EVALUATION OF THE NEUTRAL RED ASSAY AS A STRESS RESPONSE INDICATOR IN MUSSELS (MYTILUS SPP.) IN RELATION TO SEASONAL, ENVIRONMENTAL, HANDLING, HARVESTING, PROCESSING, AND POST-HARVEST STORAGE CONDITIONS

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by

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Abstract

The neutral red assay (NRA) measures retention time of the neutral red dye in the hemocyte organelle, the lysosome, which can be correlated to the condition of a mussel under stressful circumstances. Shelf life and standard meat yield also provide an indication of mussel physiological condition. The objectives of this study were to evaluate mussel stress response, as assessed by the NRA and shelf life, in relation to 1) seasonal and environmental changes, 2) handling, 3) processing, and 4) post-harvest storage practices. Neutral red retention (NRR) levels (a measurement of stress response) and shelf life in mussels were reduced in late summer compared to early summer, and increased in autumn / early winter, indicating a seasonal pattern in NRR levels. NRR also showed a in correlation to the reproductive cycle (spawning). Harvested mussels exhibited a decrease in NRR during extended air exposure (up to 8 hours), especially when held at air temperatures above and below air temperatures comparable to ambient water temperatures. NRR was altered in mussels that were washed and declumped compared to unprocessed mussels. However, the process of debyssing significantly decreased NRR in mussels. Mussels held under chilled or iced storage conditions displayed lower NRR levels compared to those of mussels held under wet storage. These results demonstrate that the NRA is a useful index of physiological stress response in mussels subjected to conditions under various culture conditions and practices. Researchers and growers to define conditions that are beneficial or detrimental to optimal mussel culture production can use the NRA.

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Table of Contents

List of Tables

List of Figures

1. Introduction

1.1 Canadian and Newfoundland mussel industries

The blue mussel (*Mytilus* spp.) culture industry in Canada has been on a rapid increase since the mid 1980's. In 2000, Canadian production of cultured mussels was 21,287 tonnes with a value of $27,213,000 (DFO 2001) (Figure 1). The major contributor to this value Prince Edward Island (PEI), where production was 17, 895 tonnes with a value of $21,703,000. However, the carrying capacity for some waters around PEI has been reached and production values have levelled off (McDonald *et al.* 2002). Production values for the rest of Atlantic Canada continue to grow, with reported landings in 2000 from Newfoundland (refers to the island portion of the province of Newfoundland and Labrador) of 1,051 tonnes, valued at $2,700,000, Nova Scotia produced 1,252 tonnes, valued at $1,442,000 and New Brunswick produced 750 tonnes, valued at $825,000 (Figure 2).

The blue mussel has the highest production value of all cultured shellfish produced in Canada including the province of Newfoundland and Labrador. Commercial culture of these mussels began over 10 years ago, following several years of experimental trials. In 1989 the aquaculture industry in Newfoundland produced 70 tonnes of mussels: by 1997 this was up to 683 tonnes, and in 1999 production reached 1,700 tonnes (DFO 2001). The industry has continued to grow each year with production figures expected to continue to rise until carrying and production capacity is reached.

With the onset of various industry support programs, such as the spatfall monitoring program, environmental and biological monitoring program, and product quality programs, the overall quality and quantity of Newfoundland blue mussels continues to improve (Clemens *et al.* 1999, Macneill *et al.* 1999). Many local growers are getting involved in processing and selling of their products, and advances in marketing Newfoundland product, have contributed o the growth of the industry in Newfoundland and its role as a major supplier of Canadian mussels. With such increasing demands on the Newfoundland mussel industry, a better understanding is needed of how production activities, such as seed collection, grading, socking, harvesting, transport, processing and shipping, affect mussels. Such knowledge is required to identify conditions and activities that may adversely impact mussels and reduce the quality of the product.

1.2 Definition and effects of stress

The terminology used in studies examining stress-related issues in mussels has changed and been redefined numerous times, often reflecting the objectives of the individual studies. Further confusion about the terms "stress" and "stress response" comes from their use without being clearly defined. It has been stated that stress is induced when an environmental change alters the biological or physiological state of the animal (Bayne *et al.* 1976, Bayne *et al.* 1979, Akberali and Trueman 1985, Koehn and Bayne 1989). The stress response of animals is dynamic, and results in the alteration of functional properties (behavioural, biochemical, or cytological) that can be quantified and

correlated specific environmental stimuli (Bayne *et al.* 1976, Koehn and Bayne 1989). Simply put, stress has been described as any environmental stimulus that disturbs the normal physiological function of an organism. Any reaction by the organism to this stress is defined as the stress response (Bayne 1985). The following definitions have been formulated for the present thesis based on adaptations of several publications on this issue:

Stress is any factor, externally or internally imposed on an organism, that induces a change at any number of functional levels (subcellular, cellular, or within the whole organism) required by the organism to maintain homeostasis.

Stress response is any alteration of a functional property (behavioural, physiological, or biochemical) that is a reaction to stress, and may be reversible or irreversible in their nature.

1.2.1 Effects of stress

There has been much research on stress in shellfish. Previous experiments have examined the stress response of wild mussels to different conditions, such as hydrocarbon exposure, salinity or temperature changes (Bayne 1973, Moore *et al.* 1979, Bayne *et al.* 1981a, b, Lowe *et al.* 1981, Tripp *et al.* 1984, Hole *et al.* 1992, Regoli 1992, Lowe *et al.* 1995a, Grundy *et al.* 1996, Moore *et al.* 1996, Hauton *et al.* 1998, 2001). However, research on stress in cultured mussels is relatively limited. Bivalves, such as mussels, are exposed to a variety of physical and environmental conditions during the culture process, including handling, mechanical grading and sorting, exposure to air of various

temperatures, fluctuating salinity, fluctuating water temperatures and turbidity (Slabyj 1980, Akberali and Trueman 1985, Prochazka and Griffiths 1991, Maguire *et al.* 1999, Sarkis *et al.* 1999). With the understanding that culture conditions can vary from the natural growing conditions and habitat compared to that of wild mussels (i.e., exposure of air, sun, rain, wind, etc. on intertidal mussels during tidal action), it can be predicted that some practices may be a stress to mussels and that some sort of stress response will be elicited.

To date, most studies on stress in mussels have examined stress responses at the whole animal level as a change in performance such as growth, condition (i.e., meat texture, yield and appearance), and / or quality (Bayne *et al.* 1976, Akberali and Trueman 1985, Koehn and Bayne 1989). The expression of stress at the whole animal level, however, is the product of a number of cascading events that start at the cellular and subcellular levels. Changes at the various functional levels continue and compound until stress conditions are stopped or altered, or if the animal is able to compensate in such a way to maintain homeostasis and avoid further stress responses.

Although a given stress response can be reversed by physiological compensation, the methods of compensation can be metabolically costly and induce other forms of stress, and collectively the resultant stress responses have detrimental effects. Animals undergoing stress response are more susceptible to changing environmental conditions, and multiple stress responses can be quickly reflected in reduced growth, reproductive failure and mortality (Bayne *et al.* 1976, Akberali and Trueman 1985, Koehn and Bayne 1989).

Stress response can be measured at the various functional levels, and possible outcomes for the animal can be predicted. Many studies have shown early stress responses in wild mussels can be measured at the cellular and subcellular level (e.g., Moore 1982, Moore 1990). These studies focused on determining changes in, or appearance of, proteins, oxygen (O_2) consumption and ammonia production, hemocyte activity, and lysosomal activities when a bivalve is exposed to specific stressors (i.e., chemicals, air, temperature changes, reproductive strategies). For example, two-dimensional gel electrophoresis has shown differences in protein profiles between intertidal and cultured Mediterranean mussels (*Mytilus galloprovincialis*). Researchers were attempting to show that a range of different environmental conditions experienced by the two populations would influence biochemical makeup (Lopez *et al.* 2001). In another study, the effects of thermal shock on Pacific oysters (*Crassostrea gigas*) and mussels (*Mytilus edulis, M. trossulus*) were found to cause certain proteins to be upregulated or induced (Hofmann and Somero 1995, Chapple *et al.* 1998, Clegg *et al.* 1998). Changes in O_2 consumption and ammonia levels have also been found to be altered in mussels in response to cyclic temperatures, season (reproductive cycle), and air exposure (Bayne and Thompson 1970, Bayne 1973, Widdows 1976, Thompson *et al.* 1978). Temperature and salinity changes have also been found to alter physiological activity and mobility in bivalve hemocytes (Fisher 1988, Newell and Barber 1988, Sparks and Morado 1988).

