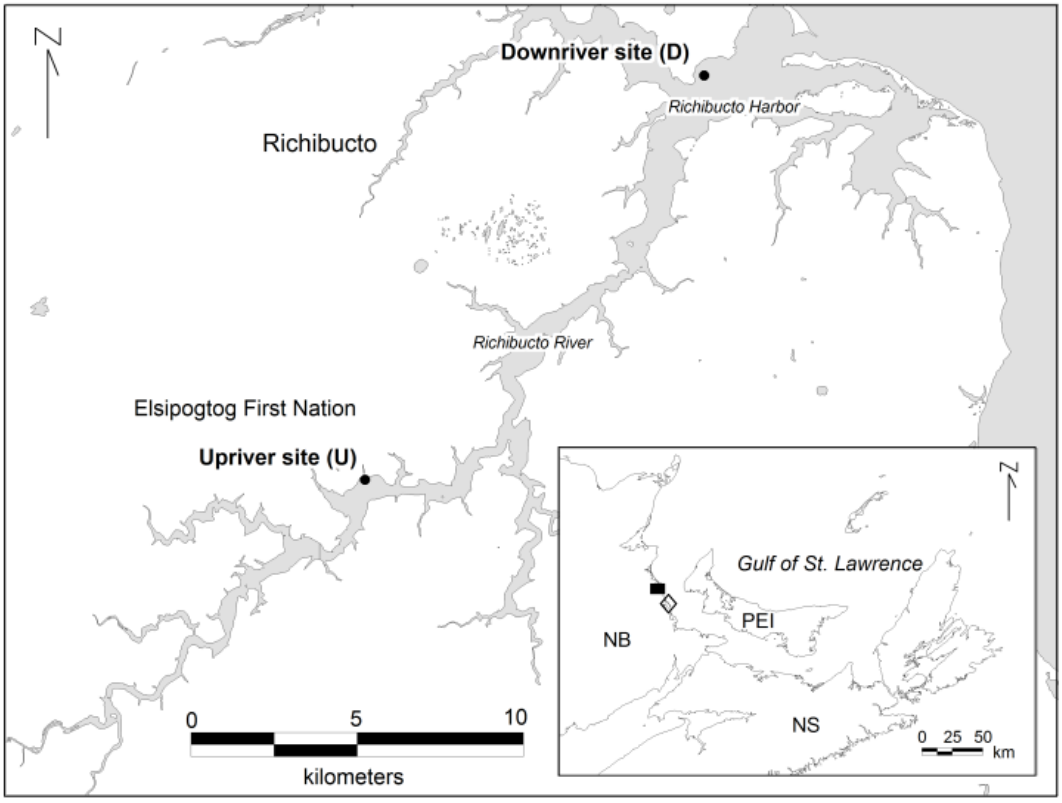

Figure 2.1 Map of the Richibucto estuary with upriver (N 46° 35' 11.277"; W 64° 58' 51.376") and downriver (N 46° 41' 42.231"; W 64° 49' 50.243") sites in eastern New Brunswick, Canada (▄ Richibouctou Harbor (inset); ◊ Bouctouche Bay).

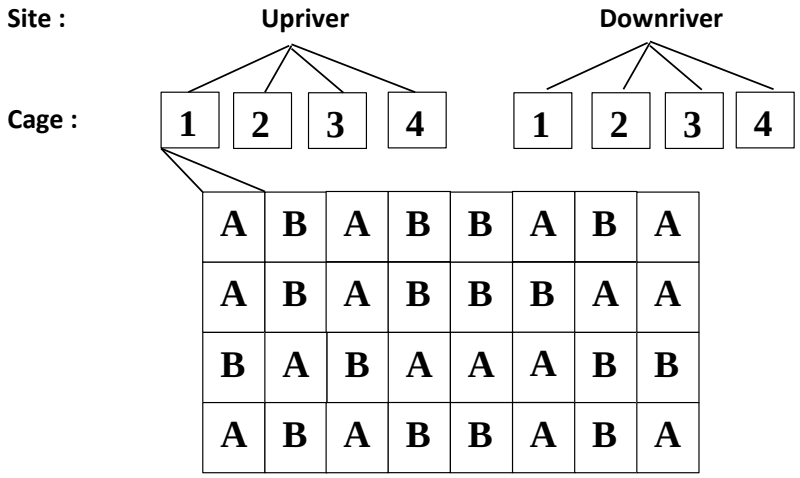

Figure 2.2 Schematic of cage used in field experiment, with oysters of two different sources ([A, B] ~ [d1, d2] for seeds, ~ [DNR, UPR] for juveniles) distributed in the 4*8 = 32 compartments per cage.

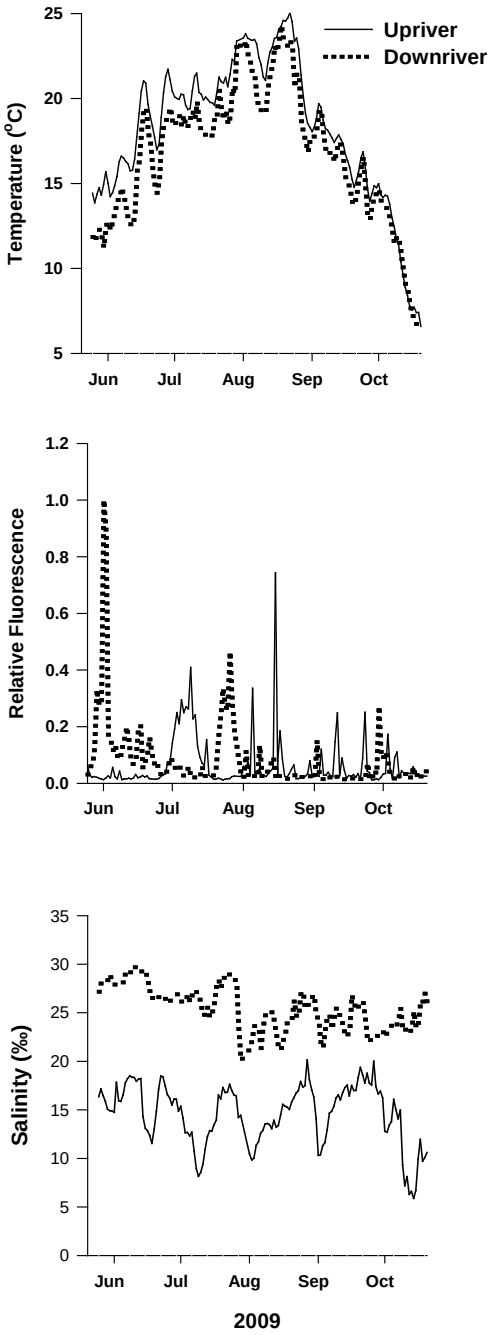

Figure 2.3 Daily mean of water temperature (°C), relative fluorescence and salinity (‰) at the upriver (-----) and downriver (·····) sites from May 25 to October 20, 2009.

Table 2.1 Number of seed oysters (d1, d2) alive and dead at the upriver and downriver sites from May to October 2009.

Site	***Source***			
	d1 (Richibouctou)		***d2 (Bouctouche)***	
	Alive	***Dead***	***Alive***	***Dead***
Upriver				
May to October	**54**	**6**	**50**	**9**
Downriver				
May to October	**58**	**1**	**55**	**7**

Table 2.2 Number of adult oysters (UPR, DNR) alive and dead at the upriver and downriver sites from May to October 2009.

Site	***Source***			
	UPR (Upriver)		***DNR (Downriver)***	
	Alive	***Dead***	***Alive***	***Dead***
Upriver				
May to October	**61**	**1**	**64**	**0**
Downriver				
May to October	**59**	**5**	**61**	**3**

Table 2.3 Estimates from statistical models for oyster productivity data from seed oysters (d1, d2) reared at the downriver and upriver sites from May to October 2009.

	Site	**Downriver**		**Upriver**	
Parameters	**Source**	**d1**	**d2**	**d1**	**d2**
Height (mm)		13.5 ± 1.1	13.3 ± 1.1	12.5 ± 1.2	12.8 ± 1.2
Width (mm)		12.1 ± 0.9	12.2 ± 0.9	11.6 ± 0.9	11.4 ± 0.9
Wet Weight (g)		4.6 ± 0.5	4.6 ± 0.5	4.4 ± 0.5	4.5 ± 0.5
Surface Area (cm^2)		5.0 ± 0.5	5.1 ± 0.5	4.6 ± 0.5	5.0 ± 0.5
Condition Index (%)		2.7 ± 0.2	2.8 ± 0.2	3.3 ± 0.2	3.2 ± 0.2

The least squares means (LSM) without a common superscript were significantly different ($P < 0.05$). Statistical comparisons were made within each row. All data are represented as the LSM ± SE.

^{1,2} indicates difference between sites;

^{A,B} indicates difference between oyster sources;

^{a,b} indicates interaction between site and oyster source.

Coding is absent if no significant treatment effect or interaction was detected.

Table 2.4 Estimates from final statistical models for oyster productivity data from market-sized oysters (DNR, UPR) reared at the downriver and upriver sites from May to October 2009.

	Site	**Downriver**		**Upriver**	
Parameters	**Source**	**DNR**	**UPR**	**DNR**	**UPR**
Height (mm)		1.8 ± 0.5^{1A}	2.6 ± 0.6^{1B}	2.7 ± 0.5^{2A}	4.8 ± 0.5^{2B}
Width (mm)*		$1.0\ [0.3, 1.2]^{1A}$	$2.3[1.5, 3.1]^{1B}$	$2.8\ [1.9, 3.6]^{2A}$	$4.4\ [3.5, 5.5]^{2B}$
Wet Weight (g)		5.6 ± 0.6^{a}	7.0 ± 0.6^{a}	7.2 ± 0.6^{a}	10.8 ± 0.6^{b}
Surface Area (cm^2)		4.0 ± 0.9	4.5 ± 0.9	4.5 ± 0.9	4.6 ± 0.9
Condition Index (%)		1.8 ± 0.1^{a}	1.7 ± 0.1^{a}	2.1 ± 0.1^{a}	2.6 ± 0.1^{b}

The least squares means (LSM) without a common superscript were significantly different ($P < 0.05$). Statistical comparisons were made within each row. All data are represented as the LSM ± SE.

^{1,2} indicates difference between sites;

^{A,B} indicates difference between oyster sources;

^{a,b} indicates interaction between site and oyster source.

Coding is absent if no significant treatment effect or interaction was detected.

* Represents median data [with 95% CI], and all other data are represented as LSM ± SE.

Chapter 3 STRESS RESPONSE OF *Crassostrea virginica* (Gmelin, 1791) OYSTERS FOLLOWING A RECIPROCAL TRANSFER BETWEEN UPRIVER AND DOWNRIVER SITES

Méthé D, Comeau LA, Stryhn H, Landry T & Davidson J. (2014) Stress response of *Crassostrea virginica* (Gmelin, 1791) oysters following a reciprocal transfer between upriver and downriver sites. Aquaculture Research. doi: 10.1111/are.12436.

3.1 Abstract

This study was designed to assess the physiological impact of transferring oysters between upriver and downriver aquaculture sites, a common practice in North America that is primarily aimed at reducing disease infections and predation on cultured stocks. In May 2009, oysters (*Crassostrea virginica*) were reciprocally transferred between an upriver and a downriver site in the Richibucto estuary in eastern Canada. Mortality, tissue and cellular stress responses were subsequently evaluated in August and October 2009. Overall, oyster mortality remained low (~ 5 %) throughout the five-month study period with no significant difference between sites or oyster sources. However, by October oysters reared at the upriver site, regardless of their origin, had significantly higher levels of lysosomal membrane destabilization (63.6 %, SE = 1.9) and digestive tubule atrophy (33.3 – 42.4 %, SE = 3.6) than oysters reared at the downriver site (47.5 %, SE = 1.8; 15.6–19.1 %, SE =

3.8, respectively). They also exhibited a greater salinity differential between their mantle/hemolymph fluids and the ambient seawater, possibly indicating more restricted exchange with the environment. In general, the transfer of upriver oysters to a downriver site had a positive impact, i.e. lower levels of lysosomal destabilization and tubule atrophy, whereas transfer of downriver oysters upriver had the opposite effect. These results suggest that upriver environmental conditions negatively impact cellular and tissue integrity in oysters without leading to mortality during the summer-autumn period.

