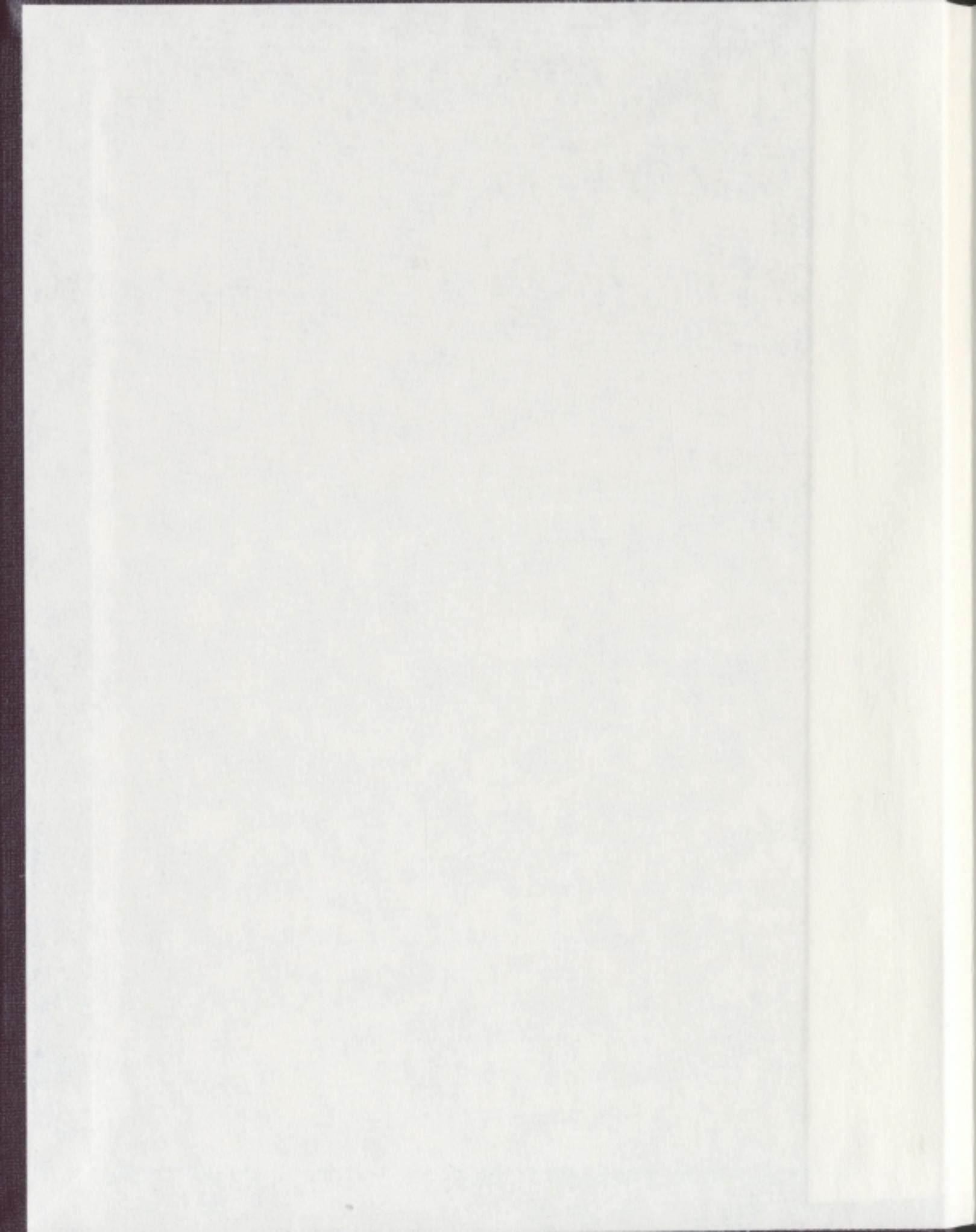


THE EFFECT OF EXTENDED HOLDING UNDER
AMBIENT WATER TEMPERATURES ON THE CONDITION,
PHYSIOLOGY AND STRESS RESPONSE OF CULTURED
BLUE MUSSELS (*MYTILUS EDULIS* L. 1758) FOLLOWING
COMMERCIAL HARVEST IN NORTHEASTERN NEWFOUNDLAND

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The effect of extended holding under ambient water temperatures on the condition,
physiology and stress response of cultured blue mussels (*Mytilus edulis* L. 1758)
following commercial harvest in northeastern Newfoundland

by

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ABSTRACT

Effects of extended holding under ambient water temperature on the physiology and stress response of cultured blue mussels (*Mytilus edulis*) in northeastern Newfoundland were examined over the course of four seasons. Changes in tissue weight, condition index, stress response, oxidative stress and immune gene expression were assessed over a period of two to three months in live-holding under ambient water temperatures. Animals in holding were compared to field control mussels collected from the original lease at each sample time. Condition and dry tissue weight decreased over time in holding during the summer, fall, and spring seasons; however the effect was only significant after one month. The summer and fall represented the most stressful seasons to maintain animals in extended live holding under ambient water temperatures as indicated by the significant decline in condition and increase in stress response as indicated by the neutral red assay. In the field, condition index and tissue weight varied seasonally and was lowest during winter peaking to a high during the spring (April). Seasonality of antioxidant and immune genes were noted however there was no consistent increased expression due to holding. Only in the spring did extended holding cause any change to digestive gland structure.

We recommend mussels be maintained in extended holding for no longer than a period of one month to reduce the impact on their physiology and an overall loss in quality. After this point there is a significant increase in stress response coupled with a decline in condition and meat weight which could result in a loss of profit for the grower.

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LIST OF ABBREVIATIONS

AMP – antimicrobial peptide
ANOVA – analysis of variance
BC – basophil cell
CAT – catalase
DC – digestive cell
DO – degenerating oocyte
GST – glutathione transferase
GSH-px – glutathione peroxidase
HE - hemocyte
MGD – *Mytilus galloprovincialis* defensin like peptide
MT – mixed tissue
NRR – neutral red retention
OV – oocyte
QPCR – quantitative polymerase chain reaction
ROS – reactive oxygen species
RT-PCR – reverse transcriptase polymerase chain reaction
SOD – superoxide dismutase
SPG – spermatagonia
SPZ - spermatozoa

CHAPTER 1: INTRODUCTION

Aquaculture can be defined as the farming and husbandry of aquatic animals and plants (Boghen 1995). The exact origins of aquaculture remain uncertain however evidence suggests the practice dates back to Chinese civilizations some four thousand years ago and that the culture of tilapia has been practiced in ancient Egypt since around 2500 B.C (Boghen 1995). Aquaculture had become well-established in Europe in the 1850s and the first documented study of aquaculture in Canada dates to 1857 (Boghen 1995). The Canadian industry grew rapidly, and is a major economic force with many finfish and shellfish species being cultivated nationally.

Various types of aquaculture systems exist and can be implemented in inland, coastal, freshwater or marine environments. The Asia-Pacific region accounts for nearly 89% of global production (in quantity) with Norway and Chile being other major producers (FAO 2005-2012). Globally a vast number of both finfish and shellfish species are cultured, and the number of species cultivated grows annually. Depending on geographic region, some of the major finfish species include carp, minnows, and salmonids with some of the major shellfish species including clams, oysters, and mussels (FAO 2005-2012).

1.1 GLOBAL MUSSEL CULTURE

Mussel farming is believed to have originated in 11th century France and today there are approximately nine different commercially important mussel species fished and farmed worldwide (Gosling 2003). In the Northern Hemisphere there are three main species of blue mussels (*Mytilus* spp.) cultured. Of these, *Mytilus edulis* has the most extensive geographic distribution, ranging from subtropical to Arctic regions. Other *Mytilus* spp. have a more restricted distribution; for example *Mytilus trossulus* is restricted to cool temperate waters, while the European mussel, *Mytilus galloprovincialis*, is generally limited to the warmer Mediterranean region and the west coast of Spain; however this species is also cultured in Pacific regions of North America including British Columbia. Culture in the Southern Hemisphere is dominated by three species of green mussel belonging to the genus *Perna*. These species typically have a subtropical to tropical distribution pattern (Gosling 2003). Of these three species, *Perna viridis* has the most widespread distribution ranging from India to Southeast Asia (Gosling 2003).

In general, bivalves live within a water temperature range of -3 °C to 44 °C. The tolerable temperature range is species specific and affects their global and local spatial distribution as well as many aspects of their physiology such as reproduction, feeding, growth, respiration, and immune function (Gosling 2003). The ability of different *Mytilus* spp. to cope with a wide range of temperatures and salinities is the main factor determining their global distribution patterns (Gosling 2003). Mussels generally inhabit rocky intertidal zones that can impose stressful conditions including high temperatures and extended periods of desiccation. As a result mussels possess coping mechanisms to

withstand these stressors, and thus can physiologically tolerate extreme fluctuations in temperature, salinity, and dissolved oxygen.

Their wide geographic distribution and relative abundance have made mussels important species for fisheries worldwide and their relative ease of culture have made them important aquaculture species as well. Chile and New Zealand are the major exporters of fresh-farmed mussel product. Several culture methods exist depending on the region (FAO 2006). Spain is the second largest producer in the world and the industry is mainly based on the Atlantic coast in the province of Galicia. The most common culture method in this region is the Spanish-raft system where mussels are grown suspended from a framework of eucalyptus wood (bateas) (Gosling 2003). The culture method used in France is an on-bottom culture technique known as the Bouchot method. In this technique lines of wooden poles are embedded in the intertidal seabed around which mussel seed is wrapped in mesh socks (FAO 2006). In Norway and North America the main culture system is an off-bottom system where mesh socks of mussel seed are suspended from a long-line and floatation system. This system contains a backline (usually 80 m to 100 m) that is anchored on both ends and supported by buoys, where mesh socks are suspended from the backline (Mallet & Myrand 1995). Mesh socks can be suspended in lines or through a continuous method marking an S-shape in the water column.

1.2 NEWFOUNDLAND MUSSEL AQUACULTURE

The mussel industry in Atlantic Canada can be traced back to the pickling and canning of wild mussels during the First World War (Mallet & Myrand 1995). Newfoundland was the most active region with three canneries, however due to competition with other industries, the canning industry closed. By the 1970s the mussel culture industry was becoming established and profitable as a result of growing markets, abundant seed supply, and favorable conditions leading to the growth and expansion of the industry in Atlantic Canada (Mallet & Myrand 1995).

In Newfoundland, Canada, *M. edulis* is the primary mussel species cultured although *M. trossulus* and *edulis-trossulus* hybrids do occur. Leases are distributed along the northeast and south coasts of the island. Production has increased steadily from 1,452 tonnes in 2001 to an estimated 2,461 tonnes in 2010 (Statistics Canada 2010) and the industry is continuing to expand. Mussels are cultured using the long-line suspended culture system and generally reach market size (55 mm to 60 mm) 20 months from socking. Harvesting occurs year-round depending on meat quality and yield, which varies seasonally (Mallet *et al.* 1990). Depending on local ice cover mussels may be harvested through the ice during winter months.

Newfoundland mussels are shipped to local, national, and international markets; unfortunately shipping and transportation of fresh product may occasionally be delayed as a result of unforeseen transportation issues (e.g. ferry closures) or weather conditions. As a result mussels may spend extended time in holding facilities under ambient conditions following harvest and before processing. It is a common occurrence for mussels to be held in a facility for up to one week before processing and transportation however in

some more extreme cases they could spend up to one month in extended live holding. Holding facilities in Newfoundland store live unprocessed mussels in holding tubs with water being pumped directly from the surrounding area. Most facilities use ambient water temperatures with no external control over water or air temperatures in the facility.

Little is known regarding how extended holding could potentially affect mussel physiology and stress response and how this may affect mussel quality or condition and meat yield. Determining the optimum time allowed in holding before there is a significant decrease in meat yield and shelf life is important to the industry.

1.3 SEASONAL VARIATION IN GROWTH AND CONDITION

Variations in condition, growth, and meat weight of *M. edulis* have been extensively studied and are generally influenced by seasonal changes in water temperature and nutrient levels. There is also a strong link between condition index and seasonal storage of metabolic reserves, reproduction, and utilization of reserves (Gabbott & Bayne 1973; Okumu & Stirling 1998; Orban *et al.* 2002). Condition is generally measured as a ratio of tissue weight (wet or dry) to shell weight or shell volume (Lucas & Beninger 1985). Generally growth and condition are highest during late spring and early summer, a period just before spawning and when nutrient levels are high and water temperatures are favourable. Dare & Edwards (1975) found tissue weight to be highest during the summer and fall, decreasing throughout winter to a low during the spring (post-spawning) in a European population of the blue mussel *M. edulis*. Condition and dry tissue weight have been shown to decrease during the winter months, a time of decreased primary production and in animals recovering from a recent spawning event (Dare & Edwards 1975). During

this time there is also a decline in carbohydrate, lipid, and protein reserves (Dare & Edwards 1975). Since the physiological condition of mussels depends greatly on their surrounding environment, there is great spatial and temporal variation and trends should thus be studied in the specific environment where culture takes place.

1.4 STRESS RESPONSE AND CONDITION

Stress has been defined as a measureable alteration of a physiological steady-state induced by environmental change causing the individual to become more susceptible to further environmental change (Bayne 1976). Exogenous factors including temperature and nutritive stress can act as stressors negatively affecting mussel condition (Bayne 1973; Bayne & Thompson 1970). Bayne (1973) analyzed the effects of temperature and nutritive stress on the physiology of the blue mussel *M. edulis* and found that changes affected oxygen consumption and ammonia excretion resulting in a decline in body condition.

Processing, handling, and storage conditions can also cause an increased stress response in bivalves including blue mussels (Harding *et al.* 2004; Tremblett 2001). Processing activities, especially mechanical stresses and post-harvest storage conditions (on ice, dry air, ambient water), can affect the physiology of cultured shellfish. Zhang & Li (2006) found that extended storage (up to 42 days) of the oyster *Crassostrea gigas* resulted in an increased stress response in both starved and fed animals, although to a lesser degree in the latter. They also found that simulated mechanical grading of oysters for as little as three minutes significantly increased the stress response in both starved and fed animals.

Spawning requires a large amount of energy and in bivalves results in a loss of body weight coupled with a decline in condition that can lead to an increased stress response and mortality. Cho & Jeong (2005) found a significant decline in neutral red retention in spawned oysters compared to unspawned indicating an increase in stress response as marked by lysosomal membrane instability. They also found a decrease in circulating hemocytes in the spawned animals that could potentially lead to a deficiency in the immune response. The degree to which stress affects the condition of the animal depends on the stressor and the physiological state of the animal at that time. Generally stressors result in a decline in condition as animals divert energy and reserves into coping mechanisms.

1.5 DIGESTIVE GLAND STRUCTURE AND FUNCTION

The digestive gland in mussels is a brown to black organ that consists of a series of blind-ending ducts and tubules that connect to the stomach via a series of primary and secondary ducts (Gosling 2003). The epithelium of digestive tubules is comprised of two main cell types, basophil cells (secretory function) and digestive cells (Gosling 2003). Material is absorbed through the digestive cells by pinocytosis and is digested in vacuoles within the cells after which nutrients are released directly into the body cavity (Gosling 2003). The digestive gland is also an important storage organ of metabolite reserves particularly under stressful conditions or during gametogenesis when reserves might be low (Bayne *et al.* 1976). Changes in digestive gland cellular structure can be observed as a result of contaminants or starvation. In starved mussels the cytoplasm of the digestive cell has a reduced volume and density, basophil cells appear denser, and digestive cell

structure breaks down (Thompson *et al.* 1974). In response to stress the tubules appear thinner as the epithelia break down; there is also a visible enlargement of secondary lysosomes and an accumulation of lipids within the cells (Gosling 2003).

1.6 BIVALVE ANTIOXIDANT ENZYMES

Reactive oxygen species (ROS) are partially reduced oxygen species that are a natural product of cellular respiration. A state of oxidative stress occurs when antioxidant defenses are overwhelmed by pro-oxidant species that interfere with normal cellular function causing lipid, enzyme, or DNA peroxidation possibly leading to tissue damage and cell death (Abele & Pantarulo 2004; Winston & Di Giulio 1991). The most common ROS include the superoxide anion ($O_2^{\cdot-}$), the hydroxyl radical ($OH\cdot$), and hydrogen peroxide (H_2O_2) (Abele & Pantarulo 2004). The hydroxyl radical can be produced either during thermal reactions or by ionizing with a metal such as iron. As one of the most harmful oxygen radicals it can potentially react with most large macromolecules and cause enzyme inactivation or lipid peroxidation (Manduzio *et al.* 2005). Hydrogen peroxide can pass freely through cellular membranes therefore intracellular concentrations may be quite low. However, if the molecule is not broken down into less reactive intermediaries it can be readily converted to the extremely reactive hydroxyl radical (Manduzio *et al.* 2005).

In general there are two main categories of defense against oxygen radicals: (1) antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-px), and glutathione-s-transferase (GST), and (2) antioxidant compounds including glutathione, and vitamin C (Manduzio *et al.* 2005). Antioxidant

enzymes and compounds function to scavenge potential free radicals and neutralize their potential harmful effects. In general each enzyme has a particular function. The enzyme SOD reduces uncharged superoxide anions to the less reactive hydrogen peroxide molecule. Glutathione peroxidase (GSH-px) functions to reduce H₂O₂ and its activities can be divided into two categories, selenium dependent and selenium independent. Glutathione s-transferase (GST) catalyzes selenium independent GSH-px activity (Manduzio *et al.* 2005). In mussels these enzymes are expressed mainly in the gill and digestive gland and show seasonal variation in response to changes in temperature, food availability and reproductive status (Power & Sheehan 1996).

1.7 SEASONAL VARIATIONS IN BIVALVE ANTIOXIDANT DEFENSES

The gill and digestive gland are the major sites of oxidative stress (Power & Sheehan 1996; Variengo *et al.* 1991). The gill tissue is the main feeding organ in most bivalves as well as one of the first internal surfaces to encounter the external environment; as such it is the first major organ to be exposed to environmental stressors and contaminants. As with many aspects of physiology, the levels of antioxidant enzymes follow seasonal cycles that are governed mainly by changes in temperature and nutrient levels. Several studies have examined the seasonal variation of antioxidant compounds in both the gill tissue and digestive gland of mussels. Power & Sheehan (1996) found seasonal variation in antioxidant enzymes such as GST, SOD and GSH-px in both the digestive gland and gill tissue of the blue mussel *M. edulis*. They found enzyme levels of GST to be at higher concentrations in the gill compared to digestive gland with elevated levels were during the winter months. Levels of the GSH-px enzyme were found to be generally lower in

both the gill and digestive gland during late winter to early spring (Power & Sheehan 1996; Viarengo *et al.* 1991). Enzyme levels of SOD are lowest throughout the fall and early winter in the digestive gland of the blue mussel (Viarengo *et al.* 1991). Antioxidant enzyme levels generally appear to be at their lowest levels during the winter likely as a result of a shift in metabolic requirements.