Unfortunately the first observable stress responses are often the hardest to examine, as they occur at the subcellular level.

1.2.1.1 Stress responses at the subcellular level

The lysosome is a subcellular organelle found in hemocytes (blood cells) of bivalves, and may be a site for the earliest detectable change of a primary event such as stress response to a stimulus (Moore 1985). Lysosomes are involved in a wide range of physiological functions including host defence, digestion, regulation of secretory processes, cellular defence mechanisms, cell death, protein and organelle turnover, accumulation and sequestration of xenobiotics, and mediation of tissue-specific hormones (Moore 1982, Chu 1988, Lowe *et al.* 1995a, Depledge *et al.* 2000). Many marine invertebrates, including bivalves, have organs and tissues whose physiological activity has been shown to be highly dependent on well-developed lysosomal systems.

One of the fundamental biochemical properties of lysosomes is their structure-linked latency (Bayne *et al.* 1976, Moore 1980), a phenomenon whereby hydrolytic enzymes are bound within the organelle and are in a state of inactivity (Moore *et al.* 1979). The membrane stability of the lysosomes has been found to be altered under varying physiological and pathological conditions thus releasing the enzymes, which include acid and alkaline phosphatases, non-specific esterases, indoxyl esterase, lipases, lysozyme, β-hexosaminidase and β-glucuronidase (Moore *et al.* 1979, Moore 1980, 1982, Feng 1988).

Alterations of lysosomal stability in bivalves have been studied for at least 30 years. Destabilization of lysosomes in digestive glands, and more recently in hemocytes, has been examined extensively in relation to pollutants and toxic chemicals (e.g., fluoranthene, heavy metals, oil, various polycyclic aromatic hydrocarbons). Lysosomal

membrane stability, changes in enzyme activity and enlargement of the lysosomes due to fusion, have all been linked to exposure to toxins (Bayne *et al.* 1976, Lowe *et al.* 1981, Moore and Clarke 1982, Auffret 1988a,b, Lowe 1988, Regoli 1992; Lowe *et al.* 1995a, b, Grundy *et al.* 1996, Moore *et al.* 1996). In addition, some studies have shown changes in form and function of lysosomal structures and components in wild mussels exposed to non-chemical stressors, such parasites, hypoxia, hyperthermia, osmotic shock or dietary depletion (Moore *et al.* 1979, Hawkins 1980, Moore 1980, Bayne *et al.* 1981b, Moore 1990, Tremblay and Pellerin-Massicotte 1997).

Research has turned toward developing procedures that identify changes in the lysosomes that are more simple and rapid than the histological methods used previously (Moore 1976). Development of biochemical assays to measure lysosome integrity could be a useful tool for investigators or commercial producers. Such assays could provide a rapid assessment of subcellular state that, properly calibrated, could be used to assess the physiological state of the mussels. This information could be used to define optimum culture conditions and practices. Biochemical assays have been, and continue to be, developed to measure changes in lysosomal systems and the activities of lysosomal enzymes (e.g., protease assay, lysozyme assay, alkaline phosphatase assay) (Moore 1976, Lowe 1988, Regoli 1992, Lowe *et al.* 1995a,b). Most of the previous work has concentrated on lysosomes found in the digestive glands of bivalves; however this requires excision of the tissues, thus killing the animal, followed by an elaborate staining procedure (Moore 1976). In more recent years it has been discovered that the digestive cells are not the only cells where changes in lysosomal structures were induced when

challenged with crude oil (Lowe *et al.* 1995a). Live hemocytes from mussels exposed to crude oil, were extracted non-lethally, and exhibited a highly developed lysosomal system (Thompson *et al.* 1978, Moore *et al.* 1979).

Weak base substances, such as the cationic probe neutral red (in the unprotonated form), are normally accumulated by diffusion from the cellular matrix into the lysosome and trapped due to protonation by H^+ ions (Lowe and Pipe 1994, Lowe *et al.* 1995a). However, under stress conditions, the lysosomal membrane is destabilized inducing failure of the proton pump, which maintains an acidic pH within the lysosome compared with the neutral pH of the cytosol. When the pH gradient is lost between the two sides of the membrane lysosomal contents can easily pass into the cytosol (Lowe *et al.* 1995a). Lysosomal enzymes, which are normally inactive at an acidic pH, become active.

Under normal conditions when an animal is not faced with stress, granular hemocytes of molluscs appear large and irregular in shape, and the neutral red stained lysosomes appear as red / pink pinpoints within colourless cytosol (Lowe *et al.* 1995a, Depledge *et al.* 2000). Hemocytes under conditions of stress become rounder and smaller, with enlarged lysosomes. The cytosol stains pink due to the leakage of neutral red from the lysosomes with membranes damaged (Lowe *et al.* 1995a, Depledge *et al.* 2000). A disadvantage of the neutral red assay is that it is toxic to the hemocytes. After three hours of incubation, the cells swell and become vacuolated, enhancing membrane permeability and autophagy (Lowe *et al.* 1995a).

Neutral red can be used as an effective biomarker at the subcellular level, for live molluscan hemocytes, as a tool to measure pathological alteration of the lysosomal

compartment. A large amount of hemolymph, 39% to 90%, can be removed from the posterior adductor muscle, as opposed to the heart, without killing the animal (Thompson *et al.* 1978). The hemolymph can be replaced by the mussel within 4 hours. With this method there is a reduced risk of contamination of the sample with pericardial fluid or seawater from the surrounding area (Thompson *et al.* 1978). By using live hemocytes, measurements can be accomplished without causing permanent harm to the bivalve and thus allows for the individual to serve as its own control in the event of a time series study. Use of live cells also allows identification of the cellular change as it occurs, thus can serve as a model of the effects of stress at the whole animal level.

Other subcellular reactions to stress have been, and continue to be, developed in an attempt to provide earlier detection methods for stress response. Gill mucus, as one of the first lines of defence in many aquatic species (invertebrates and vertebrates), has shown an increase in enzyme levels, such as protease, when an organism is under stress from parasitic infections (Brun *et al.* 2000, Ross *et al.* 2000).

There are a number of studies examining the effects of heat shock stress on bivalves that use biochemical analysis of subcellular components. Changes in the nature and production of heat shock proteins (HSPs) are being examined in response to thermal shock and seasonal physiological variations (Hofmann and Somero 1996a, b, Chapple *et al.* 1998, Clegg *et al.* 1998). HSPs act as molecular chaperones and can protect and reverse damage to proteins. HSPs prevent proteins from becoming unfolded and aid in refolding proteins that have been partly denatured. They also aid in targeting proteins for degradation if the damage is irreversible and beyond repair. Ubiquitinated (Ub)

conjugates, which bind to proteins via an ATP-dependent process, serve as the key signal that targets damaged proteins for proteolysis in cases of irreversible damage (Hofmann and Somero 1996a, b, Clegg *et al.* 1998). Higher levels of HSPs than Ub conjugates suggest that there are more reversible denatured proteins than irreversible denatured proteins, as would be suggested by a higher ratio of Ub conjugates to HSPs. This ratio, therefore, can be used as an indicator of how well an organism (bivalve) can survive a stress factor such as heat shock (Hofmann and Somero 1995a, b). The biochemical techniques currently used to study HSPs are still under development and require specialized equipment and knowledge to perform and currently are only suitable for experimental research.

1.2.1.2 Stress responses at the cellular level - hemocytes

Hemocytes, a term that refers to the blood cells found in invertebrates, including bivalve molluscs, are eukaryotic in nature and exist as individual cells within the hemolymph and interstitial spaces of the animal. They are typically spherical within the tissues; however they may appear deformed via migration through epithelial, connective (Leydig), and other tissues (Auffret 1988a, Fisher 1988). The primary role of the hemocytes in marine bivalves is defence, functioning in inflammation, wound repair, encapsulation and phagocytosis. However they also play an important role in digestion and excretion processes (Fisher 1988, Henry *et al.* 1990).

Moore (1985) stated that it was possible to examine alterations in hemocyte activity at the early stages of cell injury before the cellular response would be integrated

and manifested into processes observed on the level of the whole animal. Several studies have shown that hemocyte activity may be altered in response to environmental conditions, such as temperature and salinity, including change in function or motility (Fisher 1988, Newell and Barber 1988). Fisher (1988) suggested that continued measurement of defence activities and related functions of the hemocytes would probably give insight into seasonality, periodicity and incidence of disease. Functional responses of hemocytes to culture conditions are unknown, but are likely to be related to the earlier subcellular biochemical alterations in lysosomal compartments. Cellular responses may be useful as indicators of stress conditions, as stress responses detected before they are expressed on the whole animal level may allow for manipulation of conditions and end stress responses before they influence the next functional level.