3.2 Keywords

lysosome membrane; stress response; neutral red retention assay; immune response; oyster; physiology; tubule atrophy; upriver

3.3 Introduction

The eastern oyster *Crassostrea virginica* is poikilosmotic or an osmocoformer whose extracellular fluid (hemolymph) remains iso-osmotic with the fluid in their mantle cavity (Loosanoff 1953; Shumway 1996). When exposed to salinity conditions outside their normal tolerance range they may remain closed for extended periods, thereby restricting their rate of exchange with the environment. Under such conditions their mantle fluid may not be in ionic equilibrium with the surrounding seawater (Coughlan *et al.* 2009). Fresh-water runoff is known to play a major role in controlling natural distribution of oysters in many estuaries (Attril *et al.* 2002; Butler,

1949; La Peyre *et al.* 2009). The optimal salinity range for oyster growth is from 14–28 gL^{-1}; although capable of surviving at lower salinities, oysters show signs of being physiologically challenged (Medcof and Needler 1941; Chanley 1958; Wang 2008). Reduced growth has been observed at ≤ 3 gL^{-1} because of valve closures. Under conditions of low salinity (0–3 gL^{-1}), survival is highest at low temperatures ($\leq 12^{\circ}$ C); however, under more optimal temperature conditions (23–27°C), the lower tolerance limit is 7.5 gL^{-1} (Loosanoff, 1953; Shumway, 1996).

While higher salinity conditions (20-28 gL^{-1}) in downriver areas promote oyster growth, they also provide a suitable environment for some disease vectors, infection and predaceous activities (Well 1961; Chu *et al.* 1993). For instance, the susceptibility of *C. virginica* to pathogens, such as *Haplosporidium nelsoni* (MSX disease), increases with salinity (Ford & Haskin 1988; Shumway 1996). Similarly, higher prevalences of Dermo (*Perkinsus marinus*) were observed in oysters reared downriver compared to those reared upriver (Albright *et al.* 2007). A laboratory study on *Ostrea edulis* maintained for seven days at a range of salinities (16, 25 and 32 gL^{-1}), demonstrated an increase in the growth of the opportunistic bacterial pathogen *Listonella anguillarum* with increasing salinity (Hauton *et al.* 2001). Moreover, predators, such as oyster drills and starfish, as well as parasites, such as boring sponges, are better adapted to higher salinity environments found in downriver areas (Gillson 2011; Wallace 1966).

The rapid development of the oyster aquaculture industry in Atlantic Canada and the recent appearance of the protozoan parasite, *H. nelsoni,* in the Bras d'Or Lakes (Cape Breton, NS), are two main reasons for farmers to consider expanding their operations upriver into lower salinity areas (McGladdery & Zurbrigg 2006). In Atlantic Canada, most of the oyster aquaculture operations are located in downriver areas where the salinity is generally conducive to the recruitment and survival of wild oysters (Bastien-Daigle *et al.* 2007). For example, the optimal salinity for normal embryonic development is 26 gL^{-1} (Davis & Calabrese 1964; MacInnes & Calabrese 1979). The ability to expand the culture of oysters upriver for initial grow-out could provide greater opportunity, flexibility and stability to the industry since upriver areas often present lower user conflicts and harbor fewer pests, competitors, predators and pathogens than downriver areas (Chintala & Fisher 1991; Ewart & Ford 1993; Shumway 1996).

The objective of this study was to assess the stress response of oysters transferred between upriver and downriver sites. Oysters were sampled in August (80 days post-transfer) and October (150 days post-transfer) to determine whether they were physiologically sensitive to being relayed along a river system. Stress was measured using the neutral red retention assay (NRA), which has been successfully applied to measure cellular responses in marine animals following environmentally induced stress or injury (Bayne *et al.* 1981; Hauton *et al.* 1998, 2001; Ringwood *et al.* 1998, 2002). The NRA technique provides an indication of the integrity of the lysosomal membrane, an indicator of cellular health status (Moore *et al.* 2006). Oysters were

also examined for epithelial atrophy of the digestive tubules, considered a general indicator of environmental stress (Winstead 1995).

3.4 Materials and methods

3.4.1 Study site and experimental design

Two sites located 20 km apart in the Richibucto estuary (eastern New Brunswick, Canada) were selected for a reciprocal transfer study (Fig. 3.1). Oysters originating from upriver (UPR) and downriver (DNR) populations were transferred to both upriver and downriver sites on May 25, 2009. UPR oysters were hand-collected from a wild population adjacent to the upriver site. DNR oysters were obtained from a Richibucto Harbor aquaculture site. The initial shell height (mean ± SE) was similar between the two oyster sources (UPR 62.5 ± 0.8 mm; DNR 69.9 ± 0.4 mm). At both study sites oysters were held in cages (60 cm × 120 cm × 10 cm) constructed of plastic-coated wire mesh with 32 compartments (4 rows), each holding an individual oyster. Cages were suspended in the upper water column near the surface. A total of 128 UPR and 128 DNR oysters were equally distributed amongst 8 cages at each site (i.e. 16 UPR and 16 DNR oysters/cage). Cages were deployed for 150 days from May 25 to October 23, 2009. Mortality was assessed by counting live/dead oysters from two subsamples (32 UPR; 32 DNR), assessed after 80 days (May to August) and 150 days (May to October). These same animals were used for NRA and digestive tubule analysis.

3.4.2 Environmental parameters

At each site, ambient salinity was measured using a Sybon automatic temperature compensated refractometer (Mail Order Pet Supplies, Toronto, ON) every time oysters were extracted from the cages (August and October 2009). Sampling occurred simultaneously (within 30 min) at both sites. Water temperature was recorded every 3 h for the duration of the study using *in situ* Minilog–TR probes (AMIRIX Systems Inc., Halifax, NS). Salinity was determined from electrical conductivity measurements taken using *in situ* YSI 6560 probe (YSI, Yellow Springs, Ohio). Relative fluorescence was measured every 3 h using *in situ* YSI 6025 chlorophyll sensor. Relative fluorescence units (RFU) were corrected for instrument drift, and converted into 0–1 scale.

3.4.3 Neutral red retention assay

Logistical challenges associated with the NRA technique limited the number of oysters that could be analyzed each day. Consequently, two weeks were required in both August and October to obtain a sufficient sample size (32) from each of the four treatment groups: upriver site (UPR oyster, DNR oyster); downriver site (UPR oyster, DNR oyster). More specifically, four oysters from each group were sampled daily over 8 days in August (11–14, 18–21) and 8 days in October (12–15, 20–23), ensuring equal representation from each treatment during the first week (50%) and the second week (50%). As a result, 4 UPR and 4 DNR oysters were sampled from each cage (middle rows) in both August (n = 128) and October (n = 128). Sampled oysters were placed in insulated containers with seawater from their respective collection site and

analyzed at the laboratory within 4 h of sampling. Water temperature was maintained in the coolers during transport and holding.

At the laboratory, the prevalence of hemocytes with destabilized lysosomes (DH) was detected using the NRA technique (Hauton *et al.* 2001; Song *et al.* 2007a). A stock solution of the neutral red dye (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) (F1631: Sigma-Aldrich, St. Louis, MO) was prepared by dissolving 2.28 mg in 1 ml of dimethyl sulphoxide (DMSO) (D128: Thermo Fisher Scientific, Waltham, MA). The dye stock solution was kept at 4°C until required. A working solution was prepared daily by dissolving 17 µl of the stock solution in 1 ml of physiological saline solution (pH 7.3), freshly prepared according to Schlieper (1972).

In the August samples, the salinity of the working solution was adjusted to that of the study site. In October, however, the technique was modified such that samples were diluted with oyster extracellular fluid instead of physiological saline. Measurements of hemolymph salinity (Sybon automatic temperature-compensated refractometer) at the time of sampling indicated that hemolymph osmolality differed substantially from the ambient level. Using filtered hemolymph instead of the physiological saline adjusted to ambient salinity, conceivably avoided placing unnecessary stress on the hemolymph cells.

Within 4 h of sampling, 0.2 ml of hemolymph was withdrawn from each oyster's pericardial cavity and transferred to a low-retention Eppendorf tube (Thermo Fisher Scientific) pre-filled with either 0.2 ml of pre-adjusted physiological saline

(August) or extracellular fluid pre-filtered through a 0.20-µm Progene cellulose acetate membrane (Ultident, St. Laurent, QC) (October). An aliquot of 40 µl was transferred to a positively charged microscope slide and incubated in a dark humidity chamber at ambient temperature (20ºC) for 15 min. Working solution (20 µl) was then added and, after an additional 15 min in the humidity chamber, a cover slip was installed; 45 min later, 50 hemocytes were examined under a compound microscope (630× magnification). Hemocytes characterized by large lysosomes or leakage of neutral red dye into the cytosol were designated as having destabilized lysosomes. The lysosomal membrane destabilization index (LDI) was calculated as the number of hemocytes with destabilized lysosomes (DH) in a total of 50 hemocytes, as follows:

$$LDI = (DH /50) * 100$$

3.4.4 Digestive tubule analysis

A subsample (8) of the 32 oysters from each treatment group (4) used for NRA analysis in August (n = 32) and October (n = 32) was also processed for histology. One oyster from each treatment group was randomly selected at each of the 8 sampling events. A 1-2 mm transverse cut was made through the midsection of the oyster body. The tissue slice was immediately fixed in Davidson's solution (48-76 h), rinsed, and stored in ethanol (70%) until further processing. Tissues were subsequently embedded in wax, sectioned (5 µm) and stained with hematoxylin and

eosin. Using light microscopy (100× magnification), histological sections were divided into two quadrats, and from each quadrat four tubules were randomly selected for tubule measurements. The tubule atrophy index (TAI) was determined by dividing the total surface area of the lumen (SL) by that of the whole tubule, including lumen (SLT), as follows:

$$TAI = (SL / SLT) * 100$$

3.4.5 Statistical data analysis

Separate statistical analyses of the LDI (and TAI) were carried out for each sampling period (August and October 2009). A statistical analysis of the hemolymph salinity (based on refractive index) was carried out for the October 2009 sampling period only. A linear mixed model with random cage effects was used because the experimental design was of a hierarchical (nested) structure (Dohoo *et al.* 2009). The statistical design had a split-plot character because two oyster sources were reared within the same cages, which in turn were located at two sites. Hence, the cages correspond to wholeplots and individual oysters correspond to subplots; furthermore, site and source were the wholeplot and subplot factors, respectively. The linear mixed model had fixed effects of site, source, sampling week and its interactions. Note that not all cages were sampled within the same week so this potentially contributed to the between-cage variability.

The assumptions of normality and homoscedasticity of linear mixed models were evaluated by residuals plots based on the standardized residuals (Dohoo *et al.* 2009).

In the presence of a significant interaction between the various factors, pairwise comparison tests with Bonferroni correction were performed to determine significance between groups. All estimates were reported as least squares mean (LSM) ± standard error (SE), unless otherwise specified.

A number of missing values in August and in October 2009 (11 and 10, respectively) were due to mortality and animals lost in the field.

A binary logistic regression analysis of the mortality data (dead/alive) was carried out for the August sampling period. A binary mixed model with random cage effects was used because the experimental design was of a hierarchical (nested) structure, as described above.

For all analyses, the significance level was set at $P \leq 0.05$. The analyses were carried out using Minitab software (Version 15.1.30) (Minitab Inc., State College, PA), SAS software (Version 9.2) (SAS Institute Inc., Cary, NC) and STATA software (Version 10.0) (StataCorp LP, College Station, TX).

3.5 Results

3.5.1 Environmental parameters

Environmental conditions at the upriver and downriver sites are presented in Fig. 2.2 (Chapter 2). Relative fluorescence was similar between the two sites (P =

0.252, paired-t test). However, water temperature was significantly different, averaging 18.1 ± 0.3 °C and 16.7 ± 0.3 °C at the upriver and downriver site, respectively ($P < 0.05$, paired-t test). Salinity was also different between sites ($P < 0.05$, paired-t test); readings were consistently lower at the upriver site (14.6 ± 0.3 gL^{-1}) compared to the downriver site (25.5 ± 0.2 gL^{-1}). This difference in salinity was more pronounced in October than in August (Table 3.1).

3.5.2 Oyster mortality

Overall mortality was low (~ 5%) during the study period (Table 3.2). A statistical analysis was carried out to compare the mortality between the two groups of oysters (UPR, DNR) reared at the upriver or downriver sites from May to August. No significant differences in oyster mortality were found between sites or oyster sources over this first period ($P = 0.627$; $P = 0.080$). Due to only four DNR mortalities being observed in the October sampling, no analysis for risk factors was carried out.

3.5.3 Hemolymph salinity

There were indications of restricted exchange between the oyster mantle fluids and the environment in October (Table 3.3). Oysters were hyperosmotic to ambient seawater at both sites ($P < 0.001$) and this hyperosmocity was significantly more pronounced in oysters reared upriver. The average hemolymph salinity of oysters reared upriver was 21.4 ± 0.6 gL^{-1}(UPR) and 25.6 ± 0.9 gL^{-1}(DNR), yet at the same time ambient salinity was 6-10 gL^{-1}.

In terms of source, the DNR oysters demonstrated a significantly higher salinity differential between their hemolymph and the ambient water than did the UPR oysters ($P < 0.001$). No interaction was found between site and source.