Pollutants and xenobiotics are also capable of producing reactive oxygen species and increase oxidative stress in bivalves (Sheehan & Power 1999). Previous studies have shown a positive correlation between levels of antioxidant defences and xenobiotics such as polychlorinated biphenyls, polycyclic aromatic carbons, phenols, and heavy metals (Sheehan & Power 1999). Levels of xenobiotics also vary seasonally thus complicating the interpretation the effect of seasonality on antioxidant defences. Studies have also shown increase in antioxidant defences in intertidal populations of mussels likely due to prolonged air exposure and elevated temperatures (Letendre *et al.* 2009). While several studies assess the impact of processing, handling, and storage on the stress response of mussels (e.g. Harding *et al.* 2004) or the impact of seasonality on antioxidant enzymes (e.g. Power & Sheehan 1996) few studies assess the impact of such activities on expression patterns of antioxidant enzymes. Furthermore ROS can have toxic effects themselves and produce increased antimicrobial activity imposing another degree of stress on the animal (Anderson 2001).

1.8 MUSSEL INNATE IMMUNE DEFENSES

Bivalves, like other marine invertebrates, have only an innate immune response with both humoral and cellular components (Tincu & Taylor 2004). The cellular response is

mediated by hemocytes, motile cells in the hemolymph that are capable of non-self-recognition, movement throughout the body cavity, and the production of antimicrobial peptides (Mitta *et al.* 2000a; Tincu & Taylor 2004). Hemocytes are capable of migrating towards and attaching to the invading pathogens and destroying the pathogen via endocytosis.

Antimicrobial peptides (AMPs) are important anti-infection compounds that are stored in hemocyte granules and released when activated by a bacterial challenge (Roch 2001). There are three main classes of AMPs found in mussels. First, the defensin peptides within the *Mytilus galloprovincialis* defensin like peptide (MGD) group consists of two isoforms, MGD1 and MGD2, similar to arthropod defensins but containing eight cysteine residues, that have been localized from *M. galloprovincialis* plasma and hemocytes (Mitta *et al.* 2000a). In mussels, defensins are active against Gram-positive bacteria. The second group, the mytilins, contain five isoforms, A, B, C, D, and G1. Mytilin A and B have been isolated from *M. edulis* plasma while B, C, D, and G1 have been isolated from *M. galloprovincialis* hemocytes. Isoforms B, C, and D show activity against both Gram-positive and Gram-negative bacteria whereas mytilin G1 is only active against Gram-positive bacteria. The third group of AMPs, myticin, consists of two isoforms, myticin A and B, both characterized from plasma and hemocytes of *M. galloprovincialis* (Mitta *et al.* 1999). The amphiphilic structure of these peptides forms pores in microbial membranes disrupting membrane integrity and causing microbial lysis (Tincu & Taylor 2004).

1.9 NEUTRAL RED RETENTION: THE STRESS TEST

Any response an animal has to a stimulus that causes a disruption of normal physiological function is a stress response. Changes to normal physiological function can be measured through a variety of methods. The neutral red retention assay is a useful indicator of animal health that assesses changes in lysosomal membrane stability over time as a measure of the stress the individual is experiencing. Several environmental factors including temperature and air exposure as well as processing practices including debyssing, grading, and post-harvest storage conditions can cause an increase in stress response and affect lysosome membrane stability (Harding *et al.* 2004).

These stressors break down lysosomal structure over time and eventually cause membrane damage and the degradation and leakage of intracellular compounds into the cytosol of the cell. Lysosomes have three means of response to stress: by increasing or decreasing the amount of various lysosome components, by changing rates of membrane fusion, or by altering membrane permeability (Zhang & Li 2006). Once the integrity of the lysosomal membrane has been compromised, the contents leak into the cytosol or lysosomes may become enlarged as a result of increased membrane fusion events (Harding *et al.* 2004). The neutral red assay is a relatively non-destructive method of evaluating the integrity of the lysosomal membrane and therefore measuring the degree of stress an animal is experiencing. If the animal is under no stress the lysosomes will retain the neutral red dye for extended periods of time however if there is an environmental or conditional stressor then the rate at which the dye leaks from the lysosomes is directly correlated to the amount of stress the animal is experiencing (Harding *et al.* 2004; Zhang & Li, 2006).

1.10 STUDY OBJECTIVES

To date there is little information on how extended holding under ambient conditions will affect the physiology and stress response of the blue mussel *M. edulis*. The purpose of this study was to identify changes in the condition of cultured blue mussels as a result of extended ambient storage assessed over four experimental seasons conducted from June 2010 to June 2011 (summer, fall, winter, and spring). Animals were held for a period of up to three months for each season and samples were taken periodically from both the holding facility as well as the original harvest site for a field control comparison. In order to better understand potential effects of extended wet-storage on the physiology of the blue mussel several parameters were studied over time in holding over four seasons.

The objectives of this study were:

1. To determine changes in mussel condition over time in holding over four seasons compared to field samples.
2. To assess the effect of extended holding on the stress response of the blue mussel through use of the Neutral Red Assay, compared to field samples.
3. To determine any effects of extended holding on oxidative stress and immune gene expression compared to field samples.
4. To recommend acceptable limits to extended wet-storage under ambient conditions

CHAPTER 2: THE EFFECT OF EXTENDED HOLDING UNDER AMBIENT TEMPERATURES ON THE CONDITION OF CULTURED BLUE MUSSELS, *MYTILUS EDULIS*

2.1 INTRODUCTION

The condition index is a useful measure of the health and viability of cultured mussels. Condition indices give two important pieces of information including (1) an economic index determining the yield and quality of the product and (2) an ecophysiological index that assesses the physiological activity of the animal, which varies under different environmental conditions (Lucas & Beninger 1985). There are several ways to calculate bivalve condition indices, one of the most useful and commonly used is the ratio of dry meat weight to dry shell weight (Orban *et al.* 2002). A low value indicates that a major physiological event has taken place such as spawning or a disease outbreak, or there has been extended maintenance under stressful environmental conditions such as prolonged exposure to increased temperatures or low nutrient availability (Lucas & Beninger 1985). The dry tissue weight to wet tissue weight ratio is a useful condition index in certain finfish species as a higher proportion of water in an individual indicates a state of depleted energy or starvation in certain finfish species (Lucas & Beninger 1985). The total live weight of the animal is not generally taken into account due to individual variation in water uptake, secretion rates, and amount of mantle fluid (Lucas & Beninger 1985).

Condition varies depending on environmental factors including fluctuations in water temperature, salinity, food availability, and reproductive status (e.g. Dare & Edwards 1975; Lutz *et al.* 1980; Orban *et al.* 2002). Bivalve condition index and dry tissue weight have been shown to be at their highest in the late spring and early summer and at their lowest immediately following a spawning event, regardless of season. During the late summer months condition is typically lower as a result of spawning and/or elevated water temperatures (Mallet *et al.* 1990). Indeed, summer mortality has been a problem for growers where losses are generally thought to be the result of elevated water temperatures. Shell strength is also a function of growth and is also susceptible to changes in food availability and temperature (Nagarajan *et al.* 2006). One method to determine shell strength is to assess the shell crack force. Crack force is defined as the maximum amount of strength required to cause breakage to a live mussel shell (Penney *et al.* 2007). Since most mussel product is shipped from Newfoundland as live fresh product it is also important that mussels maintain their shell strength over time in holding to minimize breakage and losses during processing and to maintain superior shell appearance (Penney *et al.* 2007).

Reproduction is also influenced by extrinsic environmental factors, most importantly temperature and food supply. Knowledge of spawning and condition cycles is important for mussel farmers in order to understand the optimum harvest time and extended holding time limits. Spawning generally occurs under favorable environmental conditions when temperatures are warmer and food supply is abundant (Sastry 1975). Timing of gametogenesis is coordinated with changes in the environment and as a result variations exist between populations of the same species in different local environments

(Sastry 1975). The gametogenic cycle involves the growth of spermatocytes (male) and oocytes (female), followed by vitellogenesis in the female, ripening of gametes, and lastly spawning, which is then followed by a quiescent period (Bayne 1975). This cycle closely follows the storage and utilization patterns of carbohydrates, lipids, and proteins, which are stored in the connective tissue of the gonad (Bayne 1975). There can be one major or multiple smaller spawning events which typically occur during the summer months (Thompson 1984).

Newfoundland waters are characterized by cold water temperatures and low seston concentrations, both of which can affect energy reserves in bivalves and as a result the timing of spawning and gametogenesis (Thompson 1984). Historic studies show that gametogenesis in Newfoundland mussels begins in the spring (March) and spawning takes place in late July (Thompson 1984). Changing ocean climates and differences in environmental parameters between coastal bays have resulted in deviations from this annual cycle. Recently, on the northeast coast of the island spawning has been noted up to four times in one year (Terry Mills: personal communication). The gametogenic cycle also affects condition with particularly low condition and meat weight observed immediately following a spawning event (Duinker *et al.* 2008).

In addition to its role in food processing, the digestive gland also has an important role in the storage of energy reserves (Thompson *et al.* 1974). As with reproductive organs, the digestive cells in the epithelia of the digestive tubules are also sensitive to levels of environmental contaminants and nutrient supply (Auffret 1988; Bielefeld 1991; Lowe 1988; Thompson *et al.* 1974). Previous studies have shown seasonal variations in

the biochemical composition and structure of the digestive gland as well as differences in digestive gland structure between starved and fed individuals (Thompson *et al.* 1974).

Several studies have assessed the effects of different post-harvest storage conditions on mussel physiology; however, few studies have examined potential effects of extended holding under ambient conditions for prolonged periods of time (Harding *et al.* 2004). The present study was designed to examine effects of extended holding in an ambient holding facility on the condition of unprocessed, cultured blue mussels held for a period of up to three months over four seasons (summer, fall, winter, and spring). Changes in tissue weight and condition index were assessed over time in holding and compared to field control samples. Digestive gland structure was also examined over time to determine if extended holding caused atrophy or damage to digestive gland cellular structure compared to field controls. Lastly, since reproductive status has a major impact on the condition of bivalves the gonads were studied at the initial and final samples of each season to determine reproductive state.

2.2 MATERIALS AND METHODS

2.2.1 Study site, experimental setup, and sampling protocol

Mussels from the 2008 year-class were cultivated using the long-line system and were collected from Site 13 in Bulley's Cove, Newfoundland, Canada, and transported to a commercial processing facility in Pleasantview, Newfoundland (Fig. 2.1). Lines were suspended from the water surface to a depth of approximately 15 m.

At the beginning of each experiment unprocessed mussels were split into two 1,000 L holding tanks at a density of 0.362 kg/L with a continuous flow-through of water. Unfiltered water was pumped directly from the bay at a distance of 145 m from the facility and at a depth of approximately 13 m from the surface. Water temperatures were recorded daily. The effect of extended holding on mussel condition was assessed for up to three months over four experimental seasons from June 2010 to June 2011. For each season mussels were sampled at four time points from both the holding tank and the original grow-out site (field control). Sample times included: initially upon introduction into the processing facility; after 1 week and after 1 month in holding; a final sample after 3 months in holding.

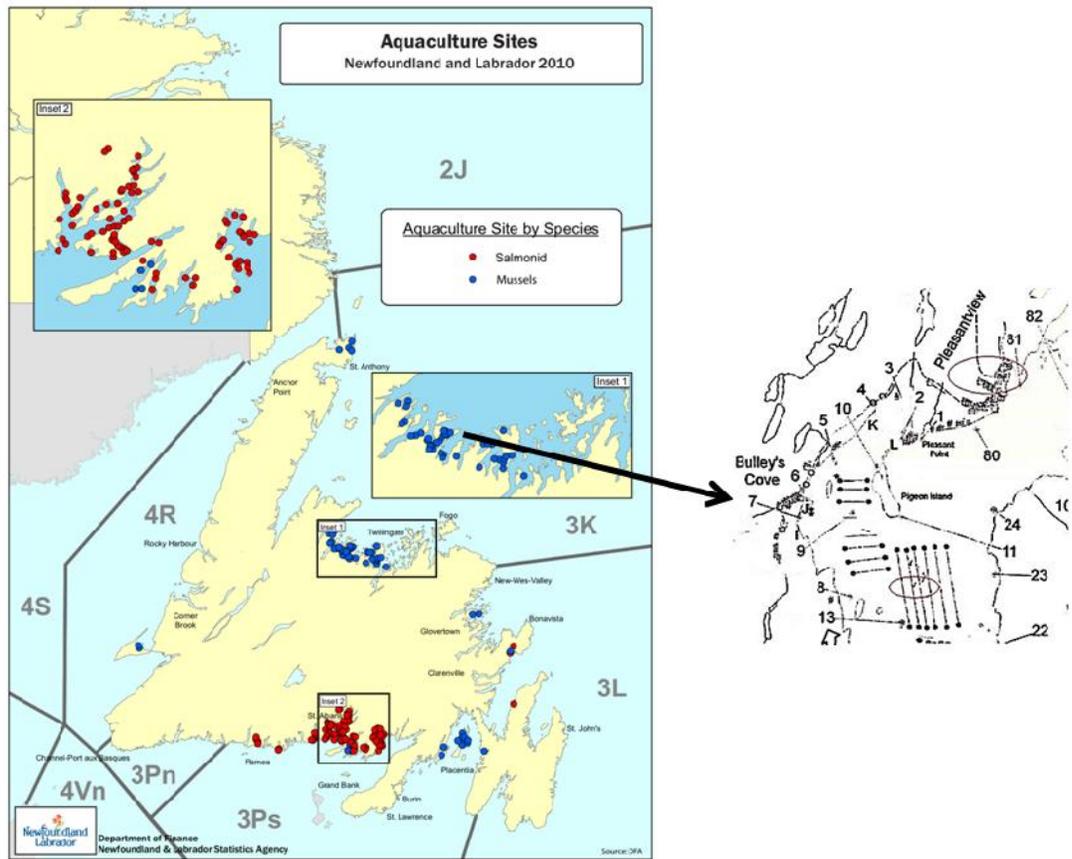


Figure 2.1: Map of Newfoundland, Canada, showing the mussel culture site located on the Northeast coast of the island in the Notre Dame Bay area. Circles indicate location of harvest lines in relation to location of processing plant in Pleasantview, Newfoundland and Labrador.

2.2.2 Morphometric analysis

At each sample time, 150 mussels were randomly sampled each from one of the holding tanks and simultaneously from the original grow-out site. Mussels were placed in coolers on ice and transported back to St. John's, Newfoundland and Labrador the next day, for measurement and analysis. For each individual the total live weight (g) was measured to the nearest 0.001 g (Ohaus Adventurer scale). Length (mm; maximum anterior-posterior axis), depth (mm; maximum dorso-ventral axis), and width (mm; maximum lateral axis) were measured using a digital caliper (MasterCraft) to the nearest 0.01 mm. To measure shell strength total shell crack force was measured using a crack force gauge (Dillon, AFG 2509N). Following crack force measurement the meat was carefully dissected away from the shell and placed in preweighed aluminum trays for dry weight analysis. Meats were dried to a constant weight at 80°C for 48 to 72 hrs (modified from Lutz *et al.* 1980). Shells were allowed to air dry for 48 to 72 hrs and their weight was measured to the nearest 0.001 g. Two condition indices were calculated (1) condition index and (2) dry tissue weight:wet tissue weight ratio:

$$(1) \frac{\text{dry meat weight}}{\text{dry shell weight}} \times 100$$

$$(2) \frac{\text{dry meat weight}}{\text{wet meat weight}} \times 100$$

2.2.4 Histology

At each sampling time, 12 mussels each from the holding tank and the field control site were fixed immediately in a solution of 5-10% buffered formalin. Of these, three mussels were used to determine stage of sexual reproduction and to observe any changes to

digestive gland structure. Formalin was decanted and replaced with a solution of 50% ethanol over a period of two days and finally replaced with 70% ethanol. Tissues were further dehydrated through an ethanol series (70% to 100%) and cleared with xylene (Leica TP1020 Tissue Processor). Tissues were then infiltrated with and embedded in paraffin wax (Leica EG1160 Paraffin Embedding Centre).

Whole mussels were sectioned (~ 7 µm) using a microtome (Leica RM2265 Rotary Microtome) and allowed to adhere to uncoated glass slides overnight prior to staining. Slides were deparaffinized and stained using hematoxylin and eosin (Leica Autostainer XL). The structure of the digestive tubules was observed in the initial and final samples of both the holding and field samples for each season. The gonadal tissue was also observed at the initial and final samples for each group to determine reproductive stage using a modified scheme from Gosling (2003) and Duinker *et al.* (2008).

Stage I: First stage following a spawning event, little gonadal tissue visible.

Stage II: Maturing. Gonads appear thicker and sex can be determined. Follicles are larger and packed tighter. Some oocytes are mature. Little connective tissue visible.

Stage III: Gonads larger still and follicles are packed together. Lumen packed with spermatozoa/fully grown oocytes. Visible yolk in oocytes, males have spermatids but majority immature sperm cells.

Stage IV: Gonad at maximum size. Testes are cream coloured and ovaries a deep orange colour. Male gonads have mature spermatozoa, females crowded with oocytes.

2.2.5 Statistical analysis

Data were analyzed using Sigmaplot 11.0 statistical and graphical software (Systat software). Data series were tested for normality and the mean (+/- standard error: SE), one-way ANOVA, and appropriate post hoc tests were calculated. Significance was set at $= 0.05$. Since the assumption of equivalence and normality were not met the Kruskal-Wallis One Way Analysis of Variance based on ranks was applied.

2.3 RESULTS

2.3.1 Sampling regime and water temperatures

Mean monthly water temperatures from June 2010 to June 2011 are shown in Figure 2.2. The summer holding experiment was carried out from June 2010 to August 2010. During this time ambient water temperature increased from an average of 8.6°C in June to a maximum of 18.2°C in August. Temperatures remained high throughout September 2010 with an average water temperature of 17.6°C. Due to high temperatures both of the experimental holding tanks suffered massive mortalities and crashed during the summer holding trial (mid-August) however a sample of 150 mussels was saved and frozen for morphometric analysis. The final field control mussels were collected at the scheduled three month sample time in September 2010.

The fall holding experiment was conducted from September 2010 to December 2010. Average water temperatures were approximately 13.6°C in September and decreased to 2.0°C in December. The winter experiment began in January of 2011 and continued until April at which time the spring experimental tanks were also set up.

During the winter, water temperatures remained stable between 0.5°C and 2.5°C, occasionally dropping below 0.0°C. Due to an error in the processing facility, after the initial experiment was set up the mussels in the experimental holding tanks were accidentally processed. Once this was noticed the workers in the facility went back to the original site (the next day) and collected another subsample of mussels from the same line. They were then transported back to the holding facility and placed in the holding tanks. The initial spring experiment was set up in April and the final sample was taken after two months in holding in June of 2011 to complete one year of sampling. During the spring season average water temperatures ranged from 2.5°C to 5.8°C.