1.2.1.3 Stress responses at the whole animal level

Stress response observed at the whole animal level is essentially an expression of all other functional changes and mechanisms within the animal that have occurred due to a stress factor. In other words, whole animal level responses are the cumulative effects of the subcellular and cellular effects. Stress response at the whole animal level is observed as changes in behaviour, growth, feeding, or ability to survive. By using the physiological parameters of reduced growth, inhibition of feeding and death, investigators can infer that a number of cumulative changes in the animal have occurred.

A number of studies have shown that stress response at the whole animal level can be rapid (acute), or prolonged over time (chronic), depending on the stress itself. In extreme case, the response cannot be reversed and leads to poor quality (physiological

condition) or death (Widdows *et al.* 1979, Slabyj 1980, Akberali and Trueman 1985, Prochazka and Griffiths 1991, Mallet and Myrand 1995).

1.2.2 Factors that induce stress responses in mussels

Cultured mussels are exposed to a number of different conditions throughout their life span. Although some of these conditions are not detrimental to the mussels, several are known to cause changes at the whole animal level. Understanding and examining the effects to which mussels are affected by certain conditions and culture practices could help elucidate what measures and techniques are required to reduce the stress at an earlier stage. Thereby, preventing adverse impacts on mussel productivity.

1.2.2.1 Susceptibility of mussels to stress

The basic biology of an organism is considered to be adapted to tolerate a normal range of conditions likely to be faced in its growing environment. This adaptation can make it more or less susceptible when challenged by different abiotic and biotic factors. The mechanism by which mussels feed-filter feeding – involves ongoing exposure to dissolved and minute particles from the water, such as hydrocarbons. These may be excreted or accumulate within the tissues, sometimes to high concentrations. Excess accumulation of certain chemicals in the mussels has been shown to damage cellular components (Bayne *et al.* 1979, Bayne *et al.* 1982, Moore and Clarke 1982, Moore 1985, Lowe 1988, Lowe *et al.* 1995a, b).

Other anatomical features of mussels can leave them vulnerable to unfavourable conditions. The outer shell encasement of the mussels if broken leaves the soft internal tissues exposed to the external environment (Dare 1974, Slabyj 1980, Warwick 1984). Byssal threads, which are secreted for the purpose of attachment from the byssus gland can be torn from the mussels during harvesting, processing or harsh environmental conditions, causing severe damage to the internal organs (Price 1982, Young 1985, Mallet and Myrand 1995).

Mussels are sessile organisms, anchored by the byssal threads, thus have limited ability to escape potentially hazardous situations (Mallet and Myrand 1995), including the environmental conditions that surround them, and without the ability to acclimate quickly, survival may not be possible. Biologically, most estuarine bivalves are able to withstand a wide range of temperatures and salinity levels, however extreme or rapid changes can be lethal. As well, seasonal reproductive cycles lead to differences in energy use and allocation, which may affect stress responses.

Blue mussels in Newfoundland have an annual reproductive cycle, which may also be a factor affecting susceptibility to other stress conditions (Thompson 1984a). Typically, the reproductive cycle for mussels in Newfoundland can be broken into five stages: i) prespawning period from April to mid-June, ii) spawning from mid-June to mid-July, iii) postspawning period from mid-July to September, iv) recovery period from October to January, and v) overwintering period from January to April. There are distinct changes in a number of physiological processes associated with the gametogenic cycle including oxygen consumption, ammonia production levels, lysosomal activity, and

plasma biochemical composition (Bayne 1973, Thompson *et al.* 1978, Tremblay *et al.* 1998a, b). In addition, seasonal mortalities, especially during summer, are considered to be physiological breakdown following loss of energy reserves during spawning (Worrall and Widdows 1984, Carver and Mallet 1991, Myrand and Gaudreault 1995).

1.2.2.2 Seasonal and environmental variations

Unlike intertidal mussels, cultured mussels are held in suspended culture their entire life until they reach market size. During this time, mussels encounter a wide variation in environmental conditions related to season, oceanic currents, and environmental factors related to the site geography and morphometry. It is possible for a single event or multivariable conditions to interact, and pose a stress factor on the mussels and thus elicit a stress response.

Seasonal variations are a major consideration as there are a number of components involved that have a compounding effect on the degree of stress response, including food availability, fluctuating water temperatures, and other hydrographic influences.

Food availability, which is limited at certain times of the year, can become a major factor of stress to mussels. For example, in the summer following spawning a reduced food supply can result in high mortality (Carver and Mallet 1991). Similarly, if energy reserves are not replenished during the autumn, a stress response may be induced by food depletion in the winter period (Hatcher *et al.* 1997).

Seasonal water temperatures are also a factor resulting in stress on mussels. This component may itself be a stress, but seasonal water temperatures also covary with the reproductive cycle discussed above. In the summer months elevated water temperatures may be a major factor in the increase of mortality rate, as they may approach lethal levels, while in winter the lower water temperatures are associated with reduced mortalities (Freeman and Dickie 1979, Sephton *et al.* 1993, Myrand and Gaudreault 1995). Seasonal conditions, such as ice cover, and increased algal coverage in relation to water temperatures also affect stress response in mussels by possibly providing protection from extreme low temperatures (Williams 1970, Freeman and Dickie 1979, Hatcher *et al.* 1997).

The effects of hydrographic conditions are also of concern as a stressor in mussels. Cultured mussels held in suspension on longline systems that are constantly under water can be exposed to rapid fluctuations in water temperature, salinity, and turbidity, brought on by excess winds, storms, and wave activity.

Spatial variability of environmental factors associated with different sites has direct influences on stress response in mussels. Sites differ in variation and timing of the reproductive cycle and physical characteristics, including food levels, ice cover, water exchange, seasonal water temperatures, and storm and tidal energy (Dickie *et al.* 1984, Mallet *et al.* 1987). Hence, differential patterns of mortalities and other stress responses may be a direct influence of location of the culture site and physiological adaptation of seed used for stocking.

1.2.2.3 The effects of culture practices on mussels

Cultured bivalves are subject to the conditions of accepted culture practices. Within the industry, practices have been developed to enhance the performance of mussels in terms of growth and survival, and shelf life following harvesting. However, understanding relatively how growth and survival can be influenced and manipulated by culture practices is limited.

1.2.2.3.1 Harvesting and barge deck holding

During harvesting mussels are brought onboard a boat using mechanical devices. The backline of a longline is partially lifted out of the water and the individual socks containing the mussels are cut and hauled onto the boat deck (Mallet and Myrand 1995). Mussels are then detached from the sleeves and placed in plastic tote boxes. Mussels are kept on barge decks or during the winter harvest on ice, for extended periods of time (hours), thus measures have to be taken to minimize stress from air exposure, extremes in air temperatures, sunlight, rain and wind, any of which can pose a stress on mussels and induce some form of response (Warwick 1984, Widdows and Shick 1985, Eertman *et al.* 1993). Although the focus of this present study was on market ready mussels, it should be noted that the activities of grading (mechanical sorting by size) and socking seed and pre-market size mussels might also induce stress response. Mussel seed are redeployed on the longline in socks and exposed to the same conditions as market sized mussels during harvesting and holding on barge decks (Mallet and Myrand 1995).

1.2.2.3.2 Processing activities

Following harvesting, cultured mussels are sent to a primary processing plant where they are washed, declumped, and debyssed before being packaged and shipped to markets. The demand for live blue mussels has focused attention on problems associated with the handling of shellfish as some of the processing procedures are harmful to the mussels and can adversely affect the shelf life of fresh product (Slabyj 1980).

The shelf life of mussels is defined as the time passed before a predetermined mortality in a group of mussels has been reached. However strong off odours have been detected in stored mussels prior to 10% mortality being attained (Slabyj 1980). The Atlantic Canada cultured mussel industry defines shelf life to be when 5% mortality has been reached in the live mussel product. Many parameters of culture practices may be manipulated in order to prolong shelf life, including post-harvest handling (Prochazka and Griffiths 1991).

The mechanical sorting, washing and declumping of mussels after harvest may induce a number of detrimental effects, such as shorter shelf life, byssus damage, liquor loss (mantle fluids), and decline in survival of air storage (Dare 1974, Slabyj and Hinkle 1976, Slabyj 1980, Prochazka and Griffiths 1991).