3.5.4 Neutral red retention assay

Microscopic assessment of the hemolymph fluid showed the level of lysosomal membrane stability in the oyster hemocytes (Fig. 3.2A-B). In panel A, destabilized membranes were characterized by enlarged lysosomes and leakage of neutral red dye into the cytosol; in panel B, stable lysosomes appeared normal and retained the neutral red dye particles.

In August (2^{nd} sampling week), oysters reared at the upriver site, regardless of source ($P = 0.75$), had a significantly higher LDI compared to any other site by week combination (Table 3.4; $F = 6.47$, $P = 0.012$). Similarly, in October, oysters reared at upriver site had significantly higher LDI compared to those reared at the downriver site ($F = 38.23$, $P < 0.001$). The significant difference in LDI between sampling weeks in August ($F = 6.47$, $P = 0.012$) and again in October ($F = 11.59$, $P = 0.001$) suggests that this index was sufficiently sensitive to reflect changes in the oysters' physiology on a weekly scale.

3.5.5 Digestive tubule analysis

In both August and October the DNR oysters transferred upriver had significantly higher levels of digestive TAI than did their counterparts downriver (Table 3.5). Interestingly, the UPR oysters transferred downriver had similar TAI levels in August, but significantly lower TAI levels in October, compared to those

that remained at the upriver site. It appears that the transfer downriver eventually led to a reduction in digestive tubule atrophy.

At the downriver site, there was no significant difference in the TAI level between the oyster sources, either in August (P = 0.111) or October (P = 0.446). Conversely, at the upriver site the DNR oysters had significantly greater TAI values compared to the UPR oysters in August, but by the end of the study the reverse was evident (P = 0.042; P = 0.031). In October, the UPR oysters reared at the upriver site showed significantly higher TAI levels than all the other treatment groups. No significant difference in TAI levels was detected between the two sampling weeks in August (P = 0.799) or in October (P = 0.643).

3.6 Discussion

3.6.1 Stress response in oysters following reciprocal transfer

Lysosomal membrane destabilization is a highly sensitive indicator of physiological health because stress-related stimuli generally impact lysosomes rapidly (Moore 1982) with recovery taking from 5–12 days (Song *et al.* 2007b; Zhang *et al.* 2006). In our study, lysosomal destabilization values were consistently above 40%, falling into a category that reflects a degraded environment (Ringwood *et al.* 1998), presence of pollutants (Viarengo *et al.* 2007) or physiological changes associated with mobilization of nutrient reserves during gametogenesis (Ringwood *et al.* 2002). Although levels of LDI were quite elevated and variable, both spatially and temporally, they were generally higher in oysters reared upriver compared to those

reared downriver. By October, the proportion of destabilized lysosomes was 63.6 ± 1.9% in oysters upriver compared to 47.5 ± 1.8% in oysters reared downriver. The underlying factors that promote membrane destabilization in oysters are still unclear. Previous laboratory work on oysters has shown no significant difference in lysosomal destabilization at water temperatures between 15 and 20°C (Hauton *et al.* 1998), suggesting that the difference in temperature at the upriver (18.1 ± 0.3 °C) and downriver (16.7 ± 0.3 °C) sites had no effect on the LDI. Salinity however, has previously been identified as a plausible cause of lysosomal destabilization (Hauton *et al.*1998; Nicholson 2001). Hemolymph cell volume is regulated through the release of amino acids from lysosomes when ambient water salinity changes; consequently lysosomal membrane permeability is elevated during those times, compromising stability (Nicholson 2001). Hauton *et al.* (1998) argued that exposing *O.edulis* to low salinity conditions leads to an osmotic imbalance between the hemolymph and the hemocyte interior creating an electrochemical stress on the lysosomal membrane. However, Ringwood *et al.* (1998) found that neither long term (2 wk) nor short term (2 d) changes in salinity resulted in differences in the lysosomal integrity of the digestive cells of *C. virginica*. Lysosomal membrane destabilization at the upriver site is likely linked to a myriad of environmental factors, other than or in combination with low salinity (Gillson 2011; Hauton *et al.* 1998).

Our conclusion that the upriver environment is physiologically stressful to oysters was further supported by the digestive tubule atrophy results. Oysters reared upriver showed significantly higher levels of tubule atrophy (33-42%) than those

grown downriver (16–19%), the latter being considered within “normal” range (Winstead, 1995). Tubule atrophy is generally observed within 12–24 hours of exposure to a stressful event, and sometimes less (Wilson and LaTouche 1978). Stable TAI values between the two week interval, in August and October suggest that this indicator is more stable at both sites, than LDI. Oysters reared at the upriver site exhibited other signs of physiological stress in addition to lysosomal destabilization and digestive tubule atrophy; in particular, they appeared to isolate themselves from their environment. At the upriver site the salinity differential between oyster hemolymph and the ambient seawater was greater than that of the oysters downriver (Table 3). Based on the higher stress responses observed, it would appear that oysters were experiencing a greater physiological challenge upriver and, therefore, were ventilating at a lower rate in order to restrict water exchange with their environment. Unsuitable environmental conditions provoke behavioral responses, such as valve closure (aka hermetization) in shellfish which limits water exchange with the environment (Berger & Kharazova 1997). Under temperate conditions, a wide salinity differential between the hemolymph and the ambient water may ultimately serve as an indicator of stress response in oysters. Overall, the various indices of physiological status, tubule atrophy, destabilized lysosomes and hyperosmotic hemolymph, all strongly suggested that conditions upriver in the fall were not conducive to the health of the oysters (Gillson 2011).

3.6.2 Implications for oyster culture

Transferring oysters from downriver to upriver aquaculture sites is a common practice in North America that is primarily aimed at reducing the risk of disease infections and predation. Our results imply that the upriver environment disrupts cellular functions at a sub-lethal level. Moreover, Ringwood *et al.* (2004) reported that high lysosomal destabilization (>35%) affects reproductive success (e.g. embryonic development) in oysters, which would impact recruitment. Over the short duration of our experiment (150 d), mortality rates in all groups were low (~10%) and comparable to rates reported by the industry (Doiron 2008). Disruptions in cell physiology may increase susceptibility to pathogens. However, it is well documented that pathogens generally thrive in a high salinity environment (Elston & Ford 2011; Ford & Chintala 2006; Shumway 1996). Perhaps the greatest challenge for oysters displaced upriver is surviving episodic drops in salinity, which is known to reduce pumping activity in this species (Loosanoff 1953). Fresh-water kills of oysters have been well documented, seriously impacting production levels in many estuaries (Andrews *et al.* 1959; Attril *et al.* 2002; Fisher *et al.* 1989; La Peyre *et al.* 2009).

3.7 Conclusions

Transferring oysters upriver in anticipation of reducing exposure to pests, predators and pathogens resulted in a significantly higher cellular immune response, negatively affected tissue integrity and initiated an isolation reflex in oysters. Levels of lysosomal membrane destabilization were significantly higher in oysters transferred upriver than those left at the downriver site. Levels of tubule atrophy in

the digestive gland of the oysters showed a similar pattern. Also, oysters reared upriver were significantly more hyperosmotic to their environment than downriver. Although an increased stress response is generally indicative of poor health, mortality rates were negligible in the present study. It remains unknown whether the physiological stresses of being transferred upriver can negatively impact oyster productivity. Previous work on *Mytilus edulis* has demonstrated that lysosomal membrane destabilization is linearly and negatively correlated with scope for growth (Widdows *et al.* 1982).

3.8 Acknowledgements

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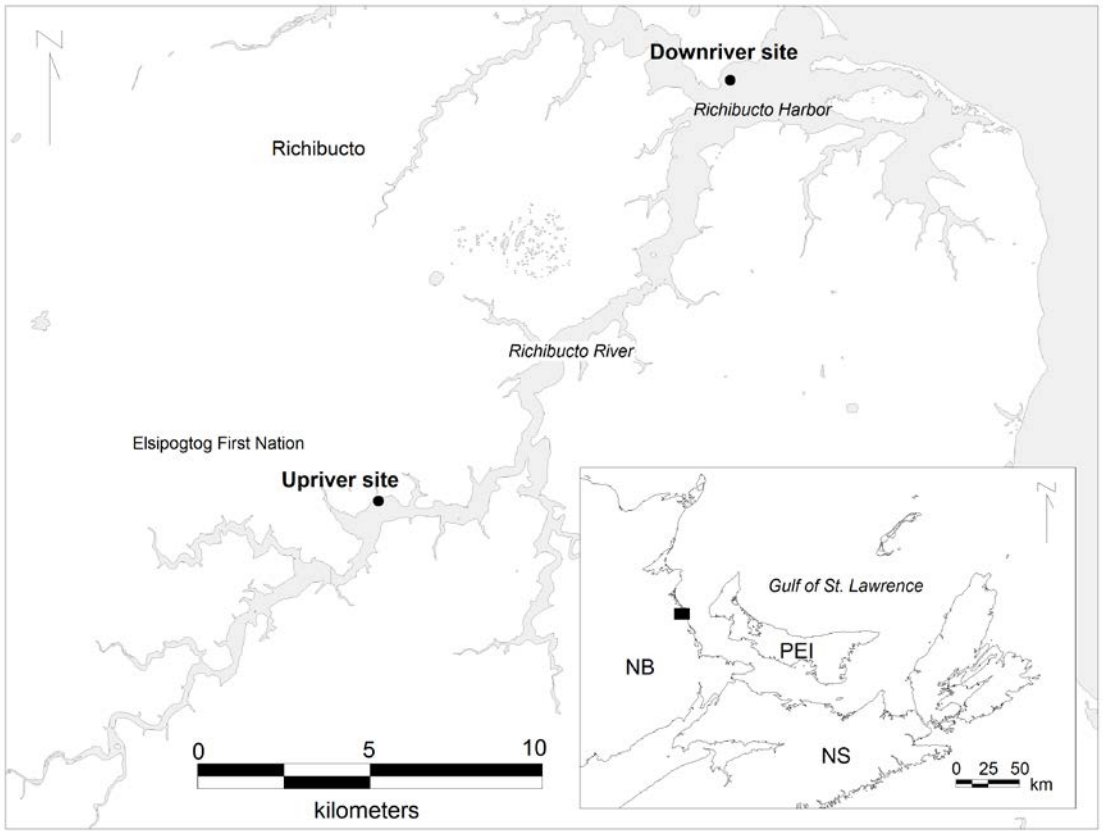
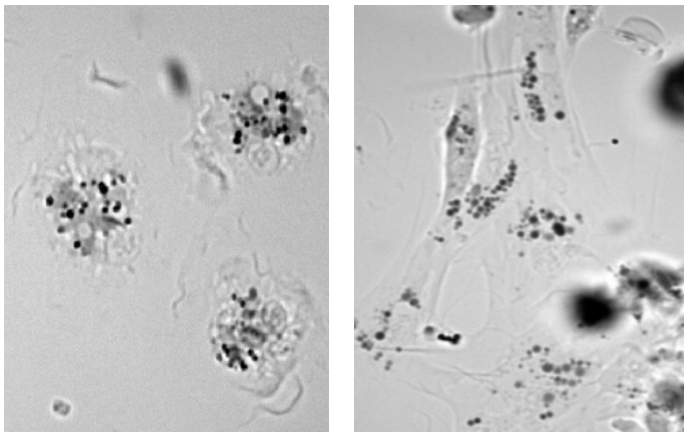


Figure 3.1 Map of the Richibucto estuary with upriver (N 46° 35.185; W 64° 58.843) and downriver (N 46° 42.231; W 64° 50.243) sites in eastern New Brunswick, Canada.



A B

Figure 3.2 Lysosomes containing neutral red dye particles: A) unstable hemocytes indicative of high stress response; B) stable hemocytes indicative of low stress response.

Table 3.1 Salinity and temperature estimates at the upriver and downriver sites during the 2-wk sampling periods in August and October 2009.

Site	Salinity (gL^{-1})			Temperature (˚C)		
	Mean	**SE**	**Min, Max**	**Mean**	**SE**	**Min, Max**
Upriver						
August	**15.4**	**0.9**	**12, 18**	**23.0**	**0.5**	**20.8, 24.5**
October	**7.1**	**0.4**	**6, 10**	**7.7**	**0.5**	**5.8, 9.8**
Downriver						
August	**25.5**	**0.7**	**23, 28**	**21.9**	**0.5**	**20.0, 23.5**
October	**23.6**	**0.5**	**22, 25**	**7.9**	**0.5**	**6.5, 9.9**

Table 3.2 Number of UPR and DNR live and dead oysters at the upriver and downriver sites from May to August and May to October 2009.

Site	*Source*			
	UPR (Upriver)		*DNR (Downriver)*	
	Live	*Dead*	*Live*	*Dead*
Upriver				
May to August	**26**	**3**	**30**	**0**
May to October	**31**	**0**	**25**	**3**
Downriver				
May to August	**30**	**2**	**31**	**1**
May to October	**32**	**0**	**30**	**1**

Table 3.3 Mean ± SE (n) ambient and hemolymph salinity and salinity differential (relative to minimum observed ambient salinity) at the upriver and downriver sites from October 12-23, 2009 (UPR = upriver oysters; DNR = downriver oysters).