2.3.1 Morphometric analysis

Significance was based on One-way ANOVA ($p < 0.05$). Statistical results are reported as either significant or not significant for each treatment per season in Tables 2.1-2.4. For all morphometric measurements, changes in tissue weight and condition indices are also graphed in Figures 2.3-2.5 for clarification and comparison between seasons for these parameters. Mean shell length, depth, width, tissue weight, and condition indices for summer holding and field control mussels are listed in Table 2.1. Mean shell length of mussels in holding remained between 58 mm and 60 mm. In contrast the field mussels grew significantly from 58.83 mm mean shell length initially to 70.13 mm over the course of the season. It is important to note the final field sample was taken at the scheduled three month time in September while the holding sample was taken from a subsample of

mussels frozen after two months in holding due to massive mortalities in both of the experimental tanks.

Mean shell length, depth, width, tissue weight, and condition indices for fall holding and field mussels are described in Table 2.2. There was little change in shell length, depth, or width over time for mussels in holding or the field during the fall holding trial with average length remaining at approximately 70 mm. The average length of the field animals varied between sample points but remained between 65 and 70 mm.

Mean shell length, depth, width, tissue weight, and condition indices for holding and field mussels during the winter season are detailed in Table 2.3. There was a significant decline in mean shell length from 70 mm to 66 mm for holding mussels between the initial sample and the subsequent samples (see error outlined in Section 2.3.1). The mean length of field mussels decreased significantly from 70.37 to 63.30 mm over the course of the season.

Mean shell length, depth, width, tissue weight, and condition indices for spring holding and field mussels are described in Table 2.4. There was little change in mean growth over time for mussels in holding or field mussels with mean shell length remaining between 59 mm and 63 mm.

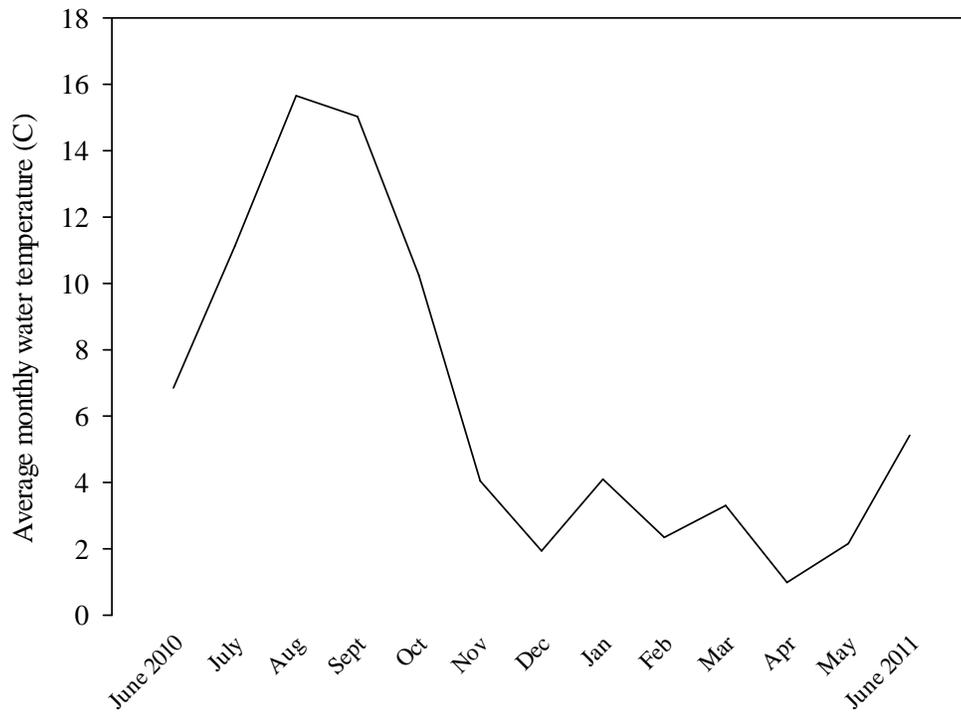


Figure 2.2: Average monthly temperature of water in holding tanks within the processing facility from June 2010 to June 2011.

Table 2.1: Morphometric measurements of *Mytilus edulis* in extended ambient holding during the summer (June to September 2010) compared to field control samples.

| Parameter | Initial | One week | | One month | | Final | |
|------------------------|---------------------------|----------------------------|---------------------------|---------------------------|----------------------------|---------------------------|---------------------------|
| | | Holding | Field | Holding | Field | Holding | Field |
| Length (mm) | 58.83 ± 0.43 ^a | 56.16 ± 0.52 ^b | 64.66 ± 0.84 ^b | 58.76 ± 0.25 ^a | 63.48 ± 0.59 ^b | 60.14 ± 0.37 ^a | 70.53 ± 0.49 ^c |
| Width (mm) | 27.94 ± 0.21 ^a | 26.96 ± 0.21 ^{ab} | 31.00 ± 0.28 ^b | 27.60 ± 0.18 ^a | 29.82 ± 0.24 ^c | 28.82 ± 0.18 ^c | 34.45 ± 0.20 ^d |
| Depth (mm) | 20.85 ± 0.16 ^a | 20.00 ± 0.18 ^b | 23.53 ± 0.27 ^b | 20.80 ± 0.15 ^a | 22.47 ± 0.24 ^c | 21.34 ± 0.15 ^a | 25.39 ± 0.19 ^d |
| Crack force (kg*cm2) | 29.05 ± 3.47 ^a | 24.85 ± 0.75 ^a | 30.87 ± 0.95 ^b | 31.55 ± 0.68 ^b | 34.31 ± 0.93 ^b | 28.07 ± 0.94 ^a | 47.65 ± 1.06 ^c |
| Wet tissue weight (g) | 7.82 ± 0.21 ^a | 6.90 ± 0.17 ^b | 13.43 ± 0.45 ^b | 8.88 ± 0.18 ^c | 11.48 ± 0.33 ^c | 4.82 ± 0.11 ^d | 16.74 ± 0.39 ^d |
| Dry tissue weight (g) | 0.84 ± 0.27 ^a | 0.68 ± 0.02 ^b | 1.32 ± 0.04 ^b | 0.79 ± 0.03 ^{ab} | 1.24 ± 0.04 ^b | 0.39 ± 0.06 ^c | 1.45 ± 0.03 ^c |
| Dry shell weight (g) | 5.00 ± 0.09 ^a | 4.57 ± 0.09 ^b | 7.09 ± 0.17 ^b | 5.09 ± 0.08 ^a | 6.64 ± 0.17 ^b | 5.52 ± 0.09 ^c | 9.36 ± 0.15 ^c |
| Condition index | 16.86 ± 0.39 ^a | 15.04 ± 0.37 ^b | 19.00 ± 0.46 ^b | 15.46 ± 0.55 ^b | 18.80 ± 0.47 ^{ab} | 6.80 ± 0.92 ^c | 15.60 ± 0.28 ^a |
| Dry weight: Wet weight | 11.49 ± 0.26 ^a | 10.39 ± 0.31 ^b | 12.09 ± 0.98 ^b | 9.01 ± 0.34 ^c | 10.96 ± 0.24 ^a | 8.71 ± 1.37 ^d | 9.06 ± 0.21 ^c |

2008 year class

Values represent mean ± SE ($n = 150$)

Different letter superscript in the same row represent statistical significance for that group (holding or field) (One-way ANOVA, $p < 0.05$)

Sampled initially, and at one week, one month, and two months in holding (summer) and simultaneously from the grow-out site (three months)

Table 2.2: Morphometric measurements of *Mytilus edulis* in extended ambient holding during the fall (September to December 2010) compared to field control samples.

| Parameter | Initial | One week | | One month | | Final | |
|------------------------|---------------------------|---------------------------|---------------------------|----------------------------|---------------------------|----------------------------|----------------------------|
| | | Holding | Field | Holding | Field | Holding | Field |
| Length (mm) | 70.53 ± 0.49 ^a | 68.94 ± 0.46 ^b | 66.89 ± 0.50 ^b | 69.98 ± 0.42 ^{ab} | 69.04 ± 0.46 ^a | 70.85 ± 0.42 ^a | 65.64 ± 0.35 ^b |
| Width (mm) | 34.45 ± 0.20 ^a | 34.14 ± 0.38 ^b | 32.21 ± 0.26 ^b | 33.55 ± 0.25 ^b | 33.44 ± 0.28 ^b | 33.81 ± 0.20 ^{ab} | 31.67 ± 0.26 ^c |
| Depth (mm) | 25.39 ± 0.19 ^a | 24.68 ± 0.19 ^b | 23.80 ± 0.20 ^b | 25.15 ± 0.18 ^a | 24.48 ± 0.19 ^b | 25.45 ± 0.16 ^{ab} | 23.27 ± 0.15 ^c |
| Crack force (kg*cm2) | 47.67±1.06 ^a | 42.12±1.07 ^b | 47.15 ± 1.14 ^a | 44.36 ± 1.19 ^{ab} | 44.36 ± 1.21 ^a | 42.77 ± 1.06 ^b | 36.81 ± 0.91 ^b |
| Wet tissue weight (g) | 16.73 ± 0.39 ^a | 17.13 ± 0.40 ^a | 14.85 ± 0.47 ^b | 19.91 ± 0.39 ^b | 19.39 ± 0.43 ^c | 20.59 ± 0.39 ^b | 14.25 ± 0.33 ^b |
| Dry tissue weight (g) | 1.46 ± 0.03 ^a | 1.48 ± 0.04 ^a | 1.15 ± 0.03 ^b | 1.25 ± 0.30 ^b | 1.38 ± 0.03 ^a | 1.05 ± 0.02 ^c | 1.05 ± 0.02 ^c |
| Dry shell weight (g) | 9.36 ± 0.14 ^a | 8.58 ± 0.15 ^b | 8.16 ± 0.16 ^b | 9.04 ± 0.17 ^{ab} | 8.98 ± 0.16 ^a | 9.01 ± 0.15 ^{ab} | 7.00 ± 0.11 ^c |
| Condition index | 15.64 ± 0.28 ^a | 17.42 ± 0.39 ^b | 14.30 ± 0.24 ^b | 13.95 ± 0.18 ^c | 15.48 ± 0.25 ^a | 11.62 ± 0.19 ^d | 15.10 ± 0.22 ^{ab} |
| Dry weight: Wet weight | 9.07 ± 0.21 ^a | 9.09 ± 0.29 ^a | 8.44 ± 0.39 ^b | 6.37 ± 0.09 ^b | 7.46 ± 0.21 ^{ab} | 5.20 ± 0.11 ^c | 7.76 ± 0.18 ^a |

2008 year class

Values represent mean ± SE ($n = 150$)

Different letter superscript in the same row represent statistical significance for that group (holding or field) (One-way ANOVA, $p < 0.05$)

Sampled initially, and at one week, one month, and three months in holding and simultaneously from the grow-out site

Trends for wet tissue weight for summer, fall, winter, and spring are shown in Figure 2.3A. Mean wet tissue weight increased up to one month in holding for the summer, fall, and spring seasons. During the summer there was a significant decline in wet tissue weight at the two month sample (Table 2.1). During the fall trial holding mussels showed a significant increase in wet tissue weight from 16.73 g initially to 20.59 g after three months (Table 2.2). During the winter wet tissue weight declined from 18.18 g initially to 15.65 g after one week in holding (Table 2.3). There was no significant change in wet tissue weight between one week and three months for mussels in holding. Wet tissue weight increased over time in holding for the spring trial as well, increasing significantly from 10.56 g at the initial time to 13.39 g at the two month sample time (Table 2.4).

Trends in dry tissue weight for summer, fall, winter, and spring holding trials are shown in Figure 2.3B. Dry tissue weight decreased over time in holding for the summer, fall, and spring seasons. Mean dry tissue weight for mussels held during summer decreased significantly from 0.78 g initially to 0.32 g at the time of the crash (Table 2.1). During the fall holding trial mean dry tissue weight decreased significantly from 1.46 g initially to 1.05 g at the final sample and during the spring there was a significant decrease from 0.87 g to 0.67 g (Table 2.2). During the winter however, there was a significant increase in dry tissue weight, increasing from 1.30 g to 1.45 g at the final three month sample (Table 2.3). Results for the spring trial revealed a decline in dry tissue weight over time in holding decreasing significantly from 0.87 g initially to 0.67 g after two months in holding (Table 2.4).

Table 2. 3: Morphometric measurements of *Mytilus edulis* in extended ambient holding during the winter (January to April 2011) compared to field control samples.

| Parameter | Initial | One week | | One month | | Final | |
|-----------------------------------|---------------------------|---------------------------|---------------------------|----------------------------|---------------------------|---------------------------|---------------------------|
| | | Holding | Field | Holding | Field | Holding | Field |
| Length (mm) | 70.37 ± 0.51 ^a | 66.82 ± 0.42 ^b | 73.76 ± 0.42 ^b | 65.49 ± 0.40 ^b | 67.45 ± 0.53 ^c | 65.78 ± 0.43 ^b | 63.30 ± 0.44 ^d |
| Width (mm) | 33.48 ± 0.19 ^a | 33.32 ± 0.33 ^a | 34.75 ± 0.25 ^b | 32.60 ± 0.18 ^{ab} | 33.28 ± 0.18 ^a | 32.21 ± 0.19 ^a | 29.76 ± 0.20 ^c |
| Depth (mm) | 25.40 ± 0.17 ^a | 24.65 ± 0.15 ^b | 26.29 ± 0.18 ^b | 24.31 ± 0.24 ^b | 24.98 ± 0.16 ^a | 24.13 ± 0.17 ^b | 22.50 ± 0.17 ^c |
| Crack force (kg*cm ²) | 47.05 ± 1.01 ^a | 42.21 ± 1.11 ^b | 53.75 ± 1.24 ^b | 36.68 ± 0.97 ^c | 41.53 ± 0.87 ^c | 43.08 ± 1.16 ^b | 33.18 ± 0.90 ^d |
| Wet tissue weight (g) | 18.18 ± 0.45 ^a | 15.65 ± 0.50 ^b | 16.23 ± 0.43 ^a | 14.62 ± 0.32 ^b | 13.02 ± 0.33 ^b | 15.30 ± 0.32 ^b | 10.44 ± 0.23 ^c |
| Dry tissue weight (g) | 1.30 ± 0.03 ^a | 1.17 ± 0.02 ^b | 1.76 ± 0.05 ^b | 1.17 ± 0.02 ^b | 1.45 ± 0.03 ^c | 1.45 ± 0.03 ^a | 0.99 ± 0.03 ^d |
| Dry shell weight (g) | 9.55 ± 0.14 ^a | 8.95 ± 0.14 ^b | 10.45 ± 0.16 ^b | 8.79 ± 0.13 ^b | 9.12 ± 0.14 ^a | 8.77 ± 0.15 ^b | 6.14 ± 0.11 ^c |
| Condition index | 13.66 ± 0.20 ^a | 13.05 ± 0.20 ^a | 16.71 ± 0.30 ^b | 13.84 ± 0.20 ^a | 15.87 ± 0.22 ^b | 16.13 ± 0.33 ^b | 16.34 ± 0.38 ^c |
| Dry weight: Wet weight | 7.43 ± 0.16 ^a | 8.08 ± 0.25 ^a | 12.06 ± 0.54 ^b | 8.31 ± 0.17 ^{ab} | 11.76 ± 0.29 ^b | 9.48 ± 0.23 ^b | 9.75 ± 0.23 ^c |

2008 year class

Values represent mean ± SE ($n = 150$)

Different letter superscript in the same row represent statistical significance for that group (holding or field) (One-way ANOVA, $p < 0.05$)

Sampled initially, and at one week, one month, and three months in holding and simultaneously from the grow-out site

Table 2.4: Morphometric measurements of *Mytilus edulis* in extended ambient holding during the spring (April to June) compared to field control samples.

| Parameter | Initial | One week | | One month | | Final | |
|-----------------------------------|---------------------------|---------------------------|---------------------------|---------------------------|----------------------------|---------------------------|----------------------------|
| | | Holding | Field | Holding | Field | Holding | Field |
| Length (mm) | 61.84 ± 0.38 ^a | 63.00 ± 0.41 ^a | 63.15 ± 0.34 ^b | 61.97 ± 0.44 ^a | 59.38 ± 0.33 ^c | 62.14 ± 0.44 ^a | 63.28 ± 0.45 ^{ab} |
| Width (mm) | 29.13 ± 0.17 ^a | 29.45 ± 0.18 ^a | 29.89 ± 0.24 ^b | 29.63 ± 0.31 ^a | 28.77 ± 0.16 ^a | 29.80 ± 0.29 ^a | 30.14 ± 0.21 ^{ab} |
| Depth (mm) | 21.98 ± 0.17 ^a | 22.15 ± 0.21 ^a | 22.53 ± 0.14 ^b | 21.87 ± 0.18 ^a | 20.78 ± 0.13 ^c | 22.15 ± 0.18 ^a | 22.37 ± 0.19 ^a |
| Crack force (kg*cm ²) | 32.28 ± 0.78 ^a | 33.97 ± 0.24 ^a | 34.28 ± 0.71 ^a | 32.41 ± 0.86 ^a | 24.63 ± 0.54 ^{ab} | 33.48 ± 0.89 ^a | 31.93 ± 0.87 ^a |
| Wet tissue weight (g) | 10.56 ± 0.27 ^a | 11.33 ± 0.24 ^a | 13.83 ± 0.25 ^b | 13.75 ± 0.36 ^b | 10.90 ± 0.20 ^a | 13.39 ± 0.30 ^b | 14.57 ± 0.34 ^b |
| Dry tissue weight (g) | 0.87 ± 0.02 ^a | 0.80 ± 0.02 ^a | 2.36 ± 0.03 ^b | 0.73 ± 0.02 ^b | 1.80 ± 0.03 ^c | 0.67 ± 0.02 ^b | 1.65 ± 0.04 ^c |
| Dry shell weight (g) | 5.82 ± 0.11 ^a | 6.21 ± 0.12 ^a | 6.42 ± 0.10 ^b | 6.07 ± 0.13 ^a | 4.80 ± 0.08 ^b | 6.14 ± 0.13 ^a | 6.39 ± 0.14 ^c |
| Condition index | 15.12 ± 0.27 ^a | 13.05 ± 0.25 ^b | 37.27 ± 0.53 ^b | 12.09 ± 0.20 ^c | 38.08 ± 0.50 ^b | 10.98 ± 0.20 ^d | 26.01 ± 0.50 ^c |
| Dry weight: Wet weight | 8.63 ± 0.20 ^a | 7.21 ± 0.14 ^b | 17.04 ± 0.22 ^b | 5.61 ± 0.20 ^c | 16.91 ± 0.28 ^b | 5.07 ± 0.10 ^c | 11.64 ± 0.27 ^c |

2008 year class

Values represent mean ± SE ($n = 150$)

Different letter superscript in the same row represent statistical significance for that group (holding or field) (One-way ANOVA, $p < 0.05$)

Sampled initially, and at one week, one month, and two months in holding and simultaneously from the grow-out site

The first measure of condition index, the ratio of dry tissue weight to dry shell weight, is shown in Figure 2.3C for each season. There was a decline in condition over time in holding for the summer, fall, and spring seasons. During the summer, condition decreased significantly from 16.86 % initially to 5.89 % (Table 2.1) after two months in holding, noting that these measurements were taken from a subsample of frozen mussels at the time of the crash. During the fall, there was a significant decline from 15.64 % initially to 11.62 % after three months in holding (Table 2.2). There was a similar trend during spring where condition decreased from 15.12 % initially to 10.98 % after two months in holding (Table 2.3). Trends for the second measure of condition index, dry to wet meat weight ratio are shown in Figure 2.3D. Over time in holding this condition index showed similar trends to the first condition index. There was a significant decrease in this parameter during the summer, fall, and spring holding trials. During the winter trial however there was a significant increase.