Debyssing, a practice employed in North America primarily, involves the removal of the byssal threads from mussels by mechanical means, where the threads are torn from the mussels. Since the byssal threads are attached to a gland at the base of the foot, this leads to some tissue damage and a stress response in the mussels. Procedures to reduce the impact of debyssing have been examined, such as a period of reimmersion following

processing to allow for mussels to be reconditioned or to recover and repair damage (Slabyj 1980, Prochazka and Griffiths 1991).

1.2.2.3.3 Storage conditions

Following processing of mussels, there is a period of time that mussels need to be kept in some form of storage, either dry (in air) or wet (in water). Consumers expect a high quality product, thus changes during post-harvest storage need to be understood so quality control procedures can be met (Brooks and Harvie 1981). Experiments to evaluate various storage conditions have examined stress responses, loss of flavour, bacterial loads, and shelf life in mussels (Boyd and Wilson 1978, Brooks and Harvie 1981, Prochazka and Griffiths 1991, Tremblett 2001). The quality of the product needs to be maintained throughout storage, therefore the shelf life of mussels under various storage conditions was explored in the present study, to determine the best conditions for holding mussels, in order to increase the time before odours, bacterial loads, and reduction in physiological condition occur.

1.3 Rationale

It is known that various culture and post-harvest conditions, both environmental and man-made, can lead to stress response and a reduction in mussel performance such as growth, feeding, condition, quality, and ability to survive. As a result, the effects of certain stress factors may compromise the overall productivity of a mussel farm, and this is detectable only after an extended period of time, at the whole animal level.

For the mussel culture industry, stress response needs to be assessed in relation to various culture conditions, so that growers can better understand the impact of various environmental factors and husbandry procedures on overall farm production. It would be an advantage to both growers and researchers to develop earlier indicators of stress response so that conditions may be changed or manipulated to avoid stress responses from progressing to the whole animal level. There is also a need for earlier and more rapid field detection methods to measure stress responses of an animal at the subcellular and cellular level, before the effects of a stress is observable on the whole animal level and farm productivity is already compromised. Methods to mitigate harmful conditions could improve mussel growth, survival, farm and post-harvest production, as well as improve production costs and product value.

Techniques, such as the neutral red assay (NRA), to measure stress responses at the subcellular level, have been developed for mussels. However their use to assess the impact of culture practices on stress response needs to be examined. These techniques need to be validated against other chemical methods, such as enzyme assays and heat shock protein expression. As well, it needs to be determined if the results of the NRA employed to evaluated culture conditions, can be used to provide recommendations about culture practices for growers in the mussel culture industry.

1.4 Objectives

1. To evaluate the neutral red assay (NRA) as a biochemical indicator of stress response in cultured mussels.
2. To evaluate basal seasonal variability in the stress response of mussels.

3. To evaluate the influence of the reproductive cycle and seasonal environmental changes on mussel stress responses.
4. To evaluate the influence of various stages of culture production including handling, processing, and post-harvest storage on mussel stress response.
5. To evaluate spatial variability in stress response of mussels from different culture sites.

1.5 Hypotheses

1. If the neutral red assay can indicate changes in stress response at the subcellular level in the lysosomes, then the effects, if any, of various conditions during culture or post-harvest storage of mussels detected using the NRA technique should be consistent with changes in the variables measured.
2. If environmental conditions (i.e., temperature, air exposure, hydrographic events) affect performance of mussels, then an increase in the magnitude of change in conditions will result in an increase in stress response.
3. If reproductive condition affects performance of mussels, then spawning will result in an increase in stress response.
4. If post-harvest processing, handling and storage affect performance of mussels, then activities will result in different levels of stress response and shelf life.

2. Materials and Methods

The experimental trials performed for the purposes of this thesis were designed to evaluate the effect that different environmental conditions and culture practices have on stress response in cultured mussels. The neutral red assay (NRA) was used to measure changes in the lysosomes of hemolymph extracted from the mussels in the various experimental treatments.

2.1 Animals

Market-sized mussels (*Mytilus* spp.) were used (i.e., >50 mm) for the different experiments and evaluation of the performance of the NRA. Mussel samples were obtained from growers located throughout Newfoundland (Figure 3). A sample consisted of randomly selected mussels, with associated biota. These were placed on ice or chilled and shipped to the lab. All mussels were received within a 24 h period of harvest at the laboratory of the Fisheries and Marine Institute of Memorial University, St. John's, Newfoundland. Upon arrival mussels were maintained in a recirculating raceway system, equipped with aeration and unfiltered seawater similar to ambient temperature and salinity before (at least 48 h and up to 7 d) and during experiments (if required for experimental purposes) (Figure 4A). Water quality parameters of temperature, dissolved oxygen, salinity and pH were assessed on a daily basis using calibrated probes and equipment. Saltwater was partially replenished daily in the system with about 25% of the water in the entire raceway system changed.

For all experiments, except those used in examining storage conditions and spatial studies, unprocessed mussels, of which >90% were *Mytilus edulis* and <10% *Mytilus trossulus* or hybrids (Penney *et al.* 2001), were obtained from Charles Arm in Notre Dame Bay (Figure 3). For the study examining storage conditions, processed mussels were obtained from a local processing plant in the Notre Dame Bay area (Figure 3). For the spatial study comparing various sites on two separate occasions, mussels were obtained from Charles Arm, Bulley's Cove, Salmonier Cove, Reach Run, and Cap Cove (Figure 3).

2.2 Hemolymph collection and sampling

In order to evaluate the stress response that was induced during different experimental trials, hemolymph needed to be properly acquired and handled for its use in the NRA.

Hemolymph was withdrawn from the posterior adductor muscle of mussels using a hypodermic needle fitted with a 21-gauge needle to reduce damage to the cells and subsequent clotting (Figure 4B). The hemolymph was drawn into an equal volume of physiological saline (0.3 mL hemolymph: 0.3 mL saline) consisting of 4.77 g HEPES, 25.48 g sodium chloride, 13.06 g magnesium sulphate, 0.75 g potassium chloride, 1.47 g calcium chloride, made up to 1 L with distilled water and pH adjusted to 7.36 (Lowe *et al.* 1995a, Depledge *et al.* 2000).

The hemolymph was then placed in siliconised Eppendorf® microcentrifuge tubes (to prevent cells from sticking to the tube walls) and placed on ice. The needle was

removed before contents of the syringe were placed in the tubes to reduce shear damage to the cells; the procedure was done slowly so not to rupture any of the cells. Eppendorf® microcentrifuge tubes were gently inverted to mix the saline and hemolymph.

Hemolymph / saline solution was then pipetted in aliquots of 40 µL onto a dry microscope slide that had been pre-treated with Poly-L-lysine solution to enhance cell adhesion (Figure 4C). In modification from the published procedure (Lowe *et al.* 1995a, Depledge *et al.* 2000), 20 µL Poly-L-lysine was used in 100 µL of distilled water; modified from 10 µL Poly-L-lysine used in 100 µL of distilled water to enhance cell adhesion to the slides. Slides were then placed in a lightproof humidity chamber for 15 min to allow cells to adhere to the slides. Excess solution was carefully removed by gently tipping the slides onto paper towel without touching the cells that had adhered to the slide.

2.3 Neutral red assay (NRA)

Precise and consistent execution of the NRA procedure is critical to the outcome of the results, as it was the primary technique used to assess stress response in mussels under various experimental treatments. The protocol adopted was a slight variation of the methods described by Lowe *et al.* (1995a).

Neutral red stock solution was made by dissolving 0.0288 g of refrigerated neutral red dye powder into 1 mL of dimethylsulphoxide (DMSO) in a lightproof vial and then refrigerated (up to 3 weeks). Neutral red working solution was prepared by pipetting 20

μL of stock solution, which had been allowed to reach room temperature, into 5 mL of physiological saline into a second lightproof vial (stable for 3 hours). Neutral red working solution was added to the hemocyte layer on the microscope slide in a 40 μL aliquot (Figure 4C).

The slides were placed in the humidity chamber for a period of 15 min to allow the neutral red dye to enter the cells. A 22 x 22 mm coverslip was then carefully applied to the slide and the first reading taken under bright field light microscopy.