Site	Ambient Salinity (gL^{-1})	Source			
		UPR		DNR	
		Hemolymph (gL^{-1})	ΔSalinity	Hemolymph (gL^{-1})	ΔSalinity
Upriver	**6-10**	**21.4 ± 0.6 (29)**	**15.4 ± 0.6**	**25.6 ± 0.9 (27)**	**19.6 ± 0.9**
Downriver	**22-25**	**29.6 ± 0.7 (32)**	**7.6 ± 0.7**	**31.7 ± 0.8 (29)**	**9.7 ± 0.8**

Table 3.4 Estimates from final statistical models for lysosomal destabilization indices from oysters reared at the upriver and downriver sites.

Variable	n	LSM[1]	SE[2]	CI[3]	P-value
August 2009					
Site * Week					**0.012[4]**
Downriver, Week 1	**29**	**41.0**	**2.3**	**36.4, 45.5[A]**	
Downriver, Week 2	**31**	**49.1**	**2.2**	**44.8, 53.6 [A]**	
Upriver, Week 1	**31**	**42.0**	**2.2**	**37.5, 46.4 [A]**	
Upriver, Week 2	**24**	**61.9**	**2.5**	**56.9, 67.0 [B]**	
October 2009					
Site					**<0.001**
Downriver	**61**	**47.5**	**1.8**	**44.0, 51.1**	
Upriver	**56**	**63.6**	**1.9**	**59.9, 67.3**	
Week					**0.001**
Week 1	**62**	**60.0**	**1.8**	**56.5, 63.5**	
Week 2	**55**	**51.2**	**1.9**	**47.4, 54.9**	

Values not sharing a common superscript differ significantly ($P < 0.05$) based on pairwise comparison tests with Bonferroni correction

[1]Least squares means
[2]Standard error
[3]95% confidence interval
[4]P-value for site*week interaction

Table 3.5 Estimates from final statistical models for tubule atrophy indices from oysters reared at the upriver and downriver sites.

Variable	n	LSM[1]	SE[2]	CI[3]	P-value
August 2009					
Site * Source					**0.010[4]**
Downriver, DNR oysters	**8**	**13.2**	**4.1**	**5.2, 21.2[A]**	
Downriver, UPR oysters	**8**	**22.3**	**4.1**	**14.3, 30.3[AB]**	
Upriver, DNR oysters	**8**	**25.4**	**3.8**	**17.9, 32.9[B]**	
Upriver, UPR oysters	**6**	**13.6**	**4.4**	**5.0, 22.2 [A]**	
October 2009					
Site * Source					**0.043[4]**
Downriver, DNR oysters	**7**	**19.1**	**3.8**	**11.7, 26.6[A]**	
Downriver, UPR oysters	**8**	**15.6**	**3.8**	**8.1, 23.0[A]**	
Upriver, DNR oysters	**8**	**33.3**	**3.6**	**26.2, 40.3[B]**	
Upriver, UPR oysters	**8**	**42.4**	**3.6**	**35.4, 49.4[C]**	

Values not sharing a common superscript differ significantly ($P < 0.05$) based on pairwise comparison tests with Bonferroni correction.

[1]Least squares means
[2]Standard error
[3]95% confidence interval
[4]P-value for site*source interaction

Chapter 4 HEMOLYMPH FLUID OSMOLALITY INFLUENCES THE NEUTRAL RED RETENTION ASSAY IN OYSTERS (*Crassostrea virginica*) HEMOLYMPHATIC CELLS

Méthé D, Comeau LA, Stryhn H, Burka JF, Landry T & Davidson J (submitted) Hemolymph fluid osmolality influences the neutral red retention assay in oysters (*Crassostrea virginica*) hemolymphatic cells. Journal of Molluscan Studies.

4.1 Abstract

The neutral red retention assay (NRA) is a subcellular biomarker of stress response, measured by the degree of lysosomal membrane destabilization within hemolymphatic cells. The purpose of this study was to investigate the impact of the hemolymph fluid osmolality on the outcome of the NRA. Eastern oysters (*Crassostrea virginica*) were subjected to different temperature and salinity regimes in order to experimentally manipulate the hemolymph fluid osmolality. It was found that the hemolymph fluid remained osmotic to ambient waters when oysters were acclimated to elevated temperatures (10–20°C). However, the fluid became hyperosmotic (~32.3 mOsm kg^{-1}) in oysters acclimated to low temperatures (0–5°C). This observation was consistent with oysters having their valves closed 90–100% of the time at low temperatures. A reduction in ambient salinity mimicking a spring freshet (6‰ salinity) resulted in hyperosmotic fluids for all temperature groups (0–

20ºC). The magnitude of the osmotic differential was inversely proportional to temperature, with the differential reaching ~645mOsm kg^{-1} at the lowest temperatures (0–5ºC). Under such conditions the application of a novel version of the NRA, one in which filtered hemolymph fluid was used, instead of the prescribed standard solution, produced measurements of lysosomal membrane destabilization in hemolymphatic cells that were 6–10% lower than those obtained using the standard NRA technique.

4.2 Keywords

osmolality, temperature, lysosomal destabilization; stress response; neutral red retention assay; oyster; spring freshet.

4.3 Introduction

In recent years, the use of biochemical, cellular and physiological biomarkers as an early warning system in the aquatic environment has increased worldwide (ICES 2013; MEDPOL 2007). The neutral red retention assay (NRA), a reliable biomarker of general stress in fish and marine bivalve, has several advantages over other indicators of stress (Domouhtsidou & Dimitriadis 2001; Izagirre & Marigomez 2009; Nicholson 2001). NRA is a simple, highly sensitive and non-invasive assay utilizing live cells (hemolymph or digestive gland) to accurately measure lysosomal membrane stability (Lowe *et al.* 1995). Lysosomes play a central role in an organism's innate defense system by destroying foreign invaders such as parasites, bacteria and viruses and are involved in numerous cellular functions including

nutrition, tissue repair, and turnover of membranes, organelles, and proteins (Settembre *et al.* 2013). They also sequester pollutants, making them a valuable biomarker of environmental and/or chemical exposure (Moore *et al.* 2008).

With the NRA now commonly used as a tool in environmental monitoring studies (MEDPOL 2007), we have a better understanding of the conditions and circumstances that may influence its validity and interpretation. Recent studies warn that seasonal changes in physiological state (e.g. gametogenic cycle, nutritional status) interfere with lysosomal stability (Izagirre *et al.* 2008; Ringwood *et al.* 2004; Ringwood *et al.* 2002; Song *et al.* 2007a). Environmental variables (i.e. salinity, temperature, algae) also affect NRA response (Dimitriadis *et al.* 2012; Hauton *et al.*1998; Keppler *et al.* 2005; Nicholson 2001; Song *et al.* 2007b). As a result, the interpretation of NRA results can be problematic, particularly in field-collected animals because of the high variability in bivalve physiology and environmental conditions (Ringwood *et al.*1999).

In this study, we investigate another factor with potential to compromise quantitative measurement of NRA responses. The assay involves the use of a prescribed physiological saline solution (Schlieper 1972) adapted to the salinity of the surrounding seawater. Most studies use this approach to determine the level of lysosomal destabilization in bivalves (Aguirre-Martinez *et al.* 2013; Harding *et al.* 2004; Hauton *et al.*1998, 2001; Peraira *et al.* 2014; Song *et al.* 2007a, 2007b). Specifically, hemocytes are typically extracted from the posterior adductor muscle sinus (or the pericardial cavity) and mixed into an equal volume of the saline

solution. However, Coughlan *et al.* (2009) warned that a saline solution adapted to the aquatic environment instead of the hemolymph fluid induces artificially high results in lysosomal destabilization data for Manila clams (*Ruditapes philippinarum).* The biological response measured in Manila clams may have been confounded with cellular damage from hyposmotic stress created by the saline solution.

We postulate that under extreme environmental conditions oysters naturally become hypo– or hyperosmotic to their surrounding environment. Oysters occupy a wide geographical range along the east coast of North American (~ 4,000km) with extreme temperatures and salinities (0–49°C; 3–30‰). For example, in the southern Gulf of St. Lawrence water temperature reaches sub-zero values during winter and in the upper reaches of its river systems, saline water is often nonexistent during spring freshets and autumn floods. Under extreme conditions oysters close their valves and limit exchange with the surrounding seawater (Comeau *et al.* 2008; Loosanoff 1958). Such behaviour presumably renders oysters hyperosmotic to ambient water, which raises doubts regarding the robustness of the standard NRA. The assay's saline solution assumes osmotic balance with the surrounding waters. Hypo– or hyperosmotic conditions cause cells to swell (shrink) in response to the osmotic influx (outflux) of water (Strange 2004). This influx/outflux of water places osmotic stress on the cells. As the use of NRA increases in monitoring studies, it is critical to know if results are, or are not, influenced by extraneous factors including osmotic stress.

The general objective of this study was to explore the validity of a modified lysosomal destabilization assay (Chapter 3), one that may be more adaptable for use in bivalves exposed to extreme environmental conditions. Specifically, the existing technique (NRA) was compared with a modified technique (NRA_{MOD}) whereby hemolymph fluid was used instead of the prescribed saline solution. The two assays were compared in *C. virginica* exposed to low water temperatures and reduced salinities, thereby mimicking a spring freshet. It was hypothesized that using hemolymph fluid that is perfectly adjusted to the osmolality of the animal tested, instead of a prepared saline solution, reduces potential for osmotic stress, an extraneous factor. To gain additional insight on the behavior and health of the oysters, their gaping activity was monitored during the entire study, while their physiological health was evaluated based on a histological examination (digestive tubule atrophy).

4.4 Materials and methods

4.4.1 Animals and experimental set up

Cultured oysters (77.5 ± 1.1mm shell height) were collected from Shédiac Bay, NB, on December 5, 2011. Water temperature at the time of collection was 5°C; salinity 27‰. Oysters were transported to the Aquatic Animal Facility at the Atlantic Veterinary College, University of Prince Edward Island (Charlottetown, PE) within 2h of collection and placed in a static aquarium (300L) with aerated artificial seawater (Instant Ocean®, Kent Marine, Acworth) at 5.0 ± 0.5°C and 27 ± 2‰ salinity (pH 7.5, dissolved oxygen [DO] > 70%, total ammonia < 1ppm). They were

fed once daily, at a concentration of 10,000 cells ml^{-1} of algal paste (Innovative Aquaculture Products, Lasqueti Island, BC). After 30 days, the oysters were distributed into 20 22-L static tanks and acclimated to experimental conditions by increasing (or decreasing) water temperature (1–2°C per day) until reaching targeted temperatures (0, 5, 10, 15 and 20°C). There were four tank replicates per temperature regime. Each tank contained 15 oysters, and water volume was approximately 2 liters per oyster. The temperature increase (or decline) was scheduled such that all groups reached the desired temperature on the same day. The oysters were fed once a day to a concentration of 150,000 cells ml^{-1} of algal paste. The water was changed every 7 days. Oyster mortality was monitored daily.

Photoperiod was set at 8 h light: 16 h dark and was continuously monitored using Hobo® UA-002 light loggers (Onset Computer Corporation, Bourne, MA), placed 2cm above the tank water. Water temperature was measured every 15min using Minilog-TR probes (AMIRIX Systems Inc., Halifax, NS). Salinity was determined every 30min, based on electrical conductivity measurements using *in situ* YSI 6560 probes (YSI, Yellow Springs, OH). The pH and dissolved oxygen were measured using a handheld YSI Pro 2030 meter. Total ammonia was determined with a HACH kit (Hach Company, Loveland, CO). Algal cell counts were determined using a hemacytometer (Bright-Line, Hausser Scientific, Horsham, PA) mounted on a Leica DM LS5 compound microscope (Leica Microsystems Inc, Concord, ON).

The experiment was carried out in two phases (I, II). Oysters that were subjected to temperature treatments (0, 5, 10, 15 and 20°C) were maintained at a

fixed salinity (27‰) during phase I (4wks). During phase II (2wks), ambient salinity was dropped to 6‰ (over a 12h period) in 2 (of 4) tanks temperature per regime.

4.4.2 Valvometry measurements

During each phase (I, II) of the study, 20 oysters distributed among replicate tanks were wired to individual sensors and monitored every minute for valve opening or gaping. Four oysters per temperature regime (0, 5, 10, 15 and 20°C at a salinity of 27‰) were included during phase I; and 2 oysters per temperature-salinity combination (0, 5, 10, 15, 20°C at 6‰; 0, 5, 10, 15, 20°C at 27‰) during phase II. At the end of the study, the wired oysters were processed for osmolality measurements, NRAs and tubule atrophy analysis.