Trends in wet tissue weight for field control mussels can be seen in Figure 2.4A. Mean wet tissue weight of summer field control mussels increased significantly over the course of the season from 12.56 g to 16.74 g at the three month sample time in September (Table 2.1). During the fall, mean wet tissue weight varied between each sample time decreasing from 16.73 g initially to 14.75 g at one week increasing again to 19.39 g at the one month sample time before decreasing again to 14.25 g at the final sample. During the winter season mean wet tissue weight declined significantly from initially 18.18 g to 6.15 g at the final sample time in April (Table 2.3). During the spring there was a significant increase in wet tissue weight from 10.56 g initially to 14.57 g at the final sample in June (Table 2.4).

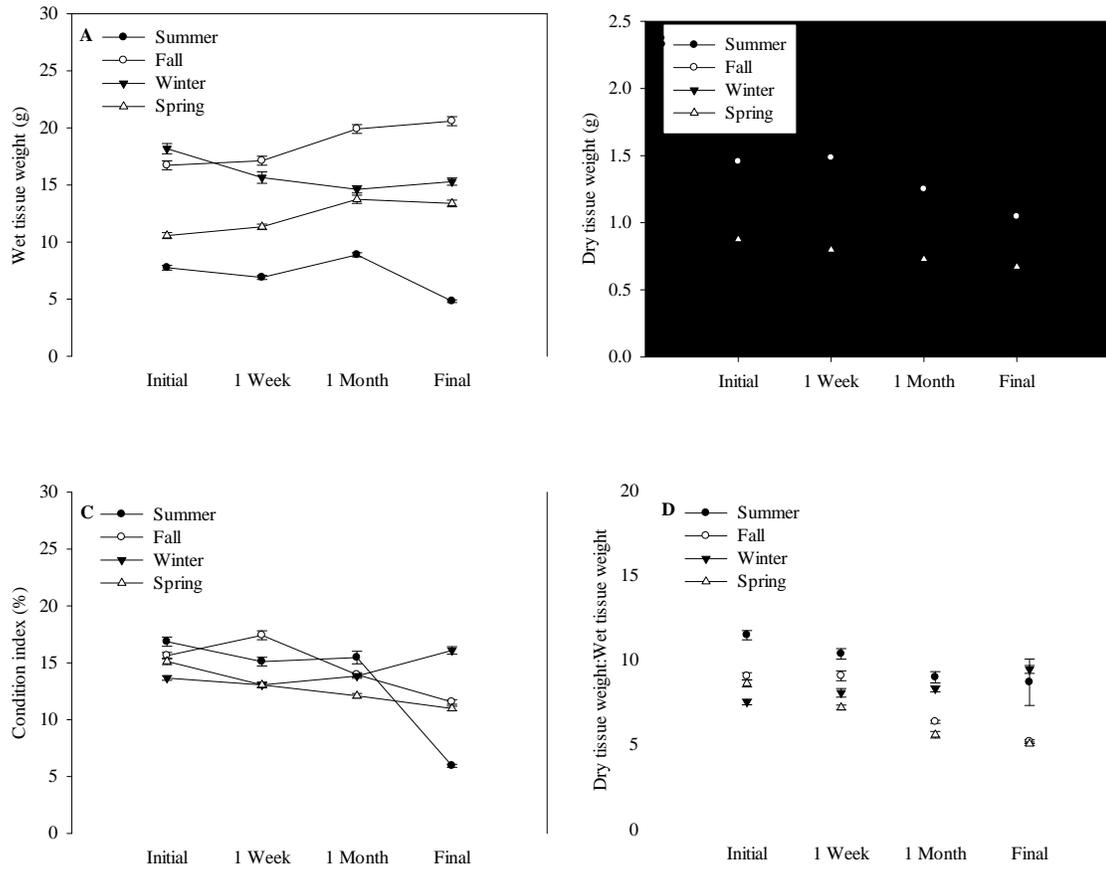


Figure 2.3: Mean wet tissue weight (A), dry tissue weight (B), dry tissue weight: dry shell weight (C), and dry tissue weight: wet tissue weight (D) for mussels held during summer (●), fall (○), winter (▼), spring (△). (mean ± SE ($n = 150$)).

Trends in dry tissue weight for field control mussels are shown in Figure 2.4B. There was a significant increase, from 0.78 g to 1.45 g, in mean dry tissue weight during the summer (Table 2.1). During the fall and winter there was a significant decline in dry tissue weight between the initial and final sample times (Table 2.2 and Table 2.3 respectively). During the spring there was an increase in dry tissue weight from 0.87 g at the initial spring sample to 2.36 g at one week, followed by a decline to 1.65 g at the final sample time in June (Table 2.4).

Trends in condition index for field control mussels are shown in Figure 2.4C. During the summer the field control mussels showed little significant variation in condition index over the season. Condition increased from 16.86 % initially to 19.00 % after one week, no change observed at the one month sample, followed by a significant decline to 15.60 % at the three month sample (Table 2.1). During the fall there was also little change in condition over the season, remaining between 14.30 % and 15.48 % (Table 2.2). During the winter there was a significant increase in condition from 13.66 % to 16.34 % at the final sample (Table 2.3). During the spring, field control mussels showed the greatest values for condition increasing from 15.12 % initially to 37.27 % during the one week sample, remaining high during the one month sample, and decreasing to 26.01 % at the final sample (Table 2.4). Trends in dry to wet meat weight ratio are similar and are shown in Figure 2.4D.

Trends in shell strength and shell mass over time for mussels in holding are shown in Figure 2.5A and 2.5B, respectively. During the summer, fall, and spring there were no significant differences in overall shell strength between the initial and final sample times.

During the winter trial however there was a significant increase in shell strength between the initial and three month sample.

Trends in shell strength and shell mass for field control mussels are shown in Figure 2.5C and 2.5D respectively. During the summer trial the freshly harvested mussels showed a significant increase in shell strength from 29.21 to 47.65 kg*cm². Field control mussels showed a significant decline in shell strength during the fall and winter. During the spring there was no significant change in shell strength over time.

2.3.2 Histology

2.3.2.1 Reproductive histology

Histological sections of mussel gonadal tissue were observed over time for both holding and field control mussels during the summer (Fig. 2.6), fall (Fig. 2.7), winter (Fig. 2.8), and spring (Fig. 2.9) to observe seasonal trends in reproduction. During the summer only male animals were noted. At the time of the initial sample in June male gonadal tissue was in Stage I of development, while after one month the gonad progressed into stage II, showing a low level of gonadal filling, for both the holding and field controls (Fig. 2.6).

At the beginning of the fall trial in September the initial female and male gonad were at stage II, with a low level of gonadal filling following a recent spawning event (Fig. 2.7). The final male and female holding samples were asynchronous, with the female gonad appearing spent (stage I) while the male showed low level of gonadal filling (stage II). The final female field control was also at stage II of development (Fig. 2.7).

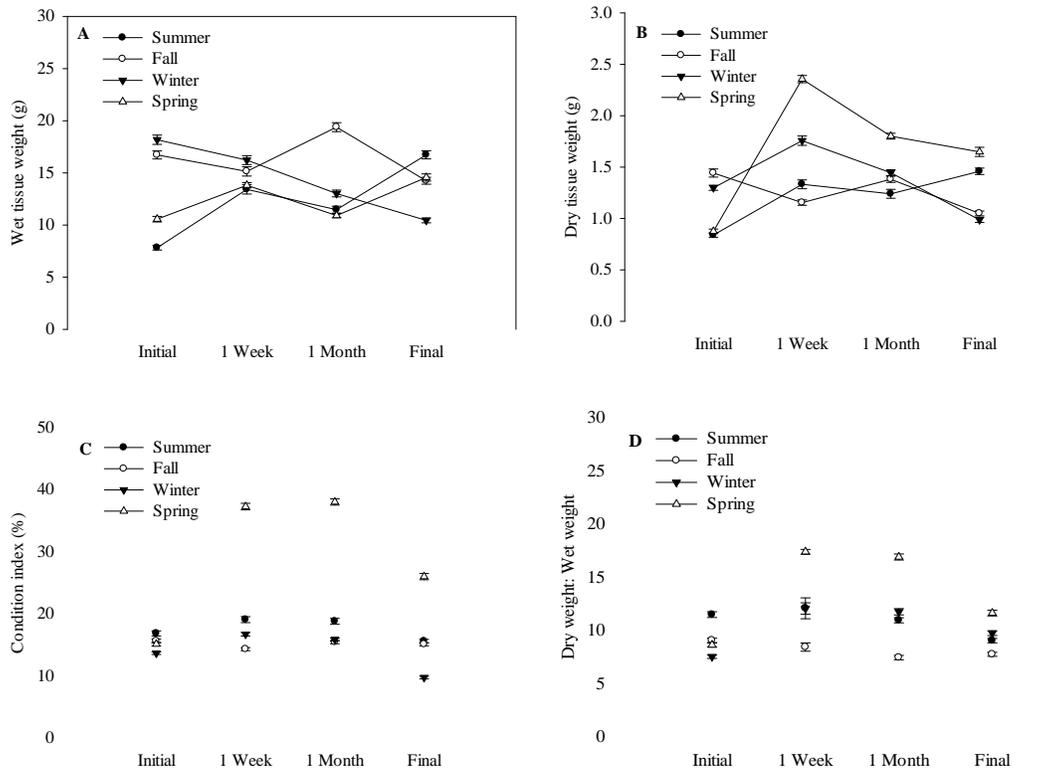


Figure 2.4: Mean wet tissue weight (A), dry tissue weight (B), condition index (C), and dry tissue weight: wet tissue weight (D) for field control mussels during summer (●), fall (○), winter (▼), spring (△). (mean ± SE ($n = 150$)).

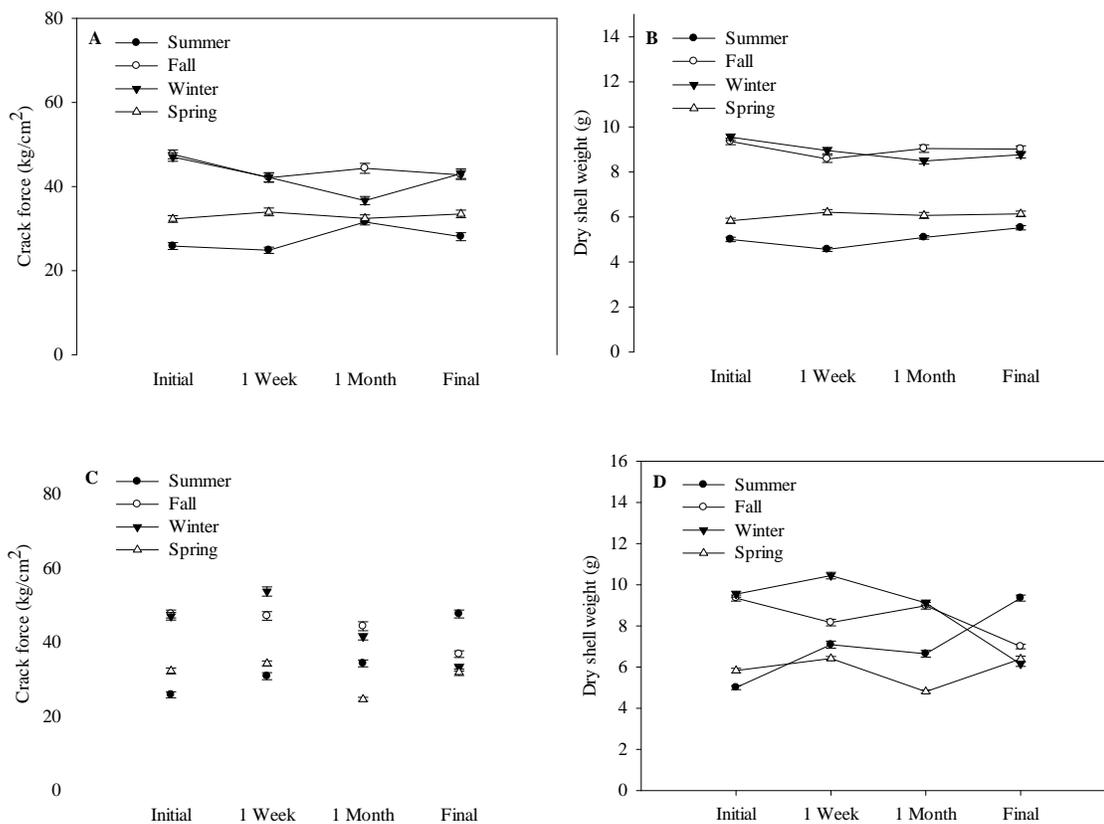


Figure 2.5: Changes in crack force (kg/cm²) (A) and dry shell weight (g) (B) for mussels in extended holding during the summer, fall, winter and spring compared to seasonal changes in crack force (kg/cm²) (C) and dry shell weight (g) (D) for field control mussels. (mean \pm SE ($n = 150$)).

The initial winter sample was taken in January, one month after the final fall sample. The initial female gonad (Fig 2.8A) was at stage III of development while the male (Fig 2.8B) remained at stage II. There was no change in male reproductive status for the final holding sample (Fig 2.8C) however the field control (Fig 2.8D) showed females with partially regressing gonadal tissue.

The initial spring sample was taken at the same time as the final winter sample in April. The initial reproductive state of the male (Fig. 2.9A) and female (Fig. 2.9B) appear to be at the same stage of development as the final winter field samples. At the final two month holding sample the female was at stage I (Fig. 2.9C) the gonad appeared to have spawned recently. The field control (male) (Fig. 2.9D) sample appeared to be at stage III of development as the gonads were full and ready to spawn.

2.3.2.2 Histology of secondary ducts of digestive gland

Changes in digestive tubule structure, specifically observations made regarding digestive and basophil cell shape and structure were assessed for the initial and final sample of each season for the mussels in both holding and field samples. Structure of secondary tubules during the initial and final sample for summer is shown in Figure 2.10. At the initial sample, ducts were full of digestive material. Digestive cells (dc) and basophil cells (bc) were distinguishable. There was little change in digestive cell structure between the initial sample in June and the final holding or control samples. Comparison of initial and final digestive gland structure during the fall is shown in Figure 2.11. There was little material present initially and after three months in holding there appears to be little breakdown of digestive cell structure at this time when compared to the field sample.

Digestive gland structure during the initial and final winter samples is shown in Figure 2.12 and there appears to be little change in digestive cell structure over time. The comparison of initial and final digestive gland structure during the spring is shown in Figure 2.13. Here degeneration of digestive cells was noted in the final holding sample and not in the field sample.

2.4 DISCUSSION

Mussel condition, meat quality, and meat yield are affected by factors such as water temperature, food availability, and reproductive status (Orban *et al.* 2002). The purpose of this experiment was to assess the physiological effects of long term holding compared to animals from the grow-out site (field control) as well as to assess changes due to seasonality. The ratio of dry meat weight to dry shell weight was used as an index as it eliminates any bias due to individual fluctuations in water content. A low condition index indicates the presence of a stressor such as poor environmental conditions, disease, or a recent spawning event (Lucas & Beninger 1985). Studies have shown that animals in a laboratory setting respond differently than animals in the field and that extended periods in a laboratory affects animals on the physiological (Bayne and Thompson 1970) and cellular level (Cajaraville *et al.* 1991).