Slides were examined every 15 minutes for the first 60 minutes, every 30 minutes until the 120 minute mark, and again at 180 minutes, the time when the experiment was terminated (Figure 4D). For each reading (n = 8), the condition of 25 hemocytes was examined under low-level illumination at magnification of 400x before being returned to the humidity chamber.

The experiments were terminated at any point during the 180 min assay once 50% or more of the hemocytes were deemed to have reached the high stress response condition: lysosomal membranes absent or only remnants could be identified, the cytosol of the hemocyte was tinged pink, and, in some cases, the hemocyte had typical, non-directional cytoplasmic projections (Auffret 1988a, Fisher 1988) (Figure 5).

2.4 Standard meat yield

As a measure of physiological / reproductive condition, standard meat yields were calculated to aid in the understanding of the seasonal variation in stress response.

Condition index was determined by steaming 1 kg of live mussels for a 10-min period, as outlined in Ibarra *et al.* (2000). The standard meat yield was calculated as:

$$CI = \frac{\text{cooked meat weight}}{(\text{cooked meat weight} + \text{shell weight})} \times 100$$

2.5 Shelf life

Shelf life was defined as the time at which 5% of the mussels were dead or moribund in a lot (3 replicates of 50 mussels). Death or morbidity was determined to have occurred when the mussels' shells failed to remain closed after being held shut for 5 s. Shelf life determination was set up by placing mussels in open-air storage at 2°C to 4°C at high relative humidity (standard holding condition for live mussels in Newfoundland), unless alternative experimental conditions applied. For this project, air storage took place in a fridge that contained a tray of water contained therein to provide humidity. All experimental treatments were run in triplicate, and shelf life examination was terminated on day 20 from the initiation of the experiment, regardless if some treatments had not reached 5% mortality.

2.6 Experimental design

2.6.1 Preliminary experiments

An experiment was designed to validate of the neutral red assay (NRA) as an indicator of stress response in mussels (neutral red retention (NRR) in lysosomes). A second experiment was designed to examine the effects of the initial transport and

laboratory holding conditions on stress response in mussels. A third experiment was carried out to examine the effects of feeding and extended holding in a laboratory raceway setting on stress response in mussels.

2.6.1.1 Evaluation of the neutral red assay using temperature shock

This experiment was conducted to assess the NRA as an indicator of stress response, by exposing mussels to a known stress condition - a thermal shock of 20°C above ambient water temperature. Mussels collected in April 2001 (overwintered / prespawning condition), were taken from holding raceways (6°C) and placed in aerated tanks at a water temperature of 26°C, a temperature close to their lethal limit and a known stress on mussels (Mallet and Myrand 1995). A control was run simultaneously with mussels maintained in holding tanks at an ambient temperature of 6°C. Exposure at these temperatures was for 8 h with the NRA performed as described above at hours 0, 4, and 8. For each temperature and sampling time, 12 animals were sampled. Mussels were not repeatedly sampled for this experiment.

2.6.1.2. Transport and laboratory holding

This experiment was designed to evaluate the NRR in mussel hemocyte lysosomes during the 24 h transport period to the laboratory, during the 2 d recovery period in seawater raceways in the laboratory, and prior to experimental use. Mussels were obtained from the growout site in the prespawning period (June 2002) and kept in a cooler with ice packs during a 24 h transport to the laboratory, where they were placed in

the raceways at ambient seawater (temperature and salinity) for a further 42 h. Samples (n = 10 mussels) were taken at harvest (T = 0), and thereafter at hours 3, 6, 16, 30, 42, and 72 following harvest period for the NRA.

2.6.1.3 Influence of feeding and extended laboratory holding

In the winter of 2002, a student from the Advanced Diploma in Aquaculture Program at the Marine Institute carried out an Independent Research Project that evaluated extended laboratory holding and the effects of feeding on NRR in mussels (Alteen 2002).

Mussels were held in raceways containing ambient seawater (temperature and salinity), undisturbed and totally submerged for a four week period. One group of mussels was fed on a daily basis, while the other group was unfed. Samples (n = 12 mussels) were collected for the NRA on the initial day of the experiment and every seven days subsequently from each of the fed and unfed groups.

2.6.2 Evaluation of temperature shock on stress response of mussels

This experiment, conducted in the spring of 2002 when ambient water temperature was in the vicinity of 5°C-6°C, was to assess various changes and extended exposure to water temperature shocks and associated stress responses in mussels. Mussels were taken from the holding raceways and placed in tanks with aerated water at temperatures of 15°C and 25°C, respectively. A control treatment consisted of mussels maintained in holding tanks at an ambient temperature of 5°C. Exposure to experimental

temperatures lasted 4 h with the NRA performed on samples collected at hours 0, 2, and 4 of exposure. At hour 4, mussels held in experimental temperatures were placed back into ambient temperature water and held for an additional 20 h with the NRA performed at hours 6, 9, 12, and 24 of the experiment. Controls were sampled at hours 0 and 24 for each experimental group. For each group and sampling hour, six mussels were sampled. In addition to the NRA, tissue and hemolymph samples were collected for additional assays on enzymes and heat shock proteins. The results of these analyses are part of a separate study on subcellular and cellular stress responses in cultured mussels and are not reported here.

To evaluate the rate at which a high level of stress response in mussels is reached during extreme temperature shock, mussels were taken from holding raceways (5°C) and placed in tanks containing aerated water at a temperature of 25°C. Exposure to the experimental temperature was for 4 h with the NRA performed on a sample of mussels (n = 10 mussels) at each of the time periods of 0, 45, 90, 135, 180, and 225 minutes of exposure. For each sampling time, 25 hemocytes were examined and given a plus / minus score based on characteristics observed, with no stress response being given a plus, a low to moderate stress response given a plus / minus, and high stress response being given a minus (Figure 5).

2.6.3 Evaluation of seasonal variation on stress response of mussels

This experiment examined the patterns of NRR in mussel lysosomes, in relation to the reproductive cycle and seasonal environmental changes. Unprocessed mussels

were sampled monthly, from June 2001 to June 2002 for a total of 12 months, with the NRA performed (n = 12 mussels). Standard meat yield and average shelf life were also determined.

2.6.4 Evaluation of rapid water temperature fluctuations on stress response of mussels

This experiment simulated the effects of rapid water temperature change, such as those caused by oceanographic events (i.e., tides, winds, currents). Unprocessed mussels were taken during early summer (June 2001), late summer (August 2001), and autumn (September 2001), and cleaned of debris and extraneous fouling. Mussels were exposed to a change in water temperature, alternating +10°C (up from initial water temperature) and −10°C (back to initial water temperature), every 6 h for a 24 h period. Samples (n = 12 mussels) from each batch were taken for the NRA just prior to being exposed to each change in water temperature.

2.6.5 Evaluation of air exposure on stress response of mussels

This experiment evaluated the impact of air temperatures and extended exposure to air as may be found during harvesting and holding on barge decks. Unprocessed mussels were taken during early summer (June 2001), summer (August 2001), early winter (January 2002), and spring (May 2002). Mussels were exposed to air temperatures below, equal to, or above ambient water temperatures depending on time of year (Table 1). Mussels were exposed to each temperature for an 8 h period with samples

(n = 12 mussels) for the NRA being taken at hours 0, 4, and 8 for each temperature exposure.

2.6.6 Evaluation of processing activities on stress response of mussels

This experiment examined the effect of various processing activities on NRR in hemocyte lysosomes of mussels. Unprocessed mussels were taken during early summer (June 2001), late summer (August 2001), early winter (January 2002), and spring (May 2002) period. Mussels were exposed to six different processing activities: 1) no processing (control), 2) washing and declumping, 3) washing, declumping and debyssing, and 4-6) 24 h reimmersion period (fully submerged) following each of the previous three activities, respectively. Samples of mussels were taken immediately following each processing activity and following the 24 h recovery period for the NRA (n = 12 mussels per treatment).

2.6.7 Evaluation of post-harvest storage conditions on stress response of mussels

This experiment evaluated NRR in lysosomes associated with post-harvest storage conditions. Processed mussels (washed, declumped, and debyssed) were obtained during early summer (June 2001), late summer (August 2001), late autumn (November 2001), and spring (May 2002). Mussels were exposed to three different storage conditions: 1) 2°C to 4°C moist air (chilled, dry storage), 2) 0°C on ice (iced, dry storage), and 3) in ambient water (7°C, 16°C, 5°C, and 6°C, for early summer, late summer, late autumn, and spring, respectively; wet storage). Samples of mussels (n =

12) from each storage condition were taken on days 0, 4, 8, and 12 for the performance of the NRA. For each of the storage conditions, the average shelf life was determined over a 20-d period, following the same guidelines used for determining shelf life as previously described in section 2.5.