Valve opening or gaping was measured from wired oysters using a method described by Comeau *et al.* (2012). Briefly, a coated Hall element sensor (HW-300a, Asahi Kasei Corp., Tokyo, Japan) was glued using Ethyl cyanoacrylate (Gel Instant Krazy Glue, Elmer's Product Canada Corp., Toronto, ON) to the left valve (maximum distance from the hinge) and a small magnet (4.8mm diameter × 0.8mm height) was glued to the right valve. The magnetic field or flux density between the sensor and magnet was a function of the gap between the two valves. The magnetic field in the form of output voltage (μV) was acquired by strain recording devices (DC 104R, Tokyo Sokki Kenkyujo Co., Tokyo, Japan) as described in Nagai *et al*. (2006). Output voltage was converted into valve opening by applying conversion algorithms specific to each sensor assembly. At the end of the monitoring phase, the adductor muscle was severed and small calibration wedges were manoeuvred between the two

valves at the point farthest from the hinge. Wedge height was 1–6mm. The relationships between voltage and wedge height (i.e. valve opening) were non-linear and strong. Valve opening (mm) data were then converted into gape angles (θ in degrees) using the following equation (Wilson *et al.* 2005):

$$\theta = 2\arcsin\left(\frac{0.5W}{SH}\right)\times 100$$

where *W* is the valve opening (mm) and *SH* (mm) is the oyster's shell height. The proportion of times the valves were open (W > 0) was computed.

4.4.3 Sampling protocol

Logistical challenges associated with the NRA technique limited the number of oysters that could be analyzed each day to 10. Two days were required to sample oysters from all the tanks. Therefore, in the first phase of the experiment one oyster was extracted from each tank (one oyster week^{-1} tank^{-1}) over 2 days week^{-1} for a period of 4 weeks (i.e. 8 sampling days). Consequently, a total of 16 oysters (4 oysters tank $^{-1}$) from each of the five treatment groups (0, 5, 10, 15 and 20°C) were sampled during the first phase of the experiment (n = 80 oysters). Their hemolymph fluid and the surrounding water were subjected to osmolality measurements (Section 4.4.4). Their stress response was also measured in terms of destabilized lysosomes (Section 4.4.5) and the level of digestive tubule atrophy (Section 4.4.6).

In the second phase of the experiment, 2 oysters were extracted from each tank (two oysters week^{-1} tank^{-1}) over 4 days week^{-1}, for a period of 2 weeks (i.e. 8 sampling days). Consequently, a total of 8 oysters (4 oysters tank $^{-1}$) from each of the

ten treatment groups (0, 5, 10, 15 and 20°C at salinities of 6 or 27‰) were sampled during the second phase of the experiment (n = 80 oysters). As described above, their hemolymph fluid and the surrounding water were subjected to osmolality and stress response measurements.

4.4.4 Osmolality measurements

At each sampling time, 20µl water was collected from each sample tank and used immediately for osmolality measurements (to the nearest 1.0 mOsm kg $^{-1}$ H_2O) using an Model 3320 osmometer (Advanced Instruments Inc., Norwood, MA). Simultaneously, 20µl mantle fluid was collected from each oyster sampled using a sterile hypodermic plastic syringe with filter (0.2µm cellulose acetate filter, Ultident Inc., St-Laurent, QC) and was also used immediately to measure osmolality. The difference in osmolality (ΔOSM) between hemolytic osmolality (HO) and ambient water osmolality (AO) was calculated as follows:

$$\Delta OSM = HO - AO$$

4.4.5 Neutral red retention assay

A stock solution of the neutral red dye was prepared by dissolving 2.28 mg of neutral red dye (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) (F1631, Sigma-Aldrich, St. Louis, MO) in 1 ml of dimethyl sulfoxide (DMSO) (D128 Thermo Fisher Scientific, Waltham, MA). The dye stock solution was kept at 4°C until required. A working solution was prepared daily by dissolving 17µl of the stock

solution in 1 ml of prepared saline solution (pH 7.3). The saline solution was prepared according to Schlieper (1972) and adjusted to the salinity of the rearing tank.

For the NRA, the salinity of the working solution was adjusted to that of the rearing tank. For the NRA_{MOD}, samples were diluted with oyster extracellular fluid instead of the prepared physiological saline.

Immediately after sampling the mantle fluid for osmolality measurements, 0.2ml of hemolymph was withdrawn from the pericardial cavity/adductor muscle sinus and transferred to a low-retention Eppendorf® tube (Thermo Fisher Scientific) pre-filled with either 0.2ml of pre-adjusted saline solution or extracellular fluid pre-filtered through a 0.2µm Progene cellulose acetate membrane (Ultident, St. Laurent, QC). An aliquot of 40µl was transferred from each tube to a corresponding microscope slide (positively charged) and incubated in a dark humidity chamber at ambient temperature (20°C) for 15min. Working solution (20µl) was then added to each slide and, after an additional 15min in the humidity chamber, a cover slip was installed; 45min later, 100 hemocytes were examined under a compound microscope (630× magnification). Hemocytes with large lysosomes or leakage of neutral red dye into the cytosol were designated as having destabilized lysosomes (DH).

The lysosomal membrane destabilization index (LDI) representing a count of hemocytes (out of a total of 100) having destabilized lysosomes was expressed as a percentage. The difference between the two NRA outcomes (ΔLDI) was calculated as follows:

$$\Delta LDI = LDI\ (NRA) - LDI\ (NRA_{MOD})$$

4.4.6 Digestive tubule analysis

During phase I, oysters were processed for histology examination to determine differences in digestive tubule atrophy, or epithelial thinning among temperature treatment groups. Once hemolymph samples were taken, a 1-2 mm transverse cut was made through the midsection of the body (Winstead 1995). The tissue slice was immediately fixed in Davidson's solution for a period of 48–76h, rinsed, and stored in ethanol (70%) until further processing. Tissues were subsequently embedded in wax, sectioned (5 µm) and stained with hematoxylin and eosin. Using light microscopy (100× magnification), histological sections were divided into two sampling areas, and from each section four tubules were randomly selected for tubule measurements. Thus, a total of 8 measurements were taken for each oyster (i.e. 4 tubules for each of the two sampling areas). The tubule atrophy index (TAI) was determined by dividing the total surface area of the lumen (SL) by that of the whole tubule, including lumen, area (SLT):

$$TAI = (SL\ /SLT) \times 100$$

4.4.7 Statistical analysis

Separate statistical analyses of ΔOsmolality (and ΔLDI, TAI) were carried out for each phase of the experiment: temperature effect (Phase I); temperature—salinity effect (excluding TAI) (Phase II). In phase II, statistical analysis was limited to the oysters subjected to 6‰, the oysters at 27‰ served as control to ensure comparability with phase I. The focus of interest in phase II were hemolymph values (not ΔOsmolality) because ambient values were intentionally out of range.

A linear mixed model with random tank effects was used due to the hierarchical (nested) structure of the experimental design (Dohoo *et al.* 2009). The models had temperature, day, week, and their interaction. In addition to the random tank effects, sampling order was also analyzed. For TAI, oyster and sampling area of the digestive gland were added to the model as random effects.

The assumptions of normality and homoscedasticity of linear mixed models were evaluated by residuals plots based on the standardized results. The deviation of residuals from normality was calculated using the Anderson-Darling normality test. Whenever model assumptions could not be met, an optimal power transformation was obtained by Box-Cox analysis (Dohoo *et al.* 2009). A cubic-root transformation was required for the TAI dataset for phase I. A log transformation was required for the haemoloymph osmolality data for phase II.

In the presence of a significant interaction between the various factors, pairwise comparison tests (with Bonferroni correction) or contrasts (with Scheffé correction, if applicable) were performed to determine significance between groups. Based on contrasts suggested by ΔOSM data from phase I, a planned temperature

contrast (0, 5ºC vs 10, 15, 20ºC) for ΔLDI data was investigated for phase II. All estimates were reported as least squares mean (LSM) ± standard error of the mean (SE); in some instances following log back-transformation the back-transformed value was interpreted as the median and confidence intervals were presented instead.

A few extreme outliers were removed from the temperature-salinity treatment dataset after they were found statistically significant by the outlier detection test (based on deletion residuals): Δosmolality (1 obs.) ΔLDI (1 obs.).

The significance level was set at $P \leq 0.05$. The analyses were carried out using Minitab software (Version 15.1.30) (Minitab Inc., State College, PA), STATA software (Version 13.0) (StataCorp LP, College Station, TX) and SAS software (Version 9.2) (SAS Institute Inc., Cary, NC).

4.5 Results

4.5.1 Valve movement

Table 4.1 shows the gaping activity of oysters during phase I of the experiment. At a constant salinity (27‰), oysters held at 0°C kept their valves closed during the entire sampling period (4 weeks). At 5°C, oysters were open 5.4 ± 3.8% (mean ± SE) of the time, while at higher temperatures (10–20°C) oysters held their valves open 24.5–39.5% of the time (Fig. 4.1).

Table 4.2 shows the gaping activity of oysters during phase II of the experiment, i.e. after exposure to a drop in ambient salinity (6‰), mimicking a spring freshet (2 week duration). As observed in phase I, oysters that were held at 0°C kept

their valves closed during the entire time. However, at the higher temperatures, oysters kept their valves opened ≤ 3.4% of the time (Fig. 4.1).

4.5.2 Hemolymph fluid osmolality

During phase I of the experiment, there were indications of a restricted exchange between the oyster hemolymph fluid and ambient water at low temperatures (Fig. 4.2). Temperature had a strongly significant impact on ΔOSM ($F = 6.18$; $P < 0.001$). When salinity was fixed at 27‰ (~810mOsm kg^{-1}) oyster fluid was hyperosmotic to ambient water at 0 and 5°C, however not at the higher water temperatures (10, 15, 20°C). At 0°C and 5°C, fluid osmolality were respectively 32.3 ± 4.5 and 19.6 ± 4.5mOsm kg^{-1} higher than ambient; while it differed by 8.3 ± 4.5mOsm kg^{-1} or less at 10, 15, 20°C.

Also in phase II of the experiment, when ambient salinity dropped to 6‰ (~180mOsm kg^{-1}), temperature had a strongly significant impact on ΔOSM ($F = 33.8$; $P < 0.001$). However, oyster fluids were hyperosmotic to ambient water at all temperatures (Fig. 4.2). Moreover, the differential was inversely proportional to water temperature (Table 4.3). At 0°C, hemolymph osmolality was 645mOsmo kg^{-1} higher than ambient water (~161mOsmo kg^{-1}), while at 10°C the differential was 493mOsmo kg^{-1} and at 20°C, 268mOsmo kg^{-1}. Pairwise comparisons of the hemolymph osmolality revealed 3 subgroups, dependent on ambient temperature: 1) 0, 5°C; 2) 5, 10°C; and 3) 15, 20°C (Table 4. 4).

In our control group (27‰), hemolymph osmolality values at 5°C were unusually low (Table 4.3) compared to those in phase I (Fig. 4.2), with no specific

explanation. In phase II (control) there is no difference with ambient water at 5°C, unlike in phase I.

4.5.3 NRA and NRA_{MOD} outcomes

During the first phase of the experiment, temperature (0, 5, 10, 15, 20°C) did not significantly affect ΔLDI outcome (Table 4.5). The ΔLDI was non-significant ($F = 1.08$; $P = 0.372$) between water temperature (salinity constant at 27‰). There was no association between ΔLDI and ΔOSM ($r = 0.15$; $P = 0.204$). A moderately positive correlation was found between LDI (NRA) and LDI (NRA_{MOD}) ($r = 0.58$, $P < 0.001$). However, during the second phase of the experiment, when ambient salinity dropped to 6 ‰, ΔLDI outcome was affected at the low temperatures. The preplanned contrast revealed two subgroups ($t = 2.41$; $P = 0.02$), dependent on ambient temperature: 1) 0, 5°C; 2) 10, 15, 20°C. At higher temperatures (10, 15, 20°C), ΔLDI values ranged between 1.2–2.4%, while at the lower temperatures (0, 5°C), 5.8–10.3%. Additionally, within a day of reaching targeted salinity (6‰) the ΔLDI was 12.4 ± 3.4%. Although it decreased on day 2 (–3.0 ± 4.4%), ΔLDI remained ≥ 2% in the days (4 out of 6) that followed. As in Phase I, a positive association was found between LDI (NRA) and LDI (NRA_{MOD}) ($r = 0.74$, $P < 0.001$).

Figure 4.3 displays scatterplot panels of ΔLDI with ΔOSM for each temperature (0, 5, 10, 15, 20°C). At 0°C, the ΔOSM is large, while at the higher temperatures, smaller and more variable; while the mean of ΔLDI points decreases, approaching zero at higher temperatures.

4.5.4 Gaping activity in relation to hemolymph osmolality and NRAs

Figure 4.4 shows that gaping activity (phase II) was uncorrelated (based on Spearman correlation) with (a) the hemolymph osmolality (spring freshet group, r = –0.38; P = 0.351 and control group, r = 0.37; P = 0.362), (b) the Δosmolality (spring freshet group r = –0.46, P = 0.247 and control group, r = –0.07, P = 0.875), and (c) the ΔLDI (spring freshet group, r = 0.01, P = 0.974 and control group, r = 0.39, P = 0.346).