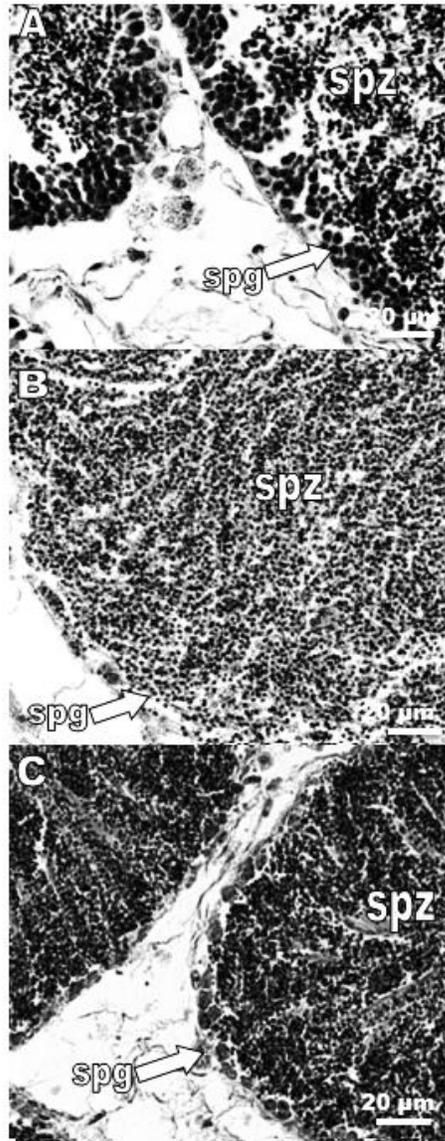


Figure 2.6: Mussel gonadal tissue development during the summer (June-August). Initially upon introduction to the facility (A: male), at one month in holding (B: male), and one month field control (C: male). (spg: spermatogonia; spz: spermatozoa)

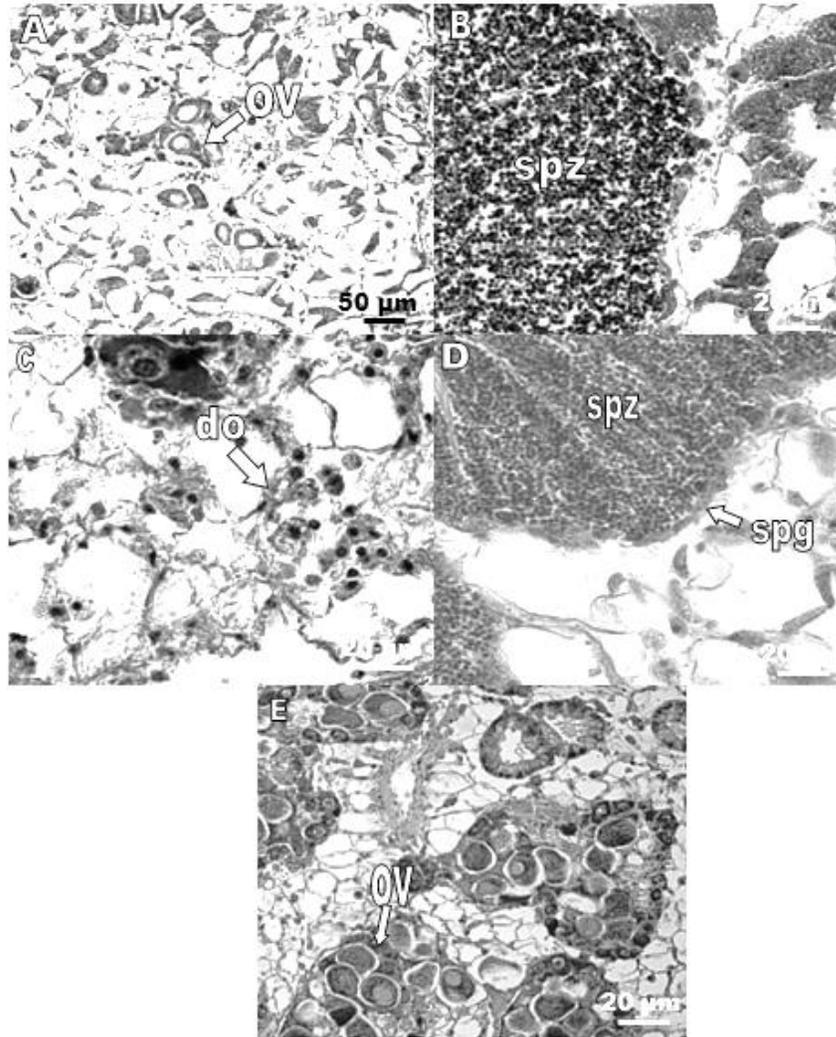


Figure 2.7: Mussel gonadal tissue development during the fall (September-December). Initially upon introduction to the facility (A; female, B; male), after three months in holding (C: female, D: male) and the final field control (E: female). (spg: spermatogonia; ov: ovocytes; do: degenerating ovocytes)

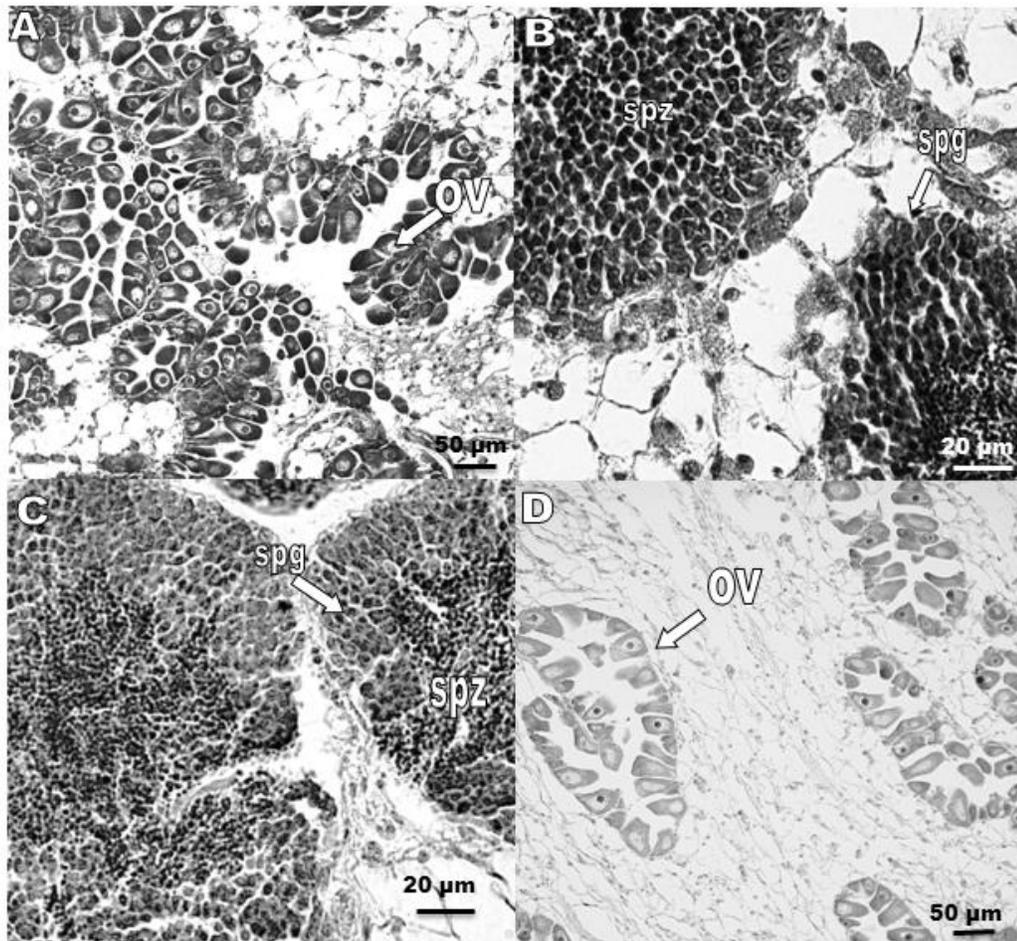


Figure 2.8: Mussel gonadal tissue development during the winter (January-April). Initially (A: female, B: male), at three months in holding (C: male), and final field control (D: female). (spg: spermatogonia; ov: ovocytes; do: degenerating ovocytes).

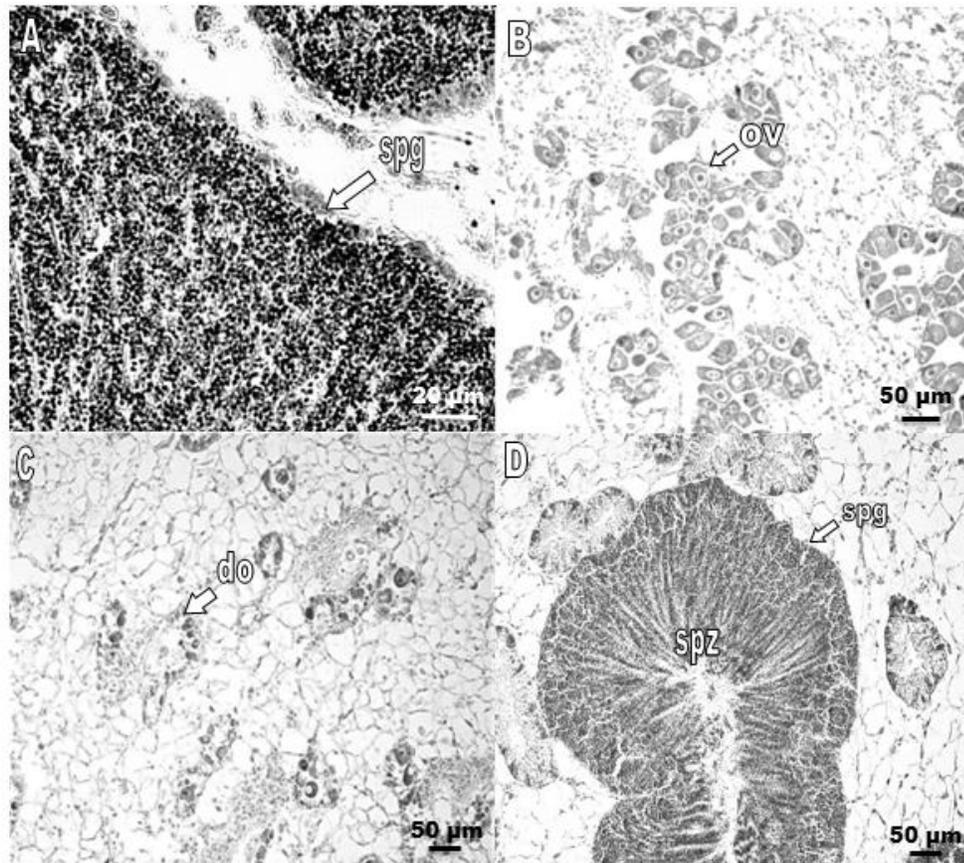


Figure 2.9: Mussel gonadal tissue development during the spring (April-June). Initially (A: male, B: female), at two months in holding (C: female), and the final field control (D: male).

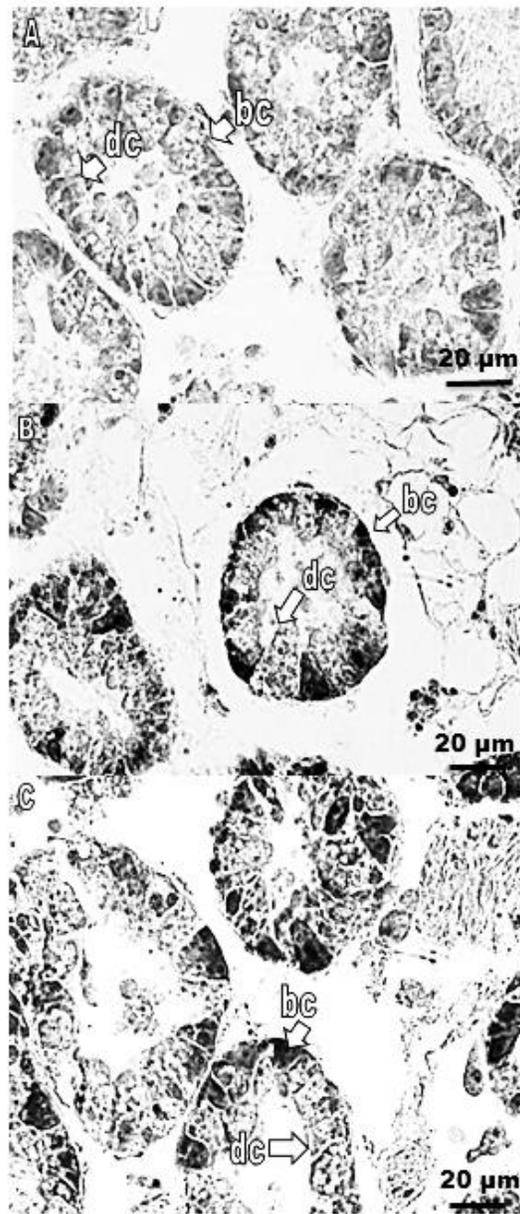


Figure 2.10: Histology of secondary ducts of *Mytilus edulis* digestive gland at the initial summer (A), final summer holding (B) and final summer field sample (C). (Digestive cells (dc) and basophil cells (bc)).

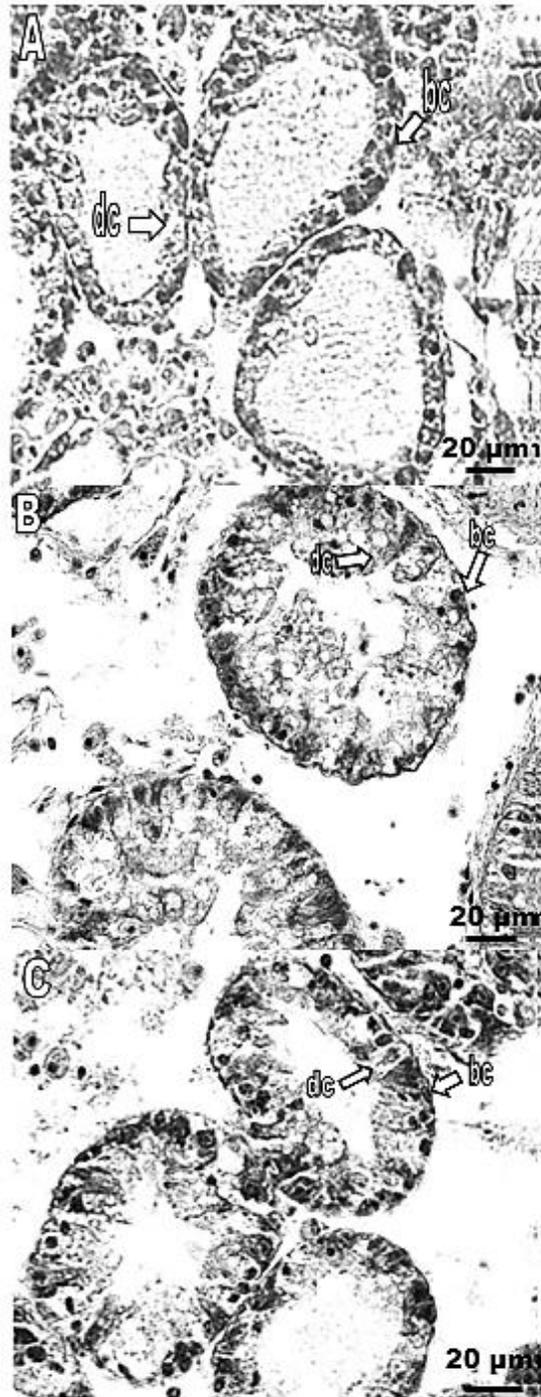


Figure 2.11: Secondary ducts of *Mytilus edulis* digestive gland from the initial fall sample (A), after three months in holding (B), and the final field sample (C). (Digestive cells (dc) and basophil cells (bc)).



Figure 2.12: Secondary ducts of *Mytilus edulis* digestive gland during the initial winter sample (A), after three months in holding (B), and the final field sample (C). (Digestive cells (dc) and basophil cells (bc)).



Figure 2.13: Secondary ducts of *Mytilus edulis* digestive gland during the initial spring sample (A), after two months in holding (B) and, the final field sample (C). (Digestive cells (dc) and basophil cells (bc)).

Our results concur with previous studies and show a significant decline in condition and dry tissue weight after two or three months in holding during the summer, fall, and spring trials suggesting that extended holding in a processing facility under ambient conditions has a negative effect on condition and meat yield, but only after extended periods of time (1 to 3 months). For all experimental seasons there was an increase in mean wet and dry tissue weight up to one month in holding followed by a significant decline at the final sample. The discrepancy between wet tissue weight and dry tissue weight can signify a state of energy depletion or starvation (Lucas & Beninger 1985). This condition index declined significantly over time in holding for the summer, fall, and spring. While condition index has been shown to vary seasonally, the present study shows that extended live-storage with no temperature control negatively impacts mussel physiological condition after a period of one month (except in winter).

Growth and condition vary seasonally with an increase observed during the spring and summer when water temperatures are warmer and nutrient levels are high (Seed 1976). Warmer temperatures stimulate higher feeding rates thus increasing growth and stimulating reproduction (Seed 1976). During the summer while the increasing temperature appeared to have a positive impact on growth in the field control animals high temperatures in combination with the holding conditions led to high mortality in the holding system. The subsequent morphometric analysis of mussels in holding revealed an extremely low condition index compared to field controls. Although there is continuous flow-through of water directly from the surrounding bay there may be the question of limited food availability, crowding, and the accumulation of waste products, which could have had a negative impact on the animals in the holding system. This could

be a result of a higher stocking density in the holding tanks compared to the grow-out site. Mussels grown in high density and low-food conditions have lower condition indices and tissue weights (Alluno-Bruscia *et al.* 2001). Limited food availability for mussels in the holding facility could also account for the low condition index and dry tissue weight compared to the field samples. Bayne and Thompson (1970) demonstrated a significant difference in condition index and a sharper decline in mean total weight between fed and unfed animals held in a laboratory setting after a period of two weeks. These stressful environmental conditions associated with extended holding may be responsible for the low condition index.

In the past the phenomenon of summer mortality has been observed in both juvenile and adult mussel stocks in Canada (Mallet *et al.* 1990) and several hypotheses exist as to the root cause. Incze *et al.* (1980) suggest that mass mortalities in the summer are the result of a combination of high temperatures and less than optimal environmental conditions. They found mortality to increase at temperatures above 20 °C, during mid-August to early September. They also observed a coincident decline in food supply as indicated by chlorophyll a concentration with mortalities most likely a resulting from depleted energy reserves or a shift in gametogenic cycle coupled with high water temperatures (Incze *et al.* 1980). During the summer experimental season different trends were observed in holding compared to the field control. In the holding facility there was no net growth in length and there was a decline in tissue weight and condition after one month. The field control mussels however showed an increase in shell length and tissue weight, indicating positive growth. It is likely that low food availability in the holding

tanks resulted in no net growth and may be the cause of mortality. It is unlikely high water temperature played an important role as the water temperature coming into the facility is relatively equal to the temperature experienced by the animals in the field. Future research could compare chlorophyll levels in holding tanks to those on the lease to evaluate whether nutrient depletion may take place in the tanks.

Winter represents a time of unfavorable conditions including low water temperatures and nutrient concentration and growth is generally slowed or absent during this time (Seed 1976). Studies of other lamellibranch species have revealed a decline in tissue weight and shell length during the winter (Dare & Edwards 1975). In contrast to the other experimental seasons, there was an increase in condition and dry tissue weight over time in holding whereas the field control experienced a significant decline in both parameters. Bayne & Thompson (1970) found that changes in dry weight over time in a laboratory setting (for a period of 48 days) depended on ambient water temperature. At higher temperatures (16°C) there was a significant decline in total dry weight whereas at 6°C there was an increase in mean dry weight over a 48 day period (Bayne & Thompson 1970). Colder temperatures and winter ice cover can limit primary production and nutrient availability resulting in low metabolic and growth rates which have been observed during winter months compared to basal rates during other times of the year (Hatcher *et al.* 1997). The decrease in metabolism during the winter months could be a result of several factors including low water temperature and ice cover that limits primary productivity could result in low food availability. Furthermore histological analysis of the holding mussels revealed ripe gonadal tissue which could account for the increase in tissue weight. It is likely in this case the decline in tissue weight and condition observed

for the field control mussels is a result of low seston matter while the increase in mean tissue weight and condition for the winter holding mussels could be due to reproductive status.

Changes in crack force (shell strength) followed changes in dry shell weight and there was no significant decline in either parameter over time as a result of extended holding for any season studied, indicating that holding does not negatively impact these parameters. However, seasonal variation was observed for the field control mussels. During the summer there was a significant increase over time as mussels in the field generally grew in size from June to September 2010. The discrepancy between holding and control mussels during the summer could be due to limited food supply in the holding tanks or increased competition between individuals for nutrients and/or space. During the winter there was a significant decline in shell strength for both holding and field controls. These results are consistent with a study by Nagarajan *et al.* (2006) who found shell strength and ventral thickness of *M. edulis* shells to be lowest during the winter months likely as a result of low temperature and reduced salinity. Furthermore Alunno-Bruscia *et al.* (2001) also found that shell mass was affected by food concentration and density. The decline in shell weight and strength in both conditions is likely a result of their natural annual cycle and extended holding did not appear to have a significant effect.