2.6.8 Spatial comparison of mussel stress response

This experiment was designed to evaluate differences in NRR in lysosomes from mussels collected from various sites in Newfoundland (Figure 3), at the same time period during the postpawning / recovery phase and the prespawning phase. Mussels were obtained from 4 sites (Charles Arm, Bulley's Cove, Salmonier Cove, and Reach Run) in November 2001, and from 5 sites (Charles Arm, Bulley's Cove, Salmonier Cove, Reach Run, and Cap Cove) in May 2002. Samples from each site were used to perform the NRA (n = 12 mussels) and determined the standard meat yield.

2.7 Data analysis

Data from all experiments were analyzed using the SPSS statistical software (version 10). Descriptive statistics, ANOVA, correlation analysis (Pearson's), and post hoc tests (Tukey's b) were performed. The level of significance was set at alpha = 0.05.

3. Results

3.1 Preliminary experiments

3.1.1 Evaluation of the neutral red assay

Mussels held at a near lethal temperature of 26°C displayed significant differences in neutral red retention (NRR) scores, a measure of stress response, among the hours of exposure (ANOVA; $F_{(2, 101)}$=14.576, $p<0.001$) and treatments (control versus experimental temperature) (ANOVA; $F_{(1, 101)}$=16.576, $p<0.001$ (Figure 6). There was a reduction in NRR in the mussel lysosomes from 88 min to 8 min over the 8-h exposure time for mussels held at 26°C, while no reduction was observed in NRR in mussels maintained at 7°C (Figure 6).

3.1.2 Transport and laboratory holding conditions

This experiment examined NRR levels during transport from the sampling site and during laboratory holding conditions, prior to experimental use. Results showed a significant difference in the number of unstressed cells throughout the transport and laboratory holding times but with no consistent patterns (one-way ANOVA; $F_{(6, 62)}$=3.574, $p=0.005$) (Figure 7). There was no difference in NRR from the beginning of the 72 h period, when the mussels were removed from the grow-out site, to the time they were used in experimental trials as the number of unstressed cells at the hours 0 and 72 were the same (Tukey's b, $p>0.05$) (Figure 7).

3.1.3 Influence of feeding and extended laboratory holding

The results of the feeding experiment are not presented. Results showed that mussels can be held unfed in the laboratory in raceways of ambient seawater conditions with no significantly different NRR compared to those of mussels that were fed under the same holding conditions, for as long as four weeks (Alteen 2002). Results from the neutral red assay, remained unchanged in mussels held in the laboratory (unfed) for up to seven day from the onset of experiments until all experiments were underway within each of the sampling seasons.

3.2 Temperature shock

The NRR scores in mussels exposed to two different temperature shocks over a 24 h time period showed significant differences among the control and experimental groups. There was a decrease in NRR from 115 min to 33 min, and 100 min to 0 min after a 4 h exposure to temperature shocks of 10°C (from 5°C to 15°C) (one-way ANOVA; $F_{(6, 33)}=2.517$, $p=0.041$) and 20°C (from 5°C to 25°C) (one-way ANOVA; $F_{(6, 35)}=13.62$, $p<0.001$) (Figure 8). After mussels were placed back into ambient water and allowed to recover for 20 h, those exposed to a 10°C temperature shock had a more pronounced recovery than those exposed to a 20°C temperature shock as NRR increased from 33 min to 90 min, and 0 min to 25 min, respectively. Mussels exposed to a 10°C differential recovered fully after 20 h, while those exposed to a 20°C differential showed significant but limited recovery after 20 h. Analysis of enzymes and heat shock proteins

conducted on the same mussel samples show similar patterns in stress responses and recovery as seen here with the NRA (Egbosimba *et al.* 2002).

The second experiment that followed lysosomal stress response levels over a short time period, exposing mussels to a thermal shock of 20°C (from 5°C to 25°C), showed a significant difference in the number of unstressed cells over the extended exposure time (one-way ANOVA; $F_{(5, 54)}=14.659$, $p<0.001$) (Figure 9). There was a decrease in the number of unstressed cells over a 4 h exposure period, with a plateau being reached between 90 and 225 min.

3.3 Seasonal variation

The monthly sampling of mussels from a single site showed a seasonal pattern in NRR, and during the course of the study showed a relationship to the reproductive cycle and spawning events (Figure 10). There was a significant difference in NRR among the months (one-way ANOVA; $F_{(11, 130)}=21.327$, $p<0.001$). There were significant decreases in NRR associated with spawning events in June 2001, September 2001 and June 2002 (Tukey's b, $p<0.05$) followed by gradual increases in NRR following spawning (from 0 min to 135 min from November 2001 to May 2002) (Figure 10A).

The average shelf life of mussels (Figure 10B) showed a significant difference among the months (one-way ANOVA; $F_{(11, 24)}=15.675$, $p<0.001$, Figure 10B). The average time mussels can survive before a group reaches 5% mortality was reduced from 17 days in June to 13 days in July during summer spawning, and increased during the postspawning and recovery periods from a low of 13 days in September to 20 days by

November. No influence on shelf life caused by subsequent spawning events that occurred in October and November was detected (Mills 2001) (Figure 10B).

The condition of the mussels, as determined by a standard meat yield, showed that meat yields decreased following spawning, from 37.2% in June to 24.8% in July, and from 28.9% in September to 26.7% in November. They increased in months following spawning from 24.8% in July to 28.9% in September, and from 26.7% in November to 42% by May of 2002 (Figure 10C).

There was a low, but non-significant correlation between NRR scores and the standard meat yield (Pearson's, $r=0.59$, $p=0.045$). There was no significant correlation between NRR scores and the average shelf life (Pearson's, $r=0.41$, $p>0.05$), NRR and monthly water temperatures ($r=0.5$, $p>0.05$), shelf life and the standard meat yield ($r=0.2$, $p>0.05$), shelf life and the water temperature ($r=-0.79$, $p>0.05$), nor standard meat yield and the water temperature ($r=-0.08$, $p>0.05$).

Monthly water temperature data for Charles Arm, Notre Dame Bay, showed a decrease from it's highest level of 16°C in August 2001, to a low of −1°C in February 2002 for the experimental year (Figure 11). Salinity remained in the range of 29 to 31 ppt, and chlorophyll *a* was lowest in May, at 0.5 µg/L, and highest in August, at 2 µg/L (Figure 12).

3.4 Rapid water temperature fluctuations

An experiment simulating the effects of fluctuating water temperatures due to such factors as the tidal cycle or shifting winds, showed a significant difference in NRR

among exposure times (one-way ANOVA; $F_{(4, 54)}$=12.831, $p<0.001$). Fluctuating water temperatures had little effect on mussel NRR after the first 6 h, with a reduction in NRR from 40 min (a score that would be considered a sign of high stress conditions) at hour 0 to 0 min at hour 6, and no change thereafter (Figure 13). However, this result was only encountered in one of three trials (June 2001, August 2001, and September 2001), as scores in the other trials, including the initial value, were zero or close to zero and showed no significant difference among the groups.

3.5 Air exposure

Experiments that exposed mussels to various air temperatures showed a significant difference in NRR among the seasons (ANOVA; $F_{(3, 371)}$=141.997, $p<0.001$), among the three ambient air temperatures at each season (ANOVA; $F_{(2, 371)}$=17.007, $p<0.001$), and among the hours of exposure for mussels held at various air temperatures (ANOVA; $F_{(2, 371)}$=136.857, $p<0.001$) (Figure 14).

Seasonally, the NRR scores showed that there was no significant difference between mussels in June 2001 (prespawned) and January 2002 (recovered), but that there were significant differences in mussels in August 2001 (postspawned) and in mussels in May 2002 (overwintered) (Tukey's b, $p<0.05$). A seasonal pattern of mussel stress response to air exposure was apparent as NRR (at time zero, temperature equal to ambient water temperature, for each season) is reduced from 64 min in early summer (June 2001) to 23 min in summer (August 2001), and increased to 81 min in early winter (January 2002), and up to130 min in spring (May 2002) (Figure 14).