4.5.5 Tubule atrophy

During phase I of the experiment, TAI values ranged between 19.5–44.4. After Bonferroni correction, there were no significant differences in mean TAI values between treatment groups (0, 5, 10, 15, 20°C at a salinity of 27‰).

4.6 Discussion

4.6.1 Valve activity

When salinity was constant (27‰), oysters were most active at 20°C, with valves open 32.1–51.6% of the time. This level is quite low in comparison to previous reported work. Comeau *et al*. (2012) found that oysters kept their valves open 68.6% of the time while feeding on natural seston in late spring. The underlying factors for this incongruity are unknown and may be related to holding stress. Oysters held at the lower temperatures (≤ 5°C) had their valves opened ~0–5% of the time, while those at temperatures ≥ 10°C, ~25–40% of the time.

Following a drop in salinity (i.e. spring freshet), most of the oysters (5 out of 8) closed themselves off completely from the surrounding environment, regardless of

water temperature. Unfavorable conditions will provoke an isolation reflex in bivalves, closing them off from the external environment for indeterminate periods of time (Loosanoof 1958). Our results suggest that oysters may isolate themselves (chronic valve closure) during prolonged periods of low salinity (spring freshets and autumn floods), regardless of water temperature. With no specific explanation, during phase II one oyster in our control group (27‰) had its valves open 13% of the time at 0ºC, while that same oyster was completely closed during phase I. Moreover, at 10ºC oysters were hardly active (0–5% of the time), unlike in phase I (8–37%).

4.6.2 Hemolymph fluid osmolality

Previous studies have shown that animals inhabiting marine environments with differing salinity gradients may become hyperosmotic to their environment (Coughlan *et al.* 2009). Our study has illustrated similar results. When salinity was kept constant (27‰), the osmolality differential (~32.3mOsm kg^{-1}) measured in oysters was more pronounced at low water temperatures and was inversely proportional to water temperature. While Coughlan *et al.* (2009) observed hyperosmocity (~75 ± 15mOsm kg^{-1}) in Manila clams at 15 ± 1°C, at that same temperature our oysters were isosmotic (~ 3mOsm kg^{-1}) to ambient. Oysters occupy a wide latitudinal range along the east coast of North America. Native populations are found in the Gulf of Mexico (27°N) and northward into the Gulf of St. Lawrence (48°N), Canada (Carriker & Gaffney, 1996). Our study suggests that an osmotic imbalance between the hemolymph fluid and the surrounding seawater is likely in northern populations at times when temperatures falls below 5°C.

We also investigated whether environmental salinity changes can induce osmotic imbalances. In theory, with many sedentary bivalves exposed to intertidal/seasonal salinity fluctuations within estuaries and river systems, the potential for an osmotic differential between the environment and body fluids exists (Shumway 1977). We found that oysters exposed to a sharp drop in ambient salinity (i.e. spring freshets) failed to reduce osmolality and become isosmotic with the ambient environment, regardless of the water temperature. Therefore an osmotic imbalance was detected in all groups, including oysters exposed to higher temperatures, which were expected to pump water into their mantle cavity and render their hemolymph fluid isosmotic to ambient. The valvometry data showed the majority of oysters closing themselves off completely from the low salinity environment. Our study thus provides compelling evidence that behavior (valve closure) is the main factor maintaining hemolymph osmolality during freshet events. There were no indications that valve closure augmented hemolymph osmolality (see Fig. 4.3a) due to a putative accumulation of metabolic wastes. Moreover, no significant difference in tubule atrophy was found between treatment groups. Hence the reported osmolality differentials under freshet conditions were likely caused by falling environmental salinities and oysters closing their valves to uphold hemolymph osmolarities. Because such behavior occurred at various water temperatures (0–20ºC), it may be interpreted as a robust avoidance strategy of stressful events.

4.6.3 Lysosomal destabilization indices

4.6.3.1 Temperature effect

There was no evidence that temperature interfered with NRA outcome despite hyperosmoticity (~32.3mOsm kg^{-1}) observed in oysters held at ≤ 5°C temperatures (ambient salinity, 27‰). NRA values were not significantly different between hemolymph cells incubated in the prescribed saline solution adapted to ambient salinity and those incubated in hemolytic fluids (isosmotic). Based on our results, the osmolality differential observed in oysters held at low temperature does not interfere with the immunoassay. Consequently, at a stable salinity the standard NRA is applicable in oysters living at high latitudes.

4.6.3.2 Combined effect of temperature and salinity

This study concludes that the standard NRA is sensitive to the combined effect of temperature and salinity. In particular, NRA values were higher when oyster hemolymph cells were incubated in prescribed saline solutions adapted to ambient salinity, compared to samples incubated in hemolymph fluids (isosmotic). This result, however, occurred only under a certain set of conditions, namely when oysters held at very low temperatures (≤ 5°C) were subjected to a period (2 weeks) of low salinity (e.g. spring freshet). In such conditions, oysters were hyperosmotic to ambient salinity; in fact the osmolality differential was in excess of ~550mOsm kg^{-1}. The effect of the temperature-salinity combination on NRA suggests possible mechanisms. Solution-related elevations in assay results might be linked to osmotic stress (e.g. intracellular colloid osmotic gradient) created by the prescribed solution. When applying a hypotonic saline solution, cells swell and therefore require release of inorganic ions (Davenport 1985; Lang 2007; Strange *et al*. 1996). Volume

regulating responses (e.g. Na, K, Cl fluxes) are immediate (within seconds) in euryhaline organisms such as oysters, and as a result osmotic stress is created on cellular membranes (Strange *et al.* 1996). Osmotic differential between the environment and hemolymph fluids is highly probable during periods of low temperatures and fluctuating salinity (e.g. spring freshets and autumn floods). Consequently, it would be advisable that investigators verify osmolality of bivalve hemolymph prior to carrying out the assay to ensure that the response measured is not confounded by osmotic stress created by hypo- or hypertonic saline solutions.

4.6.4 Application of NRA

Oyster populations inhabiting regions exposed to estuarine temperatures reaching 0 to -1°C experience chronic valve closure for extended periods of time, ~4–5 months during the winter season (Comeau *et al.* 2012). This behavior (valve closure) is expected to last until water temperature warms up to 2.6 ± 0.7°C which occurs about 15 days following the departure of ice in the spring (Comeau 2014). Starting in late March and extending over 1–2 months, most estuaries receive vast amounts of freshwater discharge (e.g. spring freshets). For instance, freshwater discharge (Richibucto River, NB) data from Environment Canada (1964–2010) showed the monthly mean reached a maximum of $84 m^3 s^{-1}$ in April (compared with $34\ m^3 s^{-1}$ over a year), reducing salinity levels in the river system to a minimum of ~0‰. Comeau *et al.* (2012) found no apparent link between oyster valve activity and salinity; however, their study site experienced limited salinity perturbations (~22–28‰). Our results show that when oysters are exposed for an extended period of time

to low salinity levels (6‰) coupled with low temperature (0–5°C), such extreme environmental conditions, including spring freshets, would impact oyster behavior (e.g. valve closure) and, subsequently, NRA outcome. During such events the stress response measured using the NRA exceeded values obtained using the modified NRA. These observations suggest erroneous increases in NRA outcomes during extremely low salinity events, such as spring freshets when oysters close their valves to maintain optimal hemolymph osmolality. This interpretation is consistent with the osmolality differential observed in closed oysters, and further supports the presence of a confounding effect (osmotic stress) caused by the prescribed solution used in the standard NRA.

One should not assume that the prescribed saline solution used in the standard NRA is suitable for all bivalves under all environmental conditions. With many studies using the standard NRA to evaluate stress responses of bivalves from polluted areas, it would be important to note the behavior of these bivalves under such conditions (Lowe *et al.*1995; Nicholson 1999, 2003; Ringwood *et al.*1998, 1999). If the use of the NRA is to continue as an indicator of stress response, it would be prudent for investigators to use appropriate measures in order not to compromise the results of the assays when bivalves close their shells to the surrounding environment.

While this study has generated some important findings under laboratory conditions, it does not provide all conditions and circumstances that exist in the field. For instance, polymorphism (or physiological adaptation) exists amongst bivalve populations inhabiting different aquatic biotopes, i.e. oysters from Chesapeake Bay

have narrower salinity tolerances than their congenics along the Atlantic coast (Pierce *et al.* 1992). Therefore, it would be highly speculative to generalize our findings. Investigators should verify hemolymph osmolality of bivalves prior to carrying out the assay to ensure that the response measured is not confounded by osmotic stress.

Because damage to lysosomal membranes represents a real pathophysiological reaction at the cellular level with potentially severe consequences for health status of the animal, assay results enhance our understanding of cellular immunological processes in disease susceptibility (Hauton *et al.* 2001). With *Perkinsus* reaching the northeastern USA and recent outbreaks of MSX in the Bras d'Or Lakes, NS, Canada, the scientific community must have assurances that current assay protocols are accurate in determining intracellular disturbances in animals from these northern regions. This study suggests an appropriate protocol for bivalves living at their northern distribution limit.

4.7 Conclusion

In conclusion, the neutral red retention assay of hemolymph using the prescribed physiological saline solution has the potential to be a source of bias when measuring stress response in oysters and other poikilosmotic/osmoconformers. It is the intent that the NRA_{MOD} will detect the earliest possible change of primary intracellular disturbances, as is the case for the NRA under normal environmental conditions. Measuring cellular response under controlled conditions (at select water temperatures) provided the first indication of a problem. The successful use of NRA

will depend on its ability to reflect the true individual differences rather than the effects of extraneous factors, such as osmotic stress created from a colloid pressure gradient on cell membranes. NRA_{MOD} using filtered mantle fluid is proposed as an alternative to NRA for measuring lysosomal membrane destabilization for oysters living in cold waters.

4.8 Acknowledgements

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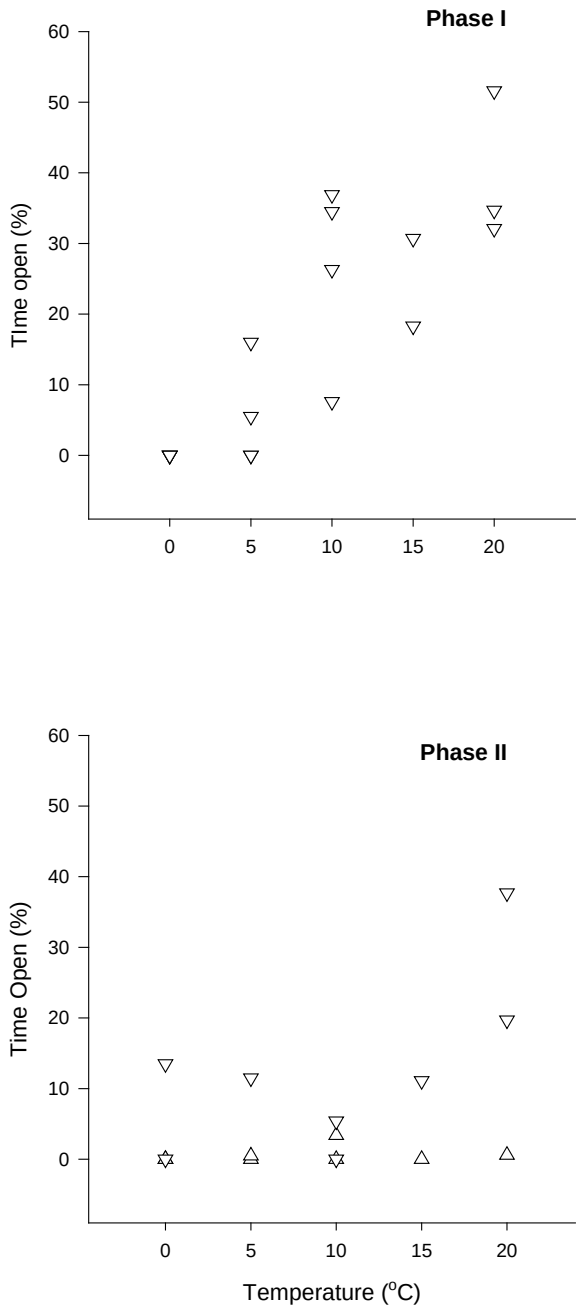

Figure 4.1 Scatterplot of gaping activity (% time valves open) of individual oysters held at different temperatures during Phase I (salinity 27‰ [810mOsmo kg^{-1}]) and Phase II (Δ spring freshet (salinity 6‰ [180mOsm kg^{-1}]);▽control (salinity 27‰ [810mOsmo kg^{-1}]) n = 17 (Phase I); n = 16 (Phase II).