Spawning events were apparent between June 2010 and June 2011 including one spawning event in the late summer of 2010, consistent with other reports concerning the timing of spawning in Newfoundland mussels (Harding *et al.* 2004; Thompson 1984; Terry Mills: personal communication). At several sample times there was complete or partial regression observed in the female gonad while none was observed in the male

gonad. A spawning event occurred prior to the initial fall sample in September as indicated by the status of the female gonad, which continued to develop over the course of the season. There appeared to be a lag in timing between the holding and field control samples. This could be a direct result of different triggers that determine the onset of spawning in mussels including nutrient availability and other favorable environmental factors. The difference in timing between treatments could also simply represent asynchronous spawning in the population as well as between sexes.

There was no clear degeneration of digestive cells over time in holding when comparing the final holding and field sample with the exception of the spring season. The final spring holding sample showed enlargement and degradation of digestive cells within the secondary ducts of the digestive gland, a pathology that was not observed in the initial or final field sample. At this sample time there was also a marked decline in condition as well as an indication of spawning, it is likely these factors contributed to the poor health of the animals at this time.

These results demonstrate that extended holding under ambient conditions had a negative impact on the condition of blue mussels after a period of one month for all seasons but the winter. We recommend that one month be the maximum time in holding during the fall, winter, and spring. It is disadvantageous to hold mussels longer than one week during the summer without temperature control. Future studies examining changing spawning patterns in this area could be beneficial to help determine the optimum harvest time before there is a sharp decline in condition and meat yield. It might also be advantageous to measure chlorophyll levels to determine the amount of food available on site and in the holding facility for comparison.

CHAPTER 3: THE EFFECT OF EXTENDED HOLDING UNDER AMBIENT TEMPERATURES ON THE PHYSIOLOGY AND STRESS RESPONSE OF THE BLUE MUSSEL, *MYTILUS EDULIS*

3.1 INTRODUCTION

Following commercial harvest, mussels are transported to a processing facility where they are stored in holding tanks before washing and processing. At times mussels may remain in the holding facility for extended periods as a result of transportation delays or other factors. Studies have shown that different post-processing storage conditions (on ice, chilled air, ambient water) and post-harvest handling can negatively affect the stress response of mussels and potentially lead to a decrease in meat quality and shelf life (Harding *et al.* 2004; Tremblett 2001). In general, storage in ambient water has the least impact on the stress response as indicated by neutral red retention (NRR) time, followed by those stored on ice and those stored in chilled air (Harding *et al.* 2004; Tremblett 2001).

Many aspects of bivalve physiology follow a seasonal cycle and depends on seasonal changes in external factors including temperature, nutrient availability, and reproduction. As such the expression of antioxidant enzymes also follows a seasonal cycle (Sheehan & Power 1999). The bivalve defense system is comprised of both antioxidant enzymes including SOD, GSH-peroxidase, GST and catalase, and antioxidant compounds including vitamin E and GSH (Sheehan & Power 1999). These enzymes and compounds have been localized in both the digestive gland and gill tissues and levels

follow similar seasonal patterns in both tissues, with a few exceptions. In general enzyme levels are lowest in the digestive gland during the winter months, which corresponds to a period of increased lipid peroxidation, however GST levels are at their highest levels in the gill tissue during this time (Sheehan & Power 1999). Several studies exist on the seasonality of antioxidant enzyme levels (Power & Sheehan 1996; Viarengo *et al.* 1991) but none exist on how antioxidant gene expression varies seasonally in response to extended storage conditions.

Hemocytes are the cellular mediators of invertebrate immune defense and thus have a wide variety of functions including recognition of pathogens and secretion of antimicrobial and cytotoxic substances into the surrounding hemolymph (Mitta *et al.* 2000b). Lysosomes are subcellular organelles found in all eukaryote cells, with the exception of red blood cells. They display a diverse array of functions including membrane repair, digestion, and reproduction. They also have an important role in aspects of the immune response and defense against bacteria by releasing hydrolytic enzymes and reactive oxygen species to destroy invading pathogens (Zhang *et al.* 2006). Factors such as decreased temperature (Camus *et al.* 2000), starvation (Zhang & Li 2006), reproduction (Song *et al.* 2007), heavy metals (Viarengo *et al.* 2000), and processing, storage, and handling (Harding *et al.* 2004) can affect lysosomal structure and function. These stressful environmental, mechanical, and reproductive conditions affect lysosome stability resulting in increased permeability or membrane fusion events (Lowe *et al.* 1995; Zhang *et al.* 2006). The neutral red assay is a useful technique that evaluates changes in lysosomal membrane structure over time. In unstressed animals, the lysosome membrane is relatively impermeable and will retain the neutral red dye for an extended period of

time. However, in stressed animals the membranes break down and destabilize at which time the dye will leak into the surrounding cytosol (Zhang *et al.* 2006). The rate at which these events occur can be measured over time using the neutral red assay as an indicator of the presence of a stressor and the degree of stress the animal is experiencing.

Expression of antimicrobial peptides varies seasonally as well. Stress can also affect the immune efficiency in many animals and stressful conditions can lead to disease outbreaks in bivalves (Malagoli *et al.* 2007). Malagoli *et al.* (2007) found that increased air exposure and mechanical stress resulted in an increase in circulating immunocytes in the mussel *M. galloprovincialis*. How a stressor affects the immune response depends on several factors including the type of stressor and the physiological state of the animal (Lacoste *et al.* 2002).

Few studies have examined how extended holding under ambient conditions affects the immune system of bivalves. This study uses the neutral red assay in conjunction with changes in expression patterns of several antioxidant enzymes in gill samples as well as three antimicrobial peptides to determine changes in the physiological stress response of animals over time in holding for four seasons with reference to field control animals.

3.2 Materials and Methods

3.2.1 Study site, experimental setup, and sampling protocol

Mussels from the 2008 year-class were collected from Site 13 in Bulley's Cove, Newfoundland, Canada and transported to a commercial processing facility in Pleasantview, Newfoundland. For details on the experimental setup of holding tanks and sample schedule please refer to Chapter 2, Section 2.2.1.

3.2.2 Neutral Red Assay

At each sample 12 mussels were randomly sampled from the holding facility and from the field site (field control) for neutral red assay analysis. Initially, 0.1 ml of hemolymph was extracted from the posterior adductor muscle into a 1 ml syringe with a 22.5 gauge needle containing 0.1 ml of mussel physiological saline (4.77 g HEPES, 25.48 g NaCl, 13.06 g MgSO₄, 0.75 g CaCl₂ in 1 L Millipore water; pH 7.3). Once the hemolymph was extracted the needle was removed to reduce shear stress on the cells and the solution was pipetted into a 1.5 ml Eppendorf tube. Once the 12 mussels had been bled, the tubes were carefully inverted to mix the suspension and 40 µl was pipetted onto the centre of a poly-L lysine coated slide (10 µl in 100 µl millipore water). The slides were then transferred to a light proof humidity chamber and incubated for 15 minutes to allow cells to adhere. Subsequently, excess fluid was drained off the slide by gently tapping the long edge on a paper towel. The neutral red solution stock solution (28.8 mg of neutral red in 1 ml of dimethyl sulfoxide (DMSO)) was diluted to a working solution (20 µl in 5 ml of physiological saline) and 40 µl was applied to each slide. Slides were then incubated in a

lightproof humidity chamber for an additional 15 minutes before an 18 x 18 mm cover slip was added and initial observations were recorded. Observations were made using a compound microscope after 15 minutes and systematically at 30 minute intervals up to 180 minutes. Cells were first located at 10 X and then examined in detail under 40 X objective. Observations were recorded by assigning a four point numerical score (1-4) to 25 cells per field of view. The scoring was assigned so that (1) represented low stress indicated by the appearance of tiny pink dots (lysosomes) in the cytosol, (2) moderate stress as indicated by an increase in lysosome size, (3) represented moderate high stress as indicated by leakage of dye from lysosomes to the cytosol and, (4) represented high stress response indicated by increased membrane degradation, lysosomal vacuolation, or rounding up of cells and visible no pseudopodia. Once 50% of the cells showed a high degree of stress response slide observations were terminated.

3.2.3 Gene expression

3.2.3.1 Tissue samples

For each gene of interest two PCR repetitions were performed. 18S rRNA was used as a normalizer gene and the same RNA/cDNA was used for both the gene of interest and 18S rRNA. For each season, holding and field control samples are grouped together on one gel and a negative control (water only) was also run to ensure no contamination of PCR products. In general, expression of 18S rRNA was consistent over the duration of each season. Relative expression is compared between each sample time, season, and the

reference gene to observe the effects of extended holding and determine any seasonal patterns.

Gill tissue was dissected from both holding and field animals and placed in a 1.5 ml Eppendorf tube containing five volumes of RNAlater (Ambion). Samples were stored on ice and transported back to St. John's, Newfoundland and Labrador where they were stored at -20 °C for RNA extraction and gene expression analysis. Hemocyte samples for RNA extraction were also collected. Approximately 1 - 2 ml of hemolymph was extracted from the posterior adductor muscle, transferred to a 2 ml Eppendorf tube and centrifuged at 800 x g for 15 minutes at room temperature. Following centrifugation the supernatant was carefully decanted, the pellet resuspended in RNAlater and the tube placed on ice for transport to the laboratory where they were stored at -20 C until RNA extraction could take place.

3.2.3.2 RNA extraction

Total RNA was extracted from mussel gill tissue using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was quantified using Nanodrop 2000 (Thermo Fisher Scientific).

Haemocyte total RNA was extracted from RNAlater treated haemocyte pellets by resuspending the pellet and transferring it to a 15 ml RNase free tube. The resuspended pellet was further diluted with 3 X the volume of mussel physiological saline and centrifuged at 4 °C for 30 minutes at 2000 x g. Once the majority of the supernatant was removed, 600 µl of lysis buffer with β -mercaptoethanol was added and the lysate transferred to a 1.5 ml RNase free Eppendorf tube for homogenization and extraction

using the RNeasy Mini kit and the manufacturer's recommended instructions for animal cells. Total RNA was eluted into a 30 µl final volume and quantified using the Nanodrop 2000 (Thermo Fisher Scientific) spectrophotometer.

3.2.3.3 cDNA synthesis and reverse transcriptase polymerase chain reaction (RT-PCR)

First strand cDNA was synthesized from 1 µg of purified total RNA using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions.

RT-PCR was conducted to determine relative expression of antioxidant enzymes (SOD, GST, GSHpx) in mussel gill tissue and antimicrobial peptides (MGD2, mytilin b, myticin a) from mussel hemocytes. Primers (Table 3.1) were designed using MytiBase (<http://mussel.cribi.unipd.it/>) and Primer 3 software (<http://primer3.sourceforge.net/>). Expression profiles of genes of interest were compared to the expression of an appropriate normalizer gene (18s ribosomal RNA). PCRs were carried out using 2.5 µl of the cDNA and were performed in an Eppendorf Vapoprotect Mastercycler Pro thermocycler (Thermo Fisher Scientific) using the Taq PCR Core Kit (Qiagen) in a total reaction volume of 25 µl. The amplification conditions were: 3 minutes at 95°C; 30 cycles of 1 minute at 95°C, 1 minute at 50°C, 30 seconds at 72°C; and 10 minutes at 72°C. Amplification products were resolved on a 2% agarose gel containing ethidium bromide (Sigma; 500 µg/ml) and electrophoresed at 100 V for one hour (BioRad) and compared to a 1 Kb+ DNA ladder (Invitrogen).

3.2. 3 Statistical analysis

Neutral red data were analyzed using Sigmaplot 11.0 statistical and graphical software (Systat software). Data was tested for normality and the mean (+/- SE), one-way ANOVA and appropriate post hoc tests were calculated. Significance was set at $\alpha = 0.05$. Since the assumption of equivalence and normality were not met the Kruskal-Wallis One Way Analysis of Variance based on ranks was applied.

3. 3 RESULTS

3.3.1 Neutral Red

Mean neutral red retention times for the summer holding and field controls are shown in Figure 3.1A. There was a significant decline in NR retention time for the holding animals from 95 minutes initially to 19.5 minutes after one month in holding. The field control mussels also experienced a significant decline from 95 minutes initially to 13.5 minutes at the one month sample.

Results for the fall season are shown in Figure 3.1B. During the fall holding trial, neutral red retention time decreased from 100 minutes initially to 30 minutes after three months in holding. There was no significant change in NR retention time between September (initial) and December (three month) for the fall field control mussels.

Table 3.1: Primers used for RT-PCR.

| Primer | %GC | T _m (°C) | Amount of oligo (nM) | Sequence | Product size (bp) |
|---------------|-----|------------------------|----------------------------|---------------------------------|----------------------|
| 18s rRNA MT F | 45 | 53.0 | 30.6 | 5' – CACGCCTGGGAATTTTCTTA – 3' | 238 |
| 18s rRNA MT R | 55 | 56.8 | 31.6 | 5' – ACCCACACCGTGATTCTCTC – 3' | |
| 18s rRNA HE F | 50 | 54.5 | 26.3 | 5' – GCTTTGCCTTTCGGTACTTG – 3' | 212 |
| 18s rRNA HE R | 55 | 55.9 | 24.4 | 5' – CGGAGTAGTCGATCGTGGAT – 3' | |
| SOD F | 50 | 55.2 | 28.9 | 5' – ACTGCCAAACCTTCCGTATG – 3' | 240 |
| SOD R | 50 | 55.2 | 31.2 | 5' – AATCCTCCACCGTTGTTGAC – 3' | |
| GSH-px MT F | 40 | 51.2 | 39.3 | 5' – TTCATCAATTTTCGGCCTTC – 3' | 237 |
| GSH-px MT R | 45 | 52.8 | 28.2 | 5' – GGGCATTTCAGGATTTCTTCA – 3' | |
| GST F | 55 | 55.4 | 28.4 | 5' – GCCATTAGGACAGGTTCCAG – 3' | 208 |
| GST R | 45 | 54.5 | 31.5 | 5' – TGCCTTTTTAGTCGCATCCT – 3' | |
| Mytilin B F | 50 | 56.9 | 25.7 | 5' – ATGAGGCAGAGGCAAGTTGT – 3' | 247 |
| Mytilin B R | 50 | 54.9 | 32.4 | 5' – TCACCTTGTTTCGGTTTCTCC – 3' | |
| Myticin A F | 45 | 54.5 | 27.3 | 5' – TTGTACGAAACCCGTCAACA – 3' | 205 |
| Myticin A R | 50 | 55.8 | 29.0 | 5' – ACAGCTGCAGGACAAAATCC – 3' | |
| MGD2 F | 55 | 57.1 | 18.7 | 5' – GATGCACGGGAGGTTACTGT – 3' | 211 |
| MGD2 R | 50 | 53.9 | 33.6 | 5' – CTGCAGCCTGCTTATGATTG – 3' | |

No significant difference was observed during the winter trial for either the holding or field samples (Fig. 3.1C). At the initial sample time mean NR retention time was 76.36 minutes. Although the average time declined to 47.27 minutes after three months in holding the effect was not significant (One-way ANOVA $p < 0.05$). The mean retention time decreased to 67.58 minutes for the three month field sample in April.

During the spring trial there was also no significant change in retention time for either the holding or field samples (Fig. 3.1D). Mean retention time was 71.25 minutes decreasing to 49.00 minutes for mussels in holding and 68.75 minutes for the field samples at the two month sample time.

3.3.2 Antioxidant gene expression

The relative expression of SOD for each season is shown in Figure 3.2. Relative mRNA expression appeared stronger at each sample time throughout the summer with lowest at the initial sample and highest levels after one month in holding (Fig. 3.2A). During the fall SOD was consistently present and appeared to be at high expression levels throughout but weaker during holding compared to the field control animals (Fig. 3.2B). During winter SOD was present at the initial sample time and absent from the following samples during the holding trial while expression was present for the field samples at one week, one month, and three month (Fig. 3.2C). During the spring expression of SOD was observed, but appeared weaker than in the summer and fall. Expression appeared stronger at the one week field sample compared to holding (Fig. 3.2D).

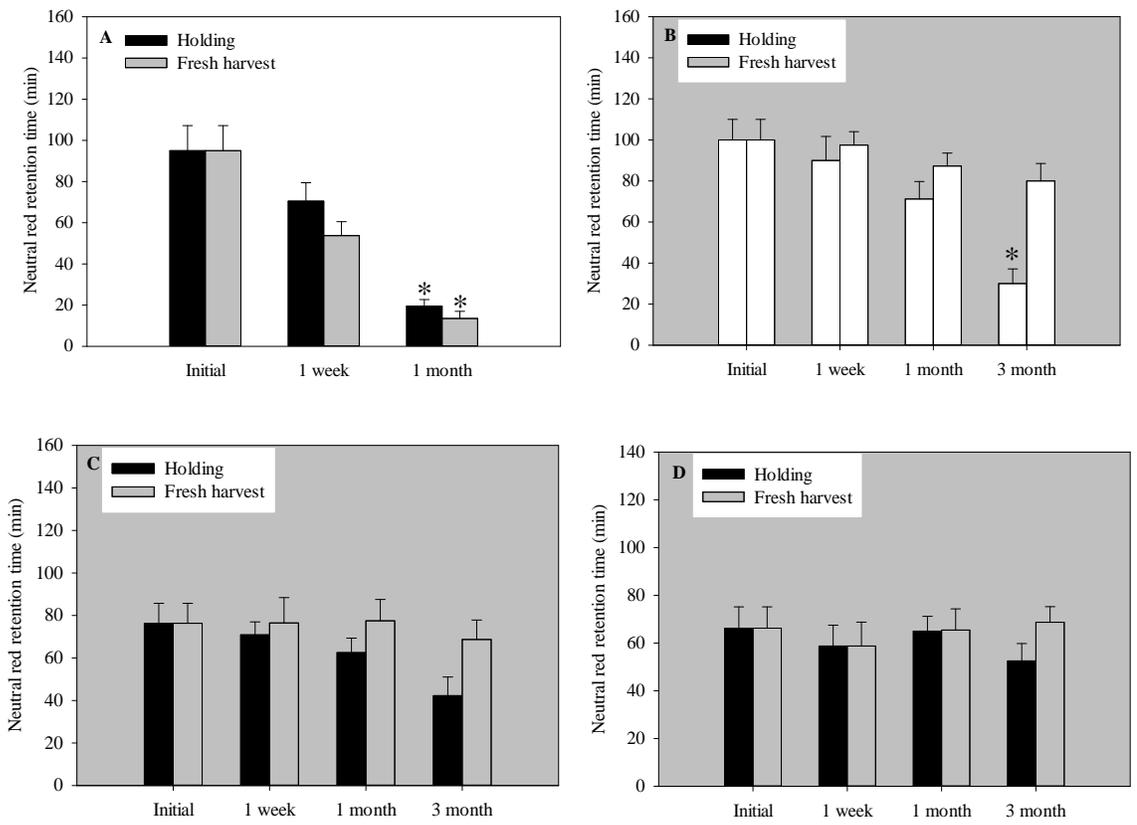


Figure 3.1: Average neutral red retention time (min) for mussels in extended holding during the summer (A), fall (B), winter (C), and spring (D). ($n = 12$, $p = 0.05$). (*) Indicates statistical significance over time for that group.