In general, NRR scores were significantly higher in mussels held in air of equal temperature to the ambient water temperature compared to any other air temperature, and that the NRR scores of mussels held in lower air temperatures than the ambient water temperature were significantly higher than the NRR scores of mussels held in an air temperatures higher than the ambient water temperature (Tukey's b, $p<0.05$). Mussels held at air temperatures that were equal to the ambient water temperatures had the highest NRR scores regardless of season or duration of exposure, compared with NRR scores of mussels held in air either below or above the equivalent ambient water temperature. For example, in spring at hour 4, the NRR score for mussels held at air temperatures equal to ambient water temperatures was 130 min. For those mussels held at air temperatures below ambient water temperatures NRR was 70 min, and for those held at air temperatures above ambient water temperatures showed a NRR score of 35 min. Also, in early summer, in summer, and in spring, mussels held at air temperatures above ambient water temperatures had reduced NRR. For example, in spring at hour 4, the NRR was 115 min for those held in air temperatures equal to ambient water temperatures, 70 min for those held at air temperatures below ambient water temperatures, and 30 min in those held in air temperatures above ambient water temperatures (Figure 14A, B, and D).

Thirdly, this experiment showed that extended air exposure on mussels reduced NRR in all seasons and conditions, as NRR scores were significantly different as exposure time increased (Tukey's b, $p<0.05$). For example, in the spring, NRR was reduced from 130 min at hour 0 to 115 min at hour 4 and to 82 min in hour 8, for mussels held in air temperatures equal to ambient water temperatures (Figure 14D).

Further examination of barge deck air temperatures showed that NRR was reduced at a faster rate when mussels were exposed to greater changes in air temperature from air temperatures that were equal to ambient water temperature of 5°C (Figure 15). For example, in hour 4, an increase of 5°C of air temperatures that were equal to ambient water temperatures reduced NRR from 115 min to 105 min, while a further increase of 5°C reduced NRR to 30 min. A final increase of 5°C reduced NRR to 25 min (Figure 15). A decrease of 5°C reduced NRR from 115 min to 70 min (Figure 15).

3.6 Processing activities

NRR results in relation to processing activities showed that the time of year influenced the mussel's lysosomes response to the type of processing activity (ANOVA; $F_{(3, 262)}$=130.647, $p<0.001$). The various processing activities significantly influenced the stress response (ANOVA; $F_{(2, 262)}$=59.122, $p<0.001$), and reimmersion following these various processing activities altered the NRR in a positive fashion (ANOVA; $F_{(1, 262)}$=7.805, $p=0.006$) (Figure 16).

Seasonally, there was no significant difference in NRR scores between mussels in June 2001 (prespawned) and late August 2001 (postspawned), but there was a significant difference in NRR between mussels collected in January 2002 (recovered) and May 2002 (overwintered) compared to all other seasons, respectively (Tukey's b, $p<0.05$). A seasonal pattern in the basal stress response level of mussels was apparent, as NRR (at hour zero, no processing for each season) increased from 1.25 min in the summer (August 2001) to 81 min in the early winter (January 2002), and up to 130 min in spring

(May 2002) (Figure 16). It should be noted, however that the experiment in early summer was run within a few days of spawning and that all scores were zero.

Experiments also showed that there was no significant difference in NRR scores in samples from mussels that were washed and declumped in comparison with those from mussels that were unprocessed. However, the NRR scores from mussels that had been debyssed was significantly different from either unprocessed mussels or washed and declumped mussels in all seasons (Tukey's b, $p<0.05$). In most cases, the process of reimmersing mussels for 24 h following a processing activity increased NRR in mussels, but only significantly for those mussels that had previously been debyssed (Tukey's b, $p<0.05$). The only exception was with no processing in the postspawning period, where NRR levels remained the same (Figure 16A).

3.7 Post-harvest storage conditions

NRR levels in response to post-harvest storage conditions are shown in Figure 17. The experiments showed that the time of year, type of storage, and duration of storage influenced stress response in mussels (seasons - ANOVA; $F_{(3, 495)}=116.617$, $p<0.001$, storage type - ANOVA; $F_{(2, 495)}=13.096$, $p<0.001$, and number of days exposure - ANOVA; $F_{(3, 495)}=124.637$, $p<0.001$).

The NRR scores showed that there were significant differences among the seasons (Tukey's b, $p<0.05$). A seasonal pattern in the basal stress response level of mussels was apparent during storage conditions when NRR (at day zero for each season) increased from 6 min in early summer (June 2001) to 21 min in summer (August 2001),

to 56 min in late autumn (November 2001), and up to 143 min in spring (May 2002) (Figure 17). As with section 3.6 (processing activities) the experiment in the early summer was run within a few days of spawning and that all scores were close to, or at zero (Figure 17A).

This experiment also showed that, in general, there were significant differences between storage conditions (Tukey's b, $p<0.05$). The mussels held in wet storage conditions (2°C-15°C) had the highest NRR, followed by those held on ice at 0°C. Those held in chilled moist air (2°C-4°C) had the lowest NRR. For example, in spring at day 4, NRR for mussels held in water was 68 min, those held on ice was 40 min, and those held in moist air was 15 min (Figure 17D). In summer (Figure 17B), at day 4, those mussels held on ice had the highest NRR. A problem with water quality in the holding system was solved by day 8.

Thirdly, this experiment showed that extended storage of processed mussels reduced NRR, no matter what the storage condition. For example, NRR in mussels stored in water during the spring was reduced from 143 min on day 0 to 34 min by day 12 (Figure 17D). In general, there was no significant difference in NRR scores between days 8 and 12 of storage. However NRR scores on days 0 and 4 were significantly different from all other sampling days, respectively (Tukey's b, $p<0.05$).

Mussel shelf life was influenced by seasonal condition and type of storage (Figure 18). Experiments that exposed mussels to various storage conditions showed that time of year (and reproductive condition), as well as type of storage, influenced shelf life. There

was a significant difference among seasons (ANOVA; $F_{(3, 22)}$=87.284, $p<0.001$), and among the different storage conditions (ANOVA; $F_{(2, 22)}$=16.072, $p<0.001$).

Seasonally, the average shelf life of mussels was not significantly different between June 2001 (prespawned) and August 2001 (postspawned), but the average shelf life was significantly different for mussels in November 2001 (recovered) and in May 2002 (overwintered) than all other seasons, respectively (Tukey's b, $p<0.05$). A seasonal pattern, with respect to wet storage, was apparent, whereby shelf life decreased from 20 days in early summer (June 2001) to 18 days in summer (August 2001), increased to 20 days in late autumn (November 2001), and decreased to 6 days in spring (May 2002) (Figure 18). In early summer, mussels that were held on ice all died within one day due to a problem with the fridge in which they were being held that caused the temperature to drop well below 0°C, thus no results for this condition were obtained (Figure 18A).

These experiments also showed that mussels stored in ambient water (2°C to 15°C), in general, had the longest shelf life, while mussels held in moist air (2°C to 4°C) had the shortest shelf life. Storage conditions were significantly different in early summer mussels (June 2001, prespawned), and summer mussels (August 2001, postspawned) (Tukey's b, $p<0.05$).

The survival of mussels declined rapidly in the early summer, late summer, and spring once a group of mussels reached 5% mortality (Figure 19A, B, and D). Mussels held in moist air had the most rapid decline in all seasons, regardless of the average shelf life (Figure 19).

3.8 Spatial comparison

Experiments to examine spatial differences among culture sites showed that there was a significant difference in NRR among the different sites in autumn (one-way ANOVA; $F_{(3, 43)}=5.464$, $p=0.003$), but not in the spring (one-way ANOVA; $F_{(4, 54)}=2.138$, $p=0.089$) (Figure 20). It should be noted Charles Arm mussels were spawning during the autumn collection (Mills 2001, personal communication).

Results also showed that there was a significant difference among the seasons (one-way ANOVA; $F_{(1, 97)}=11.07$, $p<0.001$) as determined by NRR scores. For example, NRR in mussels from Charles Arm increased from 3 min in autumn to 83 min in the spring, while NRR in mussels from Salmonier Cove decreased from 51 min in autumn to 39 min in the spring (Figure 20A and C).

The condition of the mussels from each site, as determined by the standard meat yield, showed that yields increased slightly from autumn to the spring for all sites (Figure 20B and D).

Environmental data (Figure 12) showed that water temperatures in the Notre Dame Bay (includes the sites of Charles Arm, Bulley's Cove, and Reach Run), and Salmonier Cove were highest in August (around 16°C), and were highest in Trinity Bay in September (14°C). Salinities for all three areas ranged between 29 ppt to 32 ppt. Chlorophyll *a* levels (chl *a*) in Notre Dame Bay were lowest in May at 0.5 µg/L and highest in August at 2 µg/L, with a secondary increase in October at 1.5 µg/L. Chl *a* in Trinity Bay (Cap Cove) remained constant from May to November at around 1 µg/L.