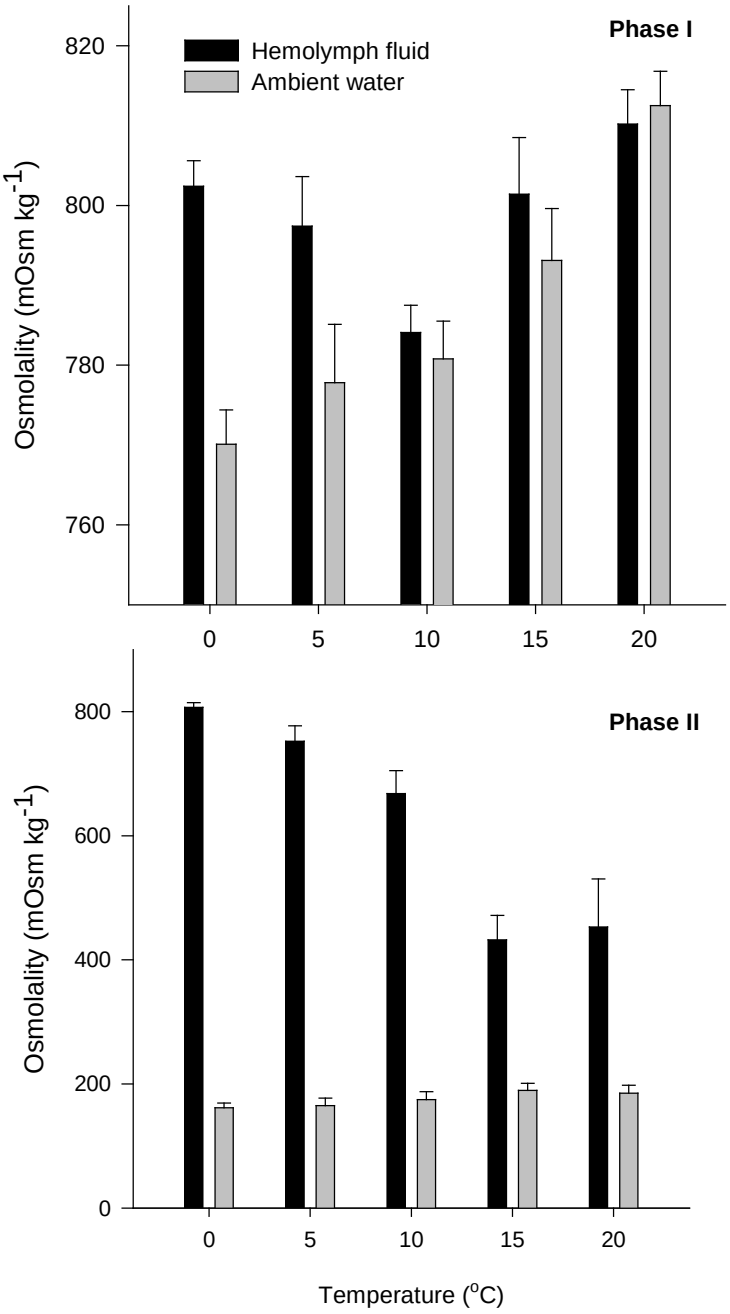

Figure 4.2 Hemolymph fluid and ambient water osmolality at different temperatures during Phase I (salinity 27‰ [810mOsmo kg^{-1}]) and Phase II (salinity 6‰ [180mOsm kg^{-1}]). Values are expressed as mean ± SE, n = 16 (Phase I); n = 8 (Phase II).

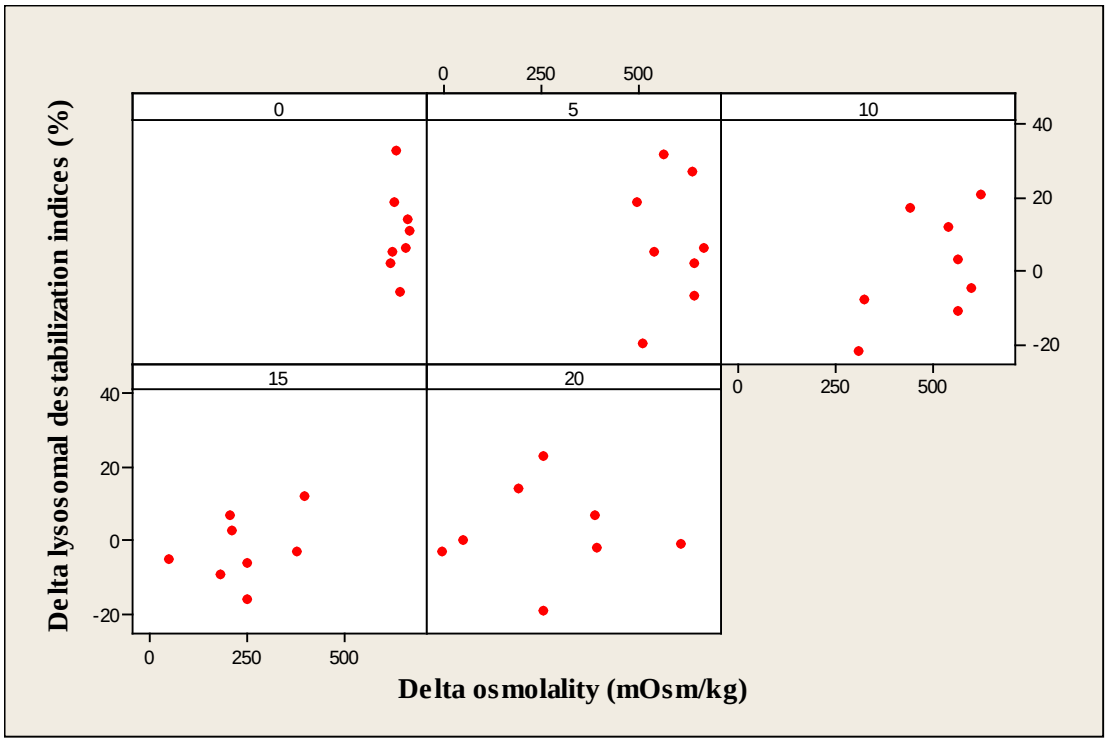

Figure 4.3 Scatterplot panels showing Δ lysosomal destabilization index (%) of individual oysters (n = 8) in relation to Δ osmolality (mOsm kg^{-1}) at each temperature during Phase II (spring freshet (6‰ salinity [180mOsm kg^{-1}]).

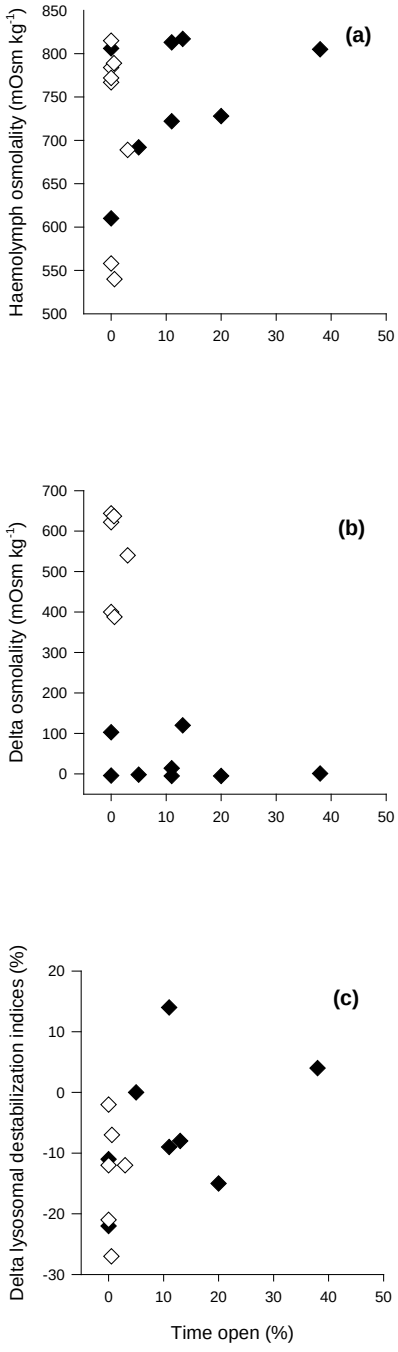

Figure 4.4 Scatterplots of gaping activity (% time valves open) of individual oysters in relation to (a) hemolymph osmolality (mOsm kg^{-1}), (b) Δ osmolality (mOsm kg^{-1}), (c) Δ lysosomal destabilization index (%) during Phase II (◊ spring freshet (6‰ salinity [180mOsm kg^{-1}]); ♦ control (27‰ salinity [810 mOsm kg^{-1}])).

Table 4.1 Gaping activity (% time open) of oysters held at different temperatures during Phase I (27‰ [810mOsmo kg^{-1}]).

		Time valves open (%)	
Variable	**n**	**Mean ± SE**	**Min, Max**
Temperature (°C)			
0	**4**	**0.0 ± 0.0**	**0.0, 0.0**
5	**4**	**5.4 ± 3.8**	**0.0, 16.0**
10	**4**	**26.3 ± 6.7**	**7.6, 36.9**
15	**2**	**24.5 ± 6.2**	**18.3, 30.7**
20	**3**	**39.5 ± 6.1**	**32.1, 51.6**

Table 4.2 Gaping activity (% time vales open) of oysters held at different temperatures during Phase II (6‰ [180mOsm kg^{-1}]).

	Time valves open (%) (n)	
Variable	**Treatment**	**Control**
	6‰ (180 mOsm kg^{-1})	**27‰ (810 mOsm kg^{-1})**
Temperature (°C)		
0	**0.0 (2)**	**6.7 (2)**
5	**0.2 (2)**	**11.4 (1)**
10	**1.7 (2)**	**2.7 (2)**
15	**0.0 (1)**	**11.1 (1)**
20	**0.6 (1)**	**29 (2)**

Table 4.3 Mean (SE) ambient and hemolymph fluid osmolality and osmolality differential of oysters held at each temperature[1] during Phase II (6‰ [180mOsmo kg^{-1}]).

Temperature (°C)	Osmolality(mOsm kg^{-1})					
	Treatment (6‰ [180 mOsm kg^{-1}])			Control (27‰ [810 mOsmo kg^{-1}])		
	Ambient	Hemolymph	ΔOsmolality	Ambient	Hemolymph	ΔOsmolality
0	161 (8)	806 (8)	645	762 (18)	822 (4)	60
5	165 (12)	752 (25)	587	760 (24)	762 (27)	2
10	175 (13)	668 (37)	493	746 (28)	749 (30)	3
15	190 (11)	432 (39)	242	765 (32)	768 (32)	3
20	185 (13)	453 (78)	268	803 (19)	802 (22)	1

[1]Sample size was 8 oysters in each category, except 20ºC (6‰), 7 oysters.

Table 4.4 Estimates from final statistical model for hemolymph fluid osmolality (mOsmo kg^{-1}) from oysters held at different temperatures during Phase II (6‰ [180mOsm kg^{-1}]).

		Hemolymph Fluid Osmolality(mOsm kg^{-1})		
Variable	**n**	**Md**	**CI**	**P-value**
Temperature (°C)				**<0.001[1]**
0	**8**	**809**	**768, 838[A]**	
5	**8**	**765**	**699, 807[AB]**	
10	**8**	**686**	**658, 711[B]**	
15	**8**	**443**	**382, 496 [C]**	
20	**7**	**421**	**353, 481[C]**	

Md, Median; CI, 95% confidence interval
Values not sharing a common superscript differ significantly ($P < 0.05$) based on pairwise comparison tests with Bonferroni correction.

[1]P-value for temperature

Table 4.5 Estimates from final statistical models for delta lysosomal destabilization indices (ΔLDI) from oysters held at different temperatures during Phase I (salinity 27‰ [810mOsmo kg^{-1}]) and Phase II (salinity 6‰ [180mOsm kg^{-1}]).

		ΔLDI			
Variables	**n**	**LSM**	**SE**	**CI**	**P-value**
Phase I (Before freshet)					
Temperature (°C)					**0.372[1]**
0	**15**	**0.2**	**3.3**	**-6.6, 6.8**	
5	**16**	**-0.4**	**3.2**	**-6.8, 5.9**	
10	**15**	**8.1**	**3.3**	**1.5, 14.7**	
15	**15**	**1.4**	**3.3**	**-5.2, 8.0**	
20	**16**	**1.3**	**3.2**	**-5.1, 7.7**	
Phase II (During freshet)					
Temperature (°C)					**0.129[1]**
0	**16**	**10.3**	**2.7**	**4.8, 15.7[A]**	
5	**14**	**5.8**	**2.9**	**0.0, 11.7[A]**	
10	**15**	**2.0**	**2.8**	**-3.6, 7.7 [B]**	
15	**15**	**1.2**	**2.8**	**-4.4, 6.9[B]**	
20	**15**	**2.4**	**2.8**	**-3.2, 8.1[B]**	
Day					**0.029[2]**
1	**10**	**12.4**	**3.4**		
2	**6**	**-3.0**	**4.4**		
3	**10**	**1.9**	**3.4**		
4	**9**	**-2.0**	**3.6**		
5	**10**	**0.5**	**3.4**		
6	**10**	**7.1**	**3.4**		
7	**10**	**9.6**	**3.4**		
8	**10**	**7.9**	**3.4**		

LSM, Least squares means; SE, Standard error; CI, 95% confidence interval. Values not sharing a common superscript differ significantly ($P < 0.05$) based on planned contrast.