Relative seasonal expression of GST is shown in Figure 3.3. During the summer expression of GST was present at the initial sample and the final one month holding sample (Fig. 3.3A). Expression was also present in the field samples however was higher at one week compared to one month (Fig. 3.3A). During the fall GST was present in all holding sample times however only in the one week field sample, and levels appeared to be high compared to the other seasons (Fig. 3.3B). GST expression was weak during the winter although present at all sample times in both treatments (Fig. 3.3C). During the spring GST expression was lowest compared to the other three seasons. Expression was visible at one week and one month in holding and also present at the one week field sample (Fig. 3.3D).

Seasonal expression of GSHpx mRNA can be seen in Figure 3.4. During the summer GSHpx was present throughout the season in both holding and field animals however expression in the initial sample appeared to be the lowest (Fig. 3.4A). Expression was also present throughout the fall and again like SOD and GST expression appeared to be the highest during this season (Fig. 3.4B). During the winter expression was present at all sample times with little difference between the holding and field animals (Fig. 3.4C). During the spring GSHpx was present at every sample time for both treatments although weakest at one month field (Fig. 3.4D). In general relative expression was lowest during the winter and spring months.

3.3.3 Expression of innate immune peptides

Seasonal variation in myticin A gene expression is shown in Figure 3.5. During the summer expression was apparent at the initial and one week sample in June for both holding and field samples. At the one month sample a band was present in both holding and field control however it was faint compared to the other sample times and the 18S rRNA gene (Fig. 3.5A). During the fall expression was visible at all sample times for both treatments (Fig. 3.5B). During the winter however there was an apparent decline in initial expression compared to the final fall sample taken one month prior. Expression was only visible after three months in holding and a faint band was also visible at the one week field sample (Fig. 3.5C). During the spring expression was visible at the initial sample and the final holding sample and the one week field sample, although faint (Fig. 3.5D).

Seasonal variation in MGD2 mRNA expression is shown in Figure 3.6. MGD2 was not present initially during the summer but was present after one week and one month holding sample and not expressed in the field samples at any time (Fig. 3.6). During the fall MGD2 was present after three months in holding as well as at the one week field sample in September. There was also a faint band present at the three month field sample in December (Fig. 3.6B). During the winter there was a faint band present at the initial sample, similar expression to the final fall sample, and at the three month field sample. No other sample times showed evidence of MGD2 expression during this season (Fig. 3.6C). During the spring MGD2 was not present from the initial sample or through time in holding. Expression gradually increased over time in the field samples however with the greatest intensity visible at the two month field sample (Fig. 3.6D).

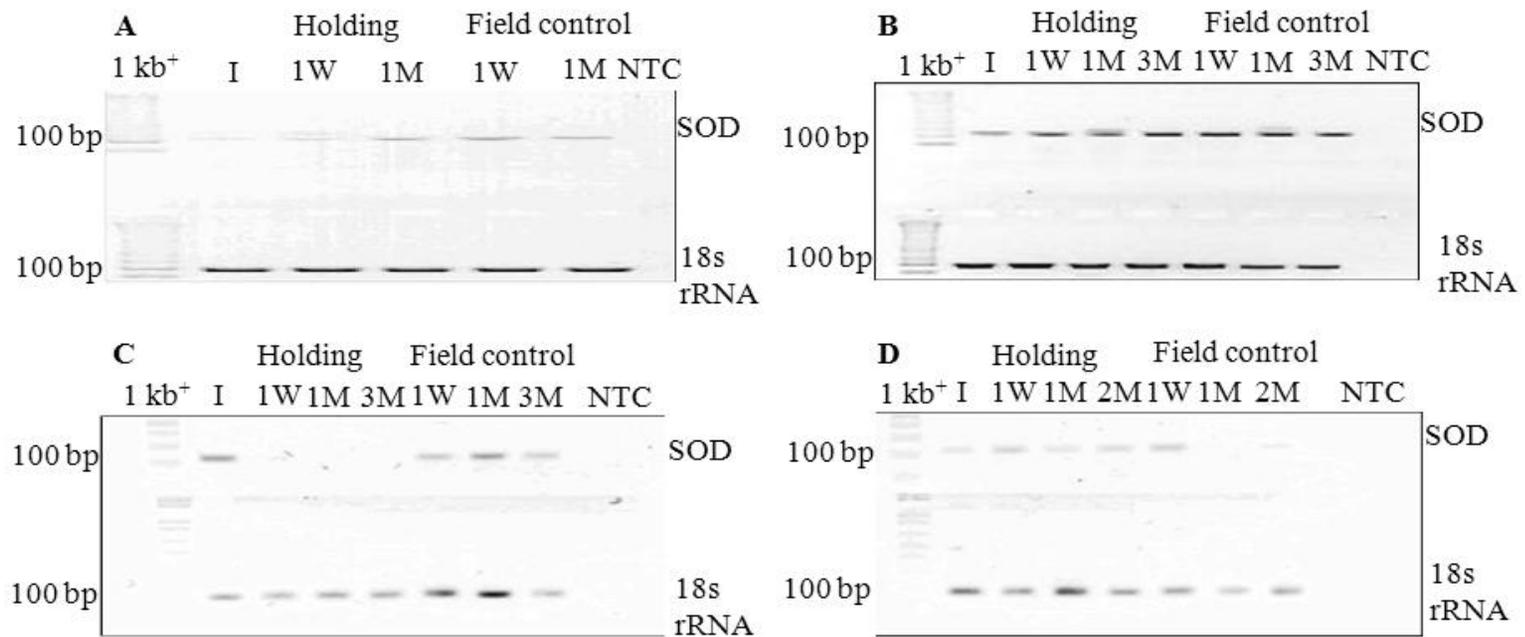


Figure 3. 2: Expression of superoxide dismutase (240 bp) and 18S rRNA MT (238 bp) in *Mytilus edulis* gill tissue for summer (A) fall (B), winter (C), and spring (D) seasons. Visualized on a 2% agarose gel (20 μ l 500 μ g/ml EtBr) displaying semi-quantitative rt-PCR amplification products. 1 kb+ DNA ladder, initial (I), one week (1W), one month (1M), two month (2M), three month (3M), negative control (NTC).

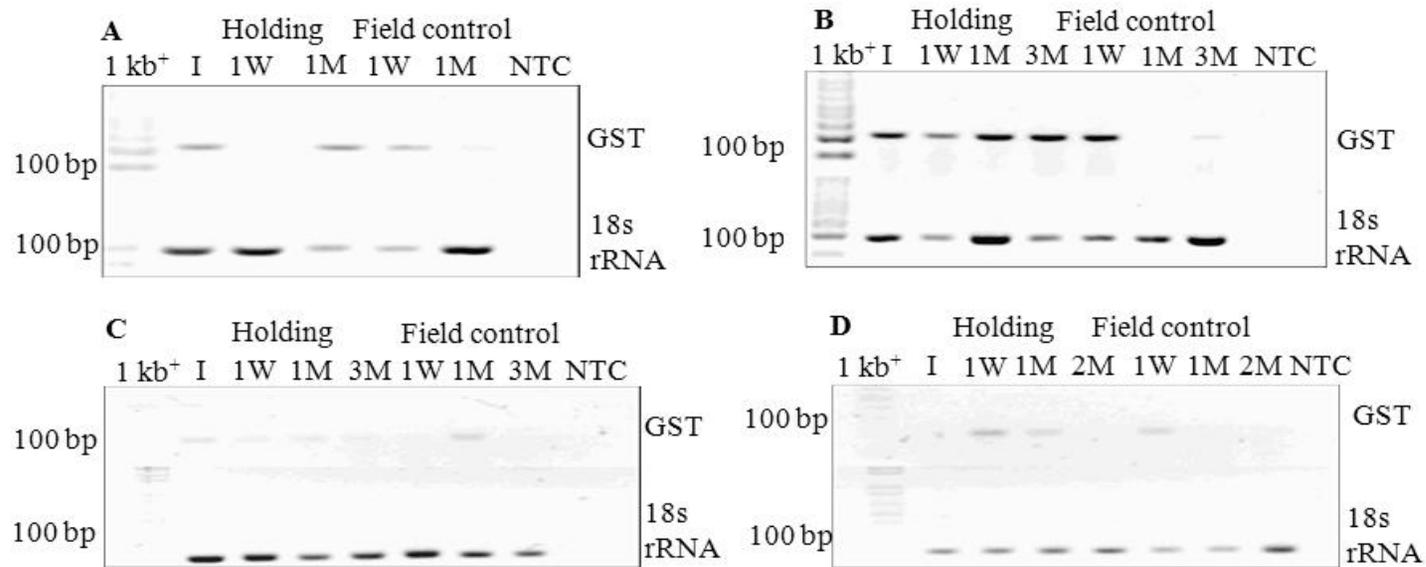


Figure 3.3: Expression of glutathione-s-transferase (208 bp) and 18S rRNA MT (238 bp) in *Mytilus edulis* gill tissue for summer (A) fall (B), winter (C) and spring (D) holding and field control mussels. Visualized on a 2% agarose gel (20 μ l 500 μ g/ml EtBr) displaying semi-quantitative rt-PCR amplification products. 1 kb+ DNA ladder, initial (I), one week (1W), one month (1M), two month (2M), three month (3M), negative control (NTC).

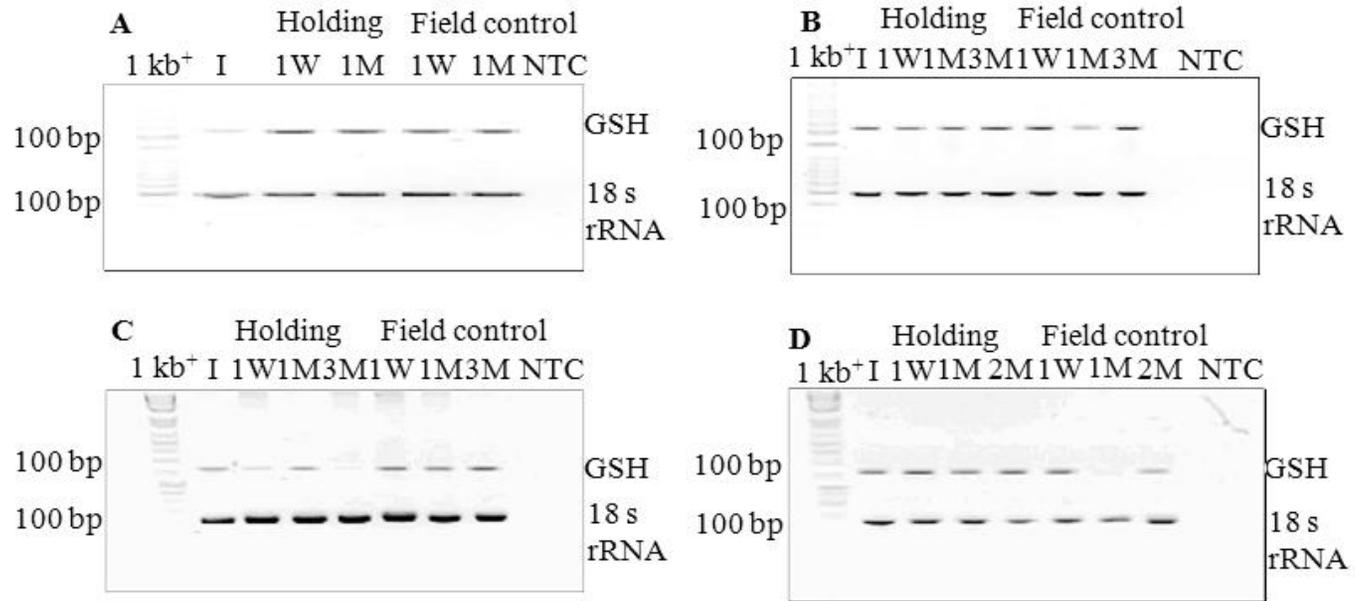


Figure 3.4: Expression of GSH-peroxidase (237 bp) and 18S rRNA MT (238 bp) in *Mytilus edulis* hemocytes summer (A), fall (B), winter (C), and spring (D) holding and field control mussels. Visualized on a 2% agarose gel (20 μ l 500 μ g/ml EtBr) displaying semi-quantitative rt-PCR amplification products. 1 kb+ DNA ladder, initial (I), one week (1W), one month (1M), two month (2M), three month (3M), negative control (NTC).

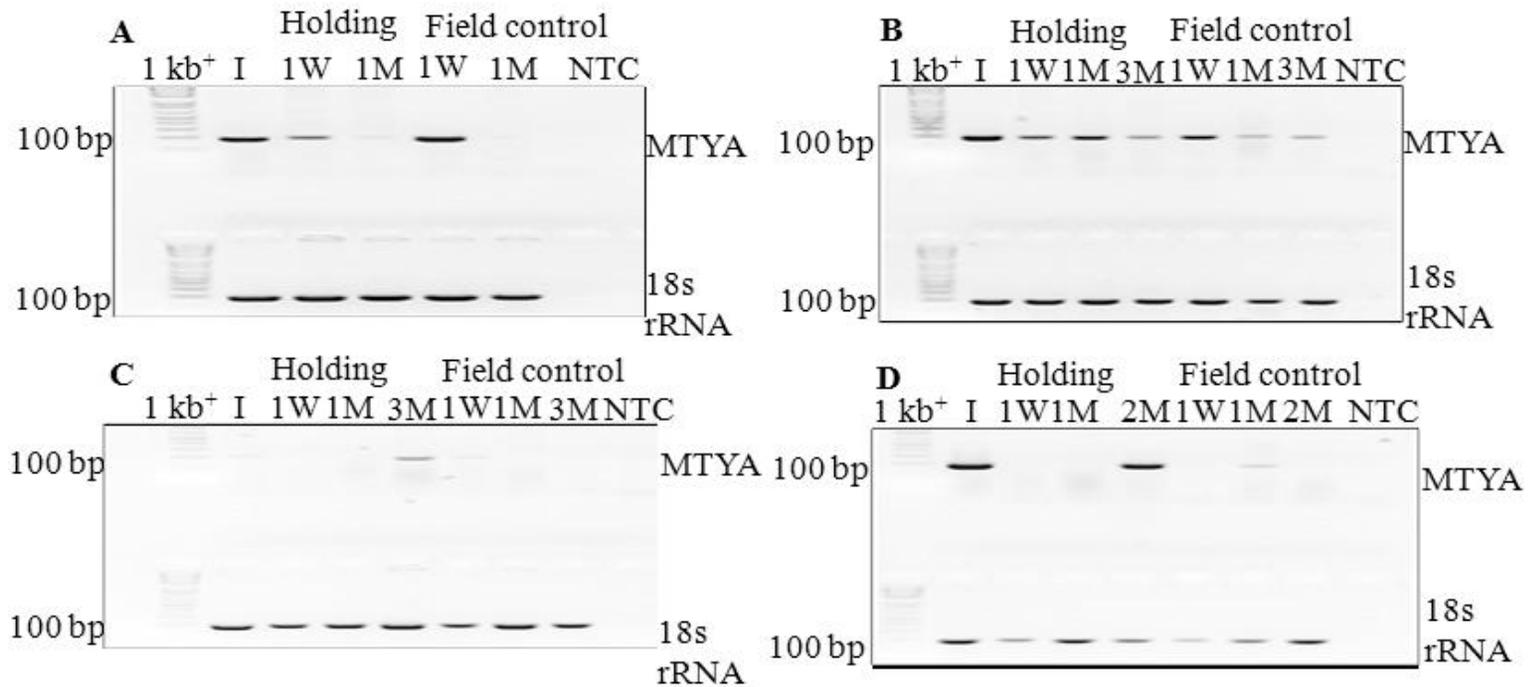


Figure 3.5: Expression of myticin a (211 bp) and 18S rRNA HE (212 bp) in *Mytilus edulis* hemocytes for summer (A), fall (B), winter (C), and spring (D) holding and field control mussels. Visualized on a 2% agarose gel (20 μ l 500 μ g/ml EtBr) displaying semi-quantitative rt-PCR amplification products. 1 kb+ DNA ladder, initial (I), one week (1W), one month (1M), two month (2M), three month (3M), negative control (NTC).

Mytilin B expression for each season is shown in Figure 3.7. Expression was present at all sample times, regardless of treatment, excluding the one week winter field and one week spring field samples. Trends in seasonal variation are difficult to determine as each season showed intense bands over time however they appear weakest during the spring.

3.3 DISCUSSION

Post-harvest storage and handling procedures have been known to negatively affect mussels and cause an increase in stress response (Harding *et al.* 2004; Tremblett 2001). Previous studies have shown that post-harvest storage in water under ambient temperature has a less stressful effect compared to storage on ice or in chilled air (Harding *et al.* 2004) however the maximum holding time studied was up to one week. The current study was designed to assess the effect of extended holding on the stress response of cultured blue mussels held in a processing facility for up to three months during the summer, fall, winter, and spring. Since environmental and mechanical stressors can also affect antioxidant enzymes and the immune response of mussels (Malagoli *et al.* 2007; Sheehan & Power 1999) this study also looked at expression of key antioxidant enzymes and antimicrobial peptides over time in holding compared to field controls as well as between seasons.