[1]P-value for temperature

[2]P-value for day

Chapter 5 SUMMARY AND GENERAL CONCLUSION

5.1 Background

The introduction of suspended culture methods and the development of a market for cocktail (65–75 mm) oysters significantly shortened the production cycle of oyster culture operations in Atlantic Canada. Moving forward, the industry is poised to benefit from a high rate of return on investment for long-term sustainability. Recently, however, the industry has been threatened by an MSX outbreak, which has resulted in upwards of 90% mortality in wild and cultured oyster stocks. To date, this outbreak has been limited to the Bras d'Or Lakes region (Stephenson *et al*. 2003). Nevertheless, the possibility of the MSX parasite spreading into NB or PEI waters and decimating oyster stocks is a real concern for the industry. The industry has not experienced massive mortality of oysters since Malpèque disease in the 1930-50s (Frazer 1938; Needler & Logie 1947). It took over 20 years for oysters to develop resistance to Malpèque disease, and the stocks have never fully recovered (Logie *et al.* 1961).

The industry is also vulnerable to other diseases that have wreaked havoc on the northeastern US seaboard, namely Dermo (*P. marinus*) and juvenile oyster disease (Boettcher *et al.* 2006; Ford 1996; Ford & Smolowitz 2007). As MSX, both of these diseases are usually expressed in oysters reared in salinities above ~18‰ (Boettcher et al. 2006; Bushek *et al.* 2012; Ford 1985). The industry is also facing an

increasing number of aquatic invasive species (AIS), some of which prey heavily on young oysters. The research presented here relates to mitigation strategies that the industry can potentially incorporate into the production cycle of their field operations to reduce the impact of diseases and AIS on their cultured stocks. This research also relates to the application of a stress response biomarker, the neutral red retention assay, to assess whether mitigation strategies are worth pursuing.

Long-term sustainability of the oyster aquaculture industry in Atlantic Canada will depend on its ability to mitigate the impact of disease infections and predation on cultured stocks. A proactive approach, that has proven to be both economically and biologically feasible for oyster operations elsewhere in North America, is the transfer of oysters upriver into lower salinity water. As part of my thesis, the benefits of incorporating an upriver site in the production cycle of an operation were investigated.

5.2 Main contributions to literature

During the study period (May-October), ambient salinity at the upriver site varied between 3–20‰ (~15‰), while it was more stable at the downriver site 20–28‰ (~26‰). Tidal influence at both sites explained ~13–14% of the observed salinity variation. The lowest observed salinity (3–4‰) in mid-October coincided with freshwater discharge (from high precipitation). Based on the productivity data, seed (~26 mm SH) transferred upriver grew and survived as well as those that remained downriver, while mortality rates of adults (~65 mm SH) were lower

upriver. Holding adult oysters upriver promoted shell growth whereas meat content was unaffected. In contrast, the transfer of adult oysters downriver resulted in a loss of meat content. No distinction was made between somatic and gonadic tissues; therefore it is difficult to discern whether these oysters would have been faced with an 'unplanned' spawning event after the transfer downriver that might explain observed loss in meat content.

For a more in-depth and thorough evaluation and to further our understanding, physiological effects of the transfers on adult oysters were also investigated. In this aspect of the field study, stress response of adult oysters transferred between upriver and downriver sites was assessed 80 and 150 days post-transfer. Stress measured using NRA revealed that regardless of their origin (upriver, downriver), adult oysters maintained upriver showed high levels of lysosomal destabilization and pronounced tissue atrophy (digestive tubules). By contrast, the transfer downriver had the opposite effect, i.e. low lysosomal destabilization and low tubule atrophy. One would have expected the opposite given that higher shell growth was observed in adult oysters upriver than downriver. Even for oysters transferred downriver, the loss in meat content observed should have been a good indicator of epithelial atrophy. Evidently other mechanisms were at play. Moreover, there may be an integrated response with shell growth, in comparison to a more punctual response with lysosomal destabilization and tubule atrophy. Nevertheless, the results of the field study strongly suggest that there exists no association between productivity parameters and the level of stress response measured by the biomarkers. This is

important information for growers, as decisions are often based on productivity parameters, such as growth and mortality, not physiological indices.

The field investigation prompted us to further explore the use of the assay to measure stress response in the oysters. The NRA is a highly sensitive biomarker of general stress, based on lysosomal destabilization; however seasonal changes in physiological state of animals and environmental conditions are known to interfere with lysosomal stability. Specifically, the prescribed saline solution for the assay assumes animals to be isosmotic to surrounding water. However, results from our field study seem to indicate that oysters were restricting exchanges with the environment. We know that oysters occupy a wide latitudinal range with extremes in temperature and salinities (-1–49ºC; 3–30‰). In the southern Gulf of St. Lawrence for instance, water temperature can reach sub-zero values in the upper reaches of its river systems in the winter. Saline water is virtually nonexistent during spring freshets and autumn floods. Under such extreme conditions, oysters are known to close their valves and limit the exchange with the ambient environment (Comeau *et al.* 2012; Comeau 2014). For instance, oysters sampled during our October sampling period were hyperosmotic to ambient seawater. Since hypo- (or hyper) osmotic conditions cause cells to swell (or shrink) in response to osmotic influx (or outflux) of water, thus exerting an osmotic stress on cells, using the prescribed saline solution that is isomotic to surrounding seawater, and not hemolymph cells, could invariably cause cellular damage (Strange 2004).

A study was undertaken to explore the validity of the above premise relating to osmotic conditions and the stress response assay. Specifically, the impact of hemolymph fluid osmolality on the outcome of the assay was investigated under laboratory conditions. Hemolymph osmolality was experimentally manipulated in adult oysters by subjecting them to different temperature (0, 5, 10, 15, 20ºC) and salinity (6, 27‰) regimes. At a constant salinity (27‰), result showed that oysters remain isosmotic to ambient waters at elevated water temperatures (10–20ºC), while hyperosmotic in low water temperatures (0–5ºC). When mimicking spring freshet conditions (6‰), hyperosmotic conditions were observed for oyster hemolymph in all temperature groups and were inversely proportional to temperature. Moreover, a close monitoring of valve activity confirmed a restricted exchange between hemolymph fluid and ambient water when oysters are exposed to 6‰ salinity (e.g. spring freshet) at all temperatures (i.e. 20ºC), when oysters would normally be active. During a low salinity event, it seems that an osmotic imbalance occurs as a result of oysters closing their valves and trying to uphold hemolymph osmolality. This behavior may be interpreted as a robust avoidance strategy to extreme environmental conditions. With these laboratory results, it became clear that the novel version of the NRA was more suitable for bivalves living at their northern distribution limit under low salinity conditions. We recommend using filtered hemolymph fluid (modified NRA), instead of the prescribed standard solution (standard NRA) adjusted to ambient seawater.

5.3 Recommendations to industry

Considering the limited scope of the field study, it would be important to determine the effects of transfers between other areas, under different environmental conditions (spring, summer, fall) prior to putting forward industry-wide recommendations. At this time, the following mitigation strategies are being proposed for Richibucto area, with the potential for application to other areas. Since no effect on oyster productivity was observed in seed oysters, spring transfers upriver are recommended. It is not detrimental to their health, when carried out after the spring freshet, likewise for adult oysters. Spring freshet might be problematic if transfers upriver are carried out in early spring because of freshwater discharge. In terms of benefits, we have not observed differences in survival of caged oysters between sites, although it is known that oysters are most vulnerable to predators at a young age. In the presence of an increasing number of predators (e.g. green crab) lurking downriver, an upriver nursery would provide additional protection to seed stocks (Gillson 2011). Seed oysters could potentially benefit from reduced exposure to infectious diseases, particularly juvenile oyster disease.

For adult oysters, by the end of the study shell growth and meat yield were greater in oysters transferred upriver compared to oysters transferred downriver. In fact, the observed low meat yields in oysters transferred downriver confirmed anecdotal evidence from fishers of the spring relay fisheries (Paul Cormier [NB Aquaculture, Fisheries and Agriculture], personal communication, July 23, 2014). Oyster transfers downriver in the spring results in reduced meat yields in the fall and

therefore must be avoided, as it is detrimental to stocks prior to the winter period. Transferring oysters to a depuration facility for cleansing, instead of downriver may warrant further investigation. However, high lysosomal membrane destabilization and epithelial atrophy observed in adult oysters upriver are indications of a diminished health condition. The benefit of increased productivity (shell growth, mortality) may outweigh the cost of reduced immune response (lysosomal destabilization, epithelial atrophy), particularly since predators and pests are less of a threat upriver. There are still many questions that need to be answered prior to making recommendations to growers in regards to transfers of adult oysters. This study did not address fall transfers of seed or adult oysters. A downriver transfer in the fall would avoid exposing oysters to low salinity events upriver during autumn flood waters, as well freshets the following spring. It would also protect gear from ice jams upriver. Based on the production cycle of an operation, it would therefore make sense for a grower to transfer oysters upriver in the spring and then to return them downriver in the fall. The grower would benefit from lower mortality and increased shell growth. Further work would be required to determine whether there would be benefits from increased somatic growth (from low reproductive effort). Growers would also benefit from fewer predators preying on oysters, in addition to less handling during the growing season, e.g. biofouling (hot-water immersion), thereby avoiding additional stress on their stocks. However, based on the reduced meat yields observed in adult oysters in spring transfers, prior to putting forward a recommendation to growers, an evaluation would need to be carried out for fall transfers.

5.4 Future research

There is still a lot of research required when applying mitigation measures. Prior to making recommendations to the industry, similar work needs to be carried at numerous other upriver and downriver sites, with varying environmental conditions and pathogenicity. In addition, parasites responsible for MSX disease, *H. nelsoni*, and Dermo, *P. marinus*, usually present in warmer months (May-Oct) are showing increased resistance to low salinity conditions. A study to document and correlate tissue growth with gametogenesis upriver is also recommended. We only hypothesized that oysters do not spawn or partially spawn upriver. Finally, previous work has demonstrated that lysosomal membrane destabilization is linearly and negatively correlated with scope for growth (Widdows *et al.* 1982), yet we found no association between lysosomal destabilization and shell or tissue growth productivity in our field study. It is possible that our field sampling occurred only during periods of high stress, which would help explain the observed growth.

Results from the laboratory study generated some important findings on the combined effect of temperature and salinity on NRA outcome. For measuring lysosomal membrane destabilization in oysters living in cold waters, the use of NRA_{MOD} (using filtered mantle fluid) is presented as an alternative to the standard NRA (using prescribed saline solution). The primary aim of the NRA biomarker is to measure stress-related responses in bivalves; however, an overestimation of the stress response can potentially occur if investigators do not fully understand the conditions

that influence bivalve behavior (valve activity). In situations where bivalves close their valves and restrict exchange with ambient waters, the prescribed saline solution provides artificially high stress response measurements. More specifically, since oysters close their valves and isolate their soft tissues from unfavorable conditions (e.g. spring freshet), an osmolality differential between internal fluids and ambient waters is possible. If the NRA is to be dependable for researchers and meaningful for growers, it is critical that it be adapted to the animal being tested, and not the surrounding environment. Moreover, the behavior of oysters, exposed to such conditions, needs to be thoroughly investigated in order to further our understanding of the stress response measured.

Future research must focus attention on environmental conditions (including anthropogenic) that could influence a behavioral response in the bivalve. Additional consideration is also warranted when using the NRA in bivalves at the geographical limit of their natural distribution. Another area requiring attention is the variability (between day effect) of the assay observed in the laboratory study. The reasons for this variability remain unknown. It certainly speaks to the sensitivity of the assay which is important to measure individual differences, however not the effect of extraneous factors. Although the laboratory study does not address all the conditions and circumstances that exist, it does warn investigators to use caution prior to carrying out the assay to ensure that the response measured is not confounded by unrelated extraneous factors.

Oyster growers are fully aware of the threats facing their industry. It is our responsibility as researchers to provide the most vital scientifically based information, in order that informed decisions can be made to address some of these threats. At this time, one of the most pressing issues is the need to be able to respond quickly to an episodic outbreak of MSX. Providing refuge to cultured oyster stocks from infectious diseases by transferring them upriver is biologically attainable.

Growers must continue to be alert to changes in their surroundings and in the health of their oyster socks. Enhanced collaboration between researchers in northeastern US and Canada is increasingly important for the long-term sustainability of the industry.

5.5 References

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