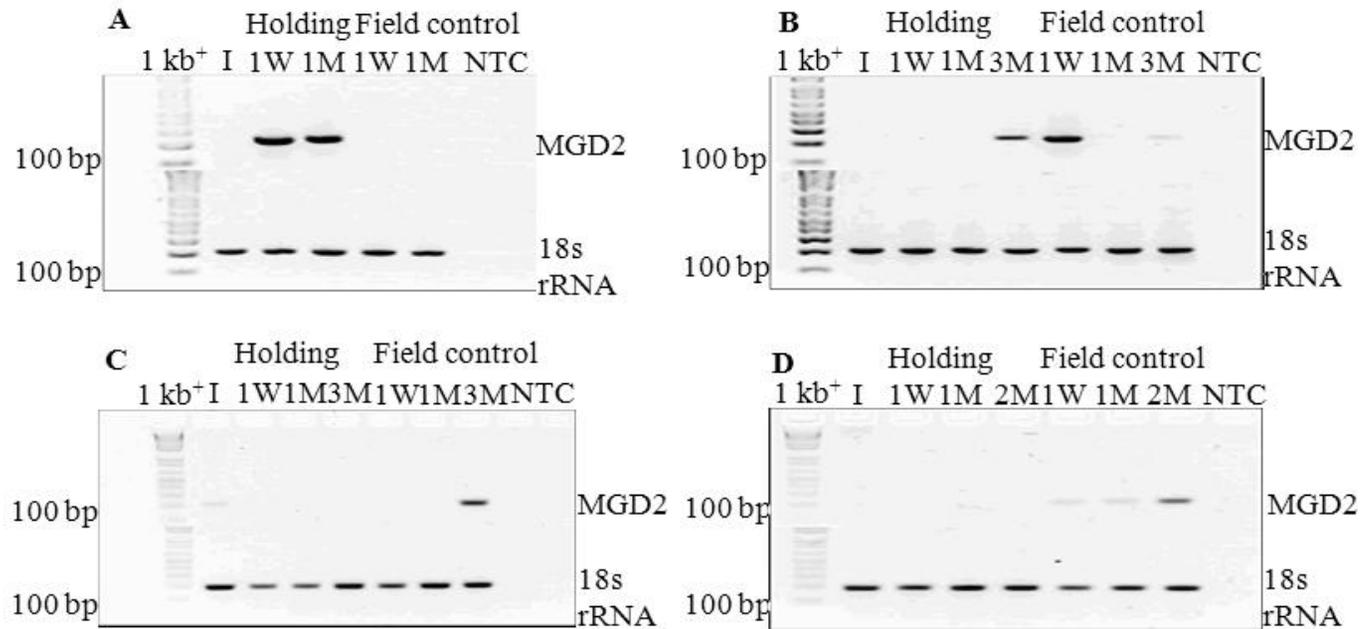


Figure 3.6: Expression of MGD2 (211 bp) and 18S rRNA HE (212 bp) in *Mytilus edulis* hemocytes for summer (A), fall (B), winter (C), and spring (D) holding and field control mussels. Visualized on a 2% agarose gel (20 μ l 500 μ g/ml EtBr) displaying semi-quantitative rt-PCR amplification products. 1 kb+ DNA ladder, initial (I), one week (1W), one month (1M), three month (3M), negative control (NTC).

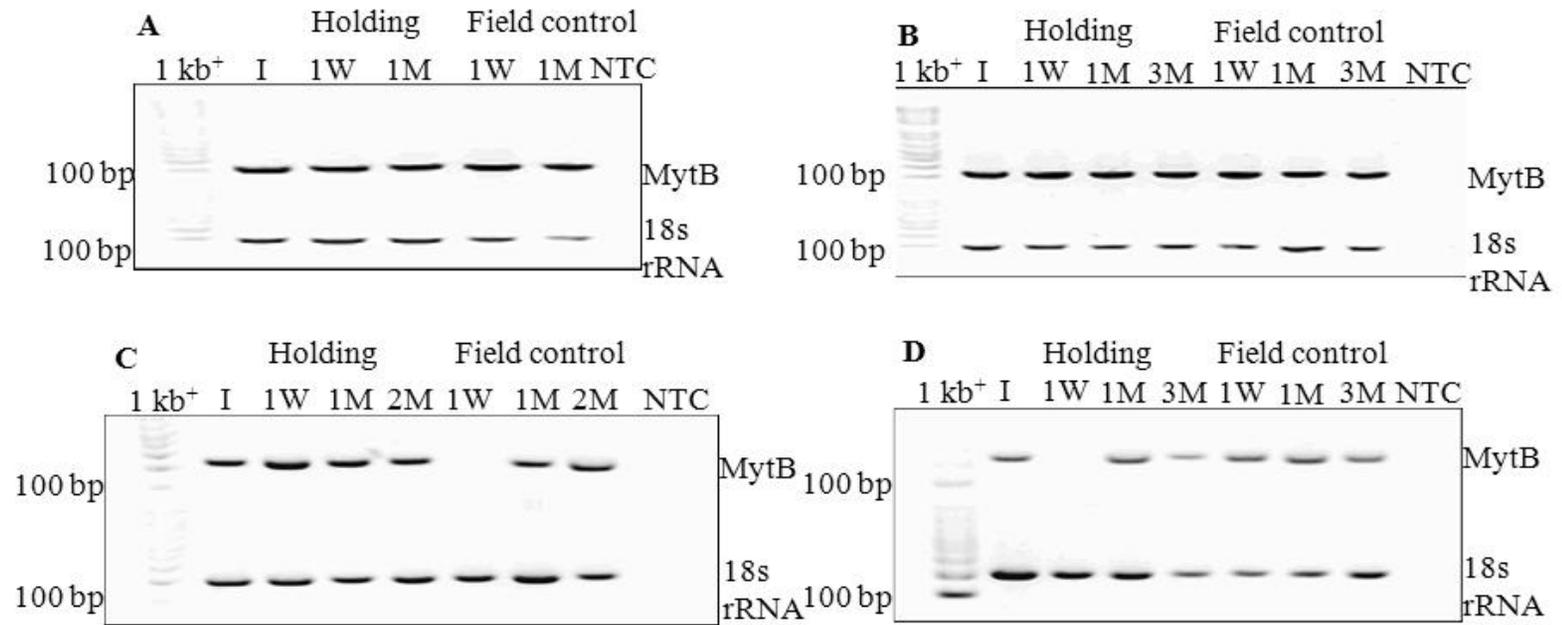


Figure 3.7: Expression of mytilin b (247 bp) and 18S rRNA HE (212 bp) in *Mytilus edulis* hemocytes for summer (A), fall (B), winter (C), and spring (D) holding and field control mussels. Visualized on a 2% agarose gel (20 μ l 500 μ g/ml EtBr) displaying semi-quantitative rt-PCR amplification products. 1 kb+ DNA ladder, initial (I), one week (1W), one month (1M), three month (3M), negative control (NTC).

Neutral red retention time is a useful technique to evaluate the stress response in bivalves. This technique has been used previously to determine seasonal effects on the stress response of blue mussels (Harding *et al.* 2004), the effects of post-harvest processing activities (Harding *et al.* 2004), and effects due to spawning (Song *et al.* 2007). This study used the neutral red assay technique to determine the effect of extended holding and seasonality on the stress response of the blue mussel followed by an evaluation of oxidative stress and immune gene expression. Rapid changes in temperature have been shown to destabilize lysosomal membrane stability and prolong recovery in oysters (Zhang *et al.* 2006). Prolonged air exposure also has the potential to negatively affect lysosome integrity although only when transferred to significantly higher or lower air temperatures, if maintaining animals at similar air and water temperatures the effect is negligible (Zhang *et al.* 2006).

Our results show a significant decline in neutral red retention during the summer for both holding samples and field controls. This is most likely a result of high water temperature, outside the acceptable physiological range; we can therefore not conclude if the increased stress response for the mussels in holding was due to extended time spent in holding alone. Results from Harding *et al.* (2004b) also show NRR to be low during the summer. During the fall the animals in extended holding also showed a significant decline in neutral red retention compared to the field control however the effect was only significant after three months. There was a decline in water temperature from approximately 15°C in September to a low of 2°C in December, however the effect was gradual and did not affect the field animals. During the winter and spring there was no sharp change in temperature: averages remained between 2°C and 5°C. As a result

neither group showed a significant decline in NRR time. Since the animals in holding ideally experience similar water temperatures and nutrient levels as the animals in the field, both should respond to the high water temperature in a similar fashion. The decline in both groups during the summer is likely a result of stressful conditions imposed by the high water temperatures in conjunction with other stressful factors associated with extended holding. The decline only observed in the fall holding and not observed in the field animals, suggests that holding in combination with the change in water temperature from the initiation of the experiment in September to the final sample in December affected the animals stress response.

Seasonal variation and response of antioxidant enzyme levels to contaminants are well established (Power & Sheehan 1996; Viarengo *et al.* 1991). This study examined seasonal variations in antioxidant mRNA expression as well as changes in expression as a result of extended storage under ambient conditions compared to field animals. Expression of antioxidant enzymes SOD and GST was highest during the late fall with lowest expression during the winter for both holding and field control. For each season relative expression of GST appeared higher for samples in holding compared to the samples in the field indicating seasonality combined with extended holding affected the response of this gene, however this was the only antioxidant where this pattern was found. Interestingly, during winter, expression of SOD and GST was not present at the same intensity in the holding animals compared to the field animals. In order to better understand the relationship of enzyme levels to gene expression within the tissue of the animal, further studies should compare both enzyme levels of specific tissues to real time expression levels within that tissue.

Expression of MGD2 and mytilin A showed seasonal variation as well as differences between holding and field animals. For both proteins, expression was stronger for animals in holding compared to the field samples and during the fall for mytilin A, suggesting the possibility of bacterial loading over time in extended holding. Interestingly, during the winter the final field sample showed an increase in MGD2 expression. The increase in antioxidant enzyme expression and innate immune gene expression during the winter field samples indicates stressful environmental conditions for the field animals at this time. This could possibly be due to winter ice cover and nutritive stress affecting their overall physiology resulting in an immune response (Hatcher *et al.* 1997). Expression of mytilin B was consistent regardless of season or treatment. These results are consistent with a previous study by Li *et al.* (2009) who found that expression of mytilin B was constant and not regulated by season. Li *et al.* (2009) also found defensin (MGD2) to be expressed during the summer months however these results are in contrast to a previous study by Roch (2001) who found defensin to be expressed constitutively during the winter months.

In conclusion the effect of extended holding on the stress response and immune function was only significant during the summer and fall seasons after a prolonged period in holding. Seasonal variations in oxidative stress genes were apparent for SOD and GST.

CHAPTER 4: GENERAL DISCUSSION

As the aquaculture industry expands and culture methods evolve, new obstacles arise, and the mussel aquaculture industry is no exception. In Newfoundland, fresh product is stored and shipped live to local, national, and international markets. As a result of living on an island with frequent weather issues transportation delays often occur. As a result growers are finding they may need to keep live animals in extended holding until transportation is possible. Commonly, live animals are held for a brief period of time following processing while awaiting transportation (Harding *et al.* 2004). Bivalves like all living organisms are subject to the environmental conditions imposed on them. Knowledge of the extrinsic environment in which bivalves are locally cultured and stored is crucial to maximizing yield and profit while minimizing losses. Growers have to not only understand how the environment affects shellfish health, but must also be aware of other potentially detrimental practices involved in long-term ambient storage that can impact shellfish health as well.

Several post-harvest and post-processing storage methods are commonly used in the mussel industry including on ice, in chilled ambient air, or wet-storage (Harding *et al.* 2004a; Tremblett 2001). It is crucial to the industry to determine which method will have the least impact on health and shelf life and also to determine the maximum time allowed in extended storage before there is a decline in overall animal health. Harding *et al.* (2004a) found ambient wet-storage to have the least drastic effect on stress response compared to the other two storage methods and found it advantageous to store processed

mussels for a period of 7 to 10 days before transportation to market. Our research assessed how extended storage under ambient conditions (no internal temperature control) affected the physiology and stress response of cultured blue mussels in order to recommend acceptable holding limits to the industry. Harvested mussels were held in a commercial facility for up to two or three months and sampled periodically at the beginning of each season (summer, fall, winter, spring). Several parameters were assessed over time in holding including changes in meat weight and condition index, stress response, and gene expression of antioxidant and immune genes and compared to field controls that were sampled simultaneously with those in holding.

An increase in stress response can be triggered by several factors including changes in temperature (Bayne 1973; Dare & Edwards 1975), reproduction (Bayne 1975) ice cover (Hatcher *et al.* 1997) and contaminants (Lowe 1988). In general an increase in stress response results in a decline in body condition, meat weight, and can lead to a decreased shelf life and meat quality. The condition of mussels is also known to vary seasonally (Dare & Edwards 1975; Lutz *et al.* 1980; Okumu & Stirling 1988) and to be affected by other factors including those named above.

4.1 SUMMER

Summer is a particularly challenging season for the shellfish industry as high temperatures can lead to an increase in stress response and mortality. *Mytilus edulis* is a cold water species preferring cooler water temperatures however in sheltered Newfoundland bays water temperature can be above their tolerable range (i.e. between 15°C and 20°C) for extended periods of time during the summer months. In many areas

in Atlantic Canada and around the world summer mortality can be an issue. During this time there can be a significant die-off of adults and juveniles due to high temperatures, spawning, or a combination of factors, although many studies suggest the susceptibility of summer mortalities is stock-dependent (Mallet *et al.* 1990). While the field animals in this experiment did not exhibit any signs of summer mortality both the holding tanks in the facility suffered mass mortalities in August 2010 after two months in holding. Comparisons between holding and field controls reveal a decline in condition over time in holding and an increase in condition observed for the field control. The discrepancy is likely the result of high temperatures combined with a limited nutrient availability.

A decline in Neutral Red Retention time for both field and holding indicates an increase in stress response in both conditions, likely as a result of increased temperature but there is also the possibility of an accumulation of biological waste in the holding tanks. Indeed the antimicrobial peptides MGD2 and myticin A were expressed more often in the holding animals compared to the field controls. A previous study by Li *et al.* (2009) also showed defensin and myticin A expression to be highest during the summer as revealed by QPCR, however this is in contrast to a previous study by Roch (2001) in which Northern blot analysis revealed MGD2 levels to be undetectable during the summer. While high temperatures appeared to have a slight negative effect on the field animals, the effect was more profound in holding suggesting that holding under ambient temperatures during the summer months should be limited to one week.

4.1 FALL

Temperature range during the fall season is more tolerable and declined at a constant rate over the three month period. Weight and condition tend to be high during the fall as well and decline with declining temperature (Bayne & Thompson 1970). Our results show a decline in condition and dry tissue weight over time in holding and an increase in wet tissue weight. The increase in wet tissue weight during this time could signify state of nutritive stress experienced by the animals as the intake of water is a compensation mechanism during a time of nutrient depletion.

The decline in neutral red retention over time further indicates that holding has a negative effect on mussel physiology during the fall. When compared to the field controls, condition and tissue weight varied at each and there was no change in neutral red retention. Expression of oxidative stress genes SOD and GSHpx appear stronger during the fall compared to the other seasons however there is no discernable difference in expression between holding and field controls. The antimicrobial peptide MGD was expressed after three months in holding and at the one week field control which suggests that extended holding affected the expression of this gene. The appearance at one week could be the result of a storm surge or fall bloom, however, since chlorophyll samples were not taken at this time it is difficult to discern the cause. Expression of AMPs myticin A and mytilin B in both holding and field control suggests no observable effect due to treatment. During the fall season it therefore appears that extended holding under ambient temperature had a negative impact on mussel physiology after a period of one month as indicated by the decline in tissue weight over time and increase in stress response.

4.3 WINTER

During the winter in northern climates, including Newfoundland, water temperatures are cold ($\sim 1^{\circ}\text{C}$), nutrient levels are low, and ice cover can be common. During this time mussels exhibit low metabolic rates and growth is generally slow or ceases altogether (Hatcher *et al.* 1997). Winter ice-cover has been an issue in Notre Dame Bay and particularly at the culture site used in this experiment (Terry Mills: personal communication). The field control growth and condition results were to be expected where tissue weight and condition declined over the three month period from January to April. The holding animals however showed markedly different results. Over time in holding there was a significant increase in both wet and dry tissue weight as well as condition over time.

Neutral red assay revealed no significant change in stress response in holding or in the field, despite the change in condition. Relative expression of SOD and GST appeared lowest during the winter months. These results are somewhat consistent with Power & Sheehan (1996) who found GST enzyme levels to peak in December and are at their lowest during the winter in the gill tissue of the blue mussel. Viarengo *et al.* (1991) also found SOD enzyme levels to be lowest during the winter in the digestive gland of mussels. In the current experiment SOD was expressed at the initial sample in January but in none of the subsequent holding samples however expression was present in each field control sample further suggesting that the field animals were possibly under a greater degree of stress compared to the holding animals. Expression of the antimicrobial peptide MGD2 was present at the initial and final field sample, and strongest at the latter. Myticin A however was strongest at the final holding sample. When combining the

cumulative results of the winter experiment we cannot conclude that extended holding has a negative impact on mussel health during this season. These results are not what we expected as the winter season generally coincides with less than ideal environmental conditions including ice cover and low food availability.

4.4 SPRING

The spring season marks a time of warming water temperatures, an increase in primary production, and consequently the onset of gametogenesis for most mussel populations in Newfoundland. These conditions result in an increase of favourable conditions resulting in an increase in body mass and condition. The morphometric trends observed during the spring season are similar to those for the summer and fall. There was an increase in wet tissue weight over time in holding coupled with a decrease in dry tissue weight again signifying a state of accumulation of water and nutrient depletion. The results for the field control are drastically different. The one week sample in April marked the highest dry tissue weight and condition index of the entire year. These animals were likely ready to spawn at this time, which would account for their increased tissue weight.

There was no change in stress response for either the holding or field mussels as indicated by the neutral red assay. Expression of SOD and GST were low in the spring compared to the other seasons and present more often in the holding samples compared to the field controls. Likewise expression of myticin a is highest at the initial and final holding samples. Expression of MGD2 however was more common in the spring field controls. These findings could be the result of a spring bloom in the area causing an increase in bacteria, however these factors were not measured over the course of this

study. As with the summer and fall, extended holding is not recommended for longer than a period of one month. After this time there is a decline in dry tissue weight making it disadvantageous and unprofitable for growers to maintain animals any longer.

4.4 CONCLUSIONS AND RECOMMENDATIONS

Our study was successful in determining a maximum allowable time for live mussels to be maintained in extended live holding. We were able to make recommendations for each season based on the results from the various parameters that were measured. Changes in condition and tissue weight over time in holding suggest that one month is the maximum time mussels should be held under ambient water temperature. After this point there is a significant decline in these parameters. During the summer the rapid decline in condition and tissue weight over time in holding indicates one week is the maximum time when water temperatures are high and unfavorable. Seasonal variations in condition and tissue weight were at their highest during the spring and at their lowest during the winter, as indicated by trends in the field control animals. The neutral red assay showed a significant increase in stress response after one month in holding during the summer and fall, further supporting that one month is the maximum recommended time to maintain animals under ambient conditions. There was no change over time in holding during the winter and the spring or for field controls however one month is still the maximum suggested time due to the decline in condition during these seasons. Expression of oxidative stress and immune genes did not reveal major changes as a result of holding, however seasonality was apparent.

Although many studies examine effects of temperature and nutritive stress on the condition and physiology of mussels most of these studies occur in a laboratory setting. To further understand if nutritive stress is a factor in the holding facility it would be advantageous to simultaneously assess the chlorophyll levels both in the facility and on the site to better understand and compare the nutrient supply. Future studies could assess the effect of extended holding under ambient conditions on biochemical parameters as well to develop a better understanding of how holding affects physiology. One ongoing study is assessing the effect of extended holding on the palatability of mussels as well as certain biochemical parameters including glycogen content and lipid profile.

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