Salmonier Cove displayed higher levels of chl *a* than the other two areas from May to November and reached highest levels of 4.5 µg/L in May and 4 µg/L in August.

4. Discussion

The need to be able to determine the effects of a stress condition on bivalves is becoming increasingly important as bivalve aquaculture develops and intensifies. In many cases, the stress response indicating that a stress event has occurred in an aquaculture situation has been traditionally observed at the whole animal level, following the internal cascade of cellular and subcellular responses. Recently however, researchers have been working on identifying stress responses at the cellular and subcellular levels in order to better predict impending whole animal effects, and provide tools to avoid effects that impact optimum shellfish production.

Protein profiles using two-dimensional gels have shown differences between intertidal and cultured mussels, with the understanding that they are influenced by a range of different environmental conditions (Lopez *et al.* 2001). The effects of heat shock protein expression, changes in O_2 consumption and ammonia production levels have been reported to be altered by to cyclic temperatures, season (reproductive cycle), and air exposure (Bayne and Thompson 1970, Bayne 1973, Widdows 1976, Thompson *et al.* 1978). Temperature and salinity changes have also been reported to alter physiological activity and mobility in bivalve hemocytes (Fisher 1988, Newell and Barber 1988, Sparks and Morado 1988).

Lysosomes of bivalves have been shown to respond to stressors of various types (Moore 1976, Moore *et al.* 1979, Bayne *et al.* 1981b, Lowe *et al.* 1981, Moore and Clarke 1982, Lowe 1988, Regoli 1992, Lowe *et al.* 1995a, b, Grundy *et al.* 1996, Moore

et al. 1996, Hauton *et al.* 1998, 2001). Air exposure, especially correlated to elevated temperatures, resulted in changes in the activities of lysosomal enzymes in the mussel, *Mytilus edulis* and the cockle, *Cerastoderma edule*, and changes in lysosomal enzymes have been reported in the oyster *Ostrea edulis* and *Mytilus edulis* due to fluctuating salinity (Moore *et al.* 1979, Bayne *et al.* 1981b, Hauton *et al.* 1998).

The research involved with the present thesis examined changes in lysosome integrity using the neutral red assay (NRA), as a method to evaluate conditions that mussels are exposed to during culture in Newfoundland. Stress response, in this regard, was determined as a measure of neutral red retention (NRR) by the lysosomes.

4.1 Transport from field to laboratory

The initial transport of mussels from the field to the laboratory, and initial holding in the laboratory in seawater raceways, prior to experimental use, showed no consistent change in stress response, in mussels. This was not surprising given that the mussels were kept in a cooler with ice (ice packs) during transport, and that the animals were kept out of water for only a brief period. Similar dry storage conditions have shown no detrimental effects on mussels in other studies (Boyd and Wilson 1978, Prochazka and Griffiths 1991). As well, a reimmersion period of at least 48 h was used to facilitate post-transportation recovery, which has also been shown to improve to mussel condition in earlier studies (Slabyj and Hinkle 1976, Slabyj 1980, Prochazka and Griffiths 1991). Thus, it was concluded that the collection and holding of mussels in the laboratory for short periods of time did not cause any observable stress, hence, the mussels could be

considered non-stressed prior to experimental manipulations aside of normal seasonal condition. However, Bayne and Thompson (1970) showed that holding mussels a few weeks in the laboratory could affect condition indices. This was consistent with observations in this project that examined wet storage of mussels over a twelve-day period, where stress response (decreased NRR) was increased. It is possible that extended holding of mussels in the laboratory prior to experimental use would have affected the initial stress response, and thus the overall experimental results.

4.2 Temperature shock

Like other experiments that exposed mussels to near lethal temperatures, the NRA demonstrated that high temperature exposure is a stress on mussels. Lysosomes in the mussel hemocytes were highly responsive to thermal stress, a phenomenon previously observed in *Ostrea edulis*, *Crassostrea gigas*, and *Pecten maximus* exposed to 25°C, and in mussels exposed to 28°C using more elaborate analytical techniques (Moore 1976, Hauton *et al.* 2001). As well, it has been demonstrated in other studies that heat shock or thermal changes have an impact on subcellular components in oysters and mussels, where heat shock proteins have either been upregulated or induced (Hofmann and Somero 1995, Chapple *et al.* 1998, Clegg *et al.* 1998). The NRA for the 4 h temperature shock of 20°C also supplied evidence that the thermal shock induced a rapid stress response in mussels at the subcellular level. A high number of hemocytes showed significant lysosomal alterations in less than 90 minutes following the initial temperature shock. This was

further corroborated by alternative biochemical indices on the same animals (Egbosimba *et al.* 2002).

Moore (1976) determined that a change in temperature of at least 13°C was required to induce significant physiological changes in wild intertidal mussels. In the present study, thermal shocks of 10°C versus a 20°C showed that there was a higher neutral red retention (NRR) (less stress response) in mussels exposed to a 10°C shock, and they were much faster to recover. This is similar to the findings in wild mussels exposed to 10°C and 20°C in air, where the latter treatment induced changes in lysosomal activity (Moore *et al.* 1979).

The NRA was able to detail changes in lysosomes very effectively in mussels that had been exposed to various thermal shocks. It was concluded that the NRA is a sensitive and rapid indicator of stress response in Newfoundland cultured blue mussels, and therefore used to examine the effects of seasonality, as well as harvesting and post-harvest practices on mussel stress responses.

4.3 Seasonal variation

Many studies have noted differences in physiological conditions of bivalves between winter and summer. There are a number of factors that change seasonally, such as reproductive condition, water temperatures, and food availability that can affect and have a major impact on mussel physiological condition. Moore (1976) reported differences in lysosomal responses in winter and summer mussels from the intertidal zone, while Bayne and Thompson (1970) found metabolic rates to be low in the summer

and autumn and higher in the winter, during periods of active gametogenesis in British mussels. Chapple *et al.* (1998) and Hofmann and Somero (1995) observed seasonal changes, in relation to temperature change, in cellular protein levels of gill tissue.

The examination of seasonal variation revealed a pattern in stress response that is related to the reproductive cycle. Neutral red retention (NRR) dropped significantly just prior to and following spawning, was slow to increase following spawning, and increased to the highest NRR score in the spring (these periods corresponded to the prespawning, postspawning, and recovery periods, respectively). Previous research has shown high lysosomal destabilization, higher metabolic demands, lower survival, reduction in feeding, reallocation of energy, lower O_2 consumption, fluctuations in plasma components, and changes in body glycogen and byssal thread production all associated with gametogenesis and spawning (Bayne and Thompson 1970, Bayne 1973, Thompson *et al.* 1978, Worrall and Widdows 1984, Young 1985, Eertman *et al.* 1993, Myrand and Gaudreault 1995, Tremblay *et al.* 1998b).

Previous studies on wild mussels in Newfoundland have shown that gametogenesis does not occur during the winter and that its progression is rapid in the spring and early summer, followed by spawning in July (Thompson 1984a). This is consistent with results from the current study, where the observed seasonal trend in stress response is related to the reproductive cycle. The basal stress response level continually decreased throughout the winter months and only began to increase again in late spring / early summer (June 2001 and June 2002), when mussels are in undergoing gametogenesis. Mussels during this immediate prespawning period would have high

metabolic demands related to gamete production and would be expected to be easily stressed (Thompson 1984b).

The seasonal variation in stress response was also examined in terms of shelf life and condition index (meat yields), both indices of mussel quality. The shelf life varied little throughout the year, with the exception of July through to September, following the major spawning period. Meat yields were highest prior to spawning and lowest following spawning. These findings can be explained by previous studies in relation to reproduction. Mussels in the period just prior to spawning have a high metabolic demand for the purpose of gamete production and are in poor condition following spawning as they undergo a number of physiological changes, which can impede survival (Bayne and Thompson 1970, Bayne 1973, Dare and Edwards 1975, Slabyj and Hinkle 1976, Warwick 1984).

Seasonal variations in basal stress response levels of the mussels, as described by the NRA, shelf life, and meat yields, may result from changes in temperature and food availability, aside from the reproductive cycle or as compounding factors. Seasonal trends in water temperature and food availability (leading to nutritive stress) have been shown to affect byssal production, lysosomal stability, and metabolic activity in wild mussels (Bayne 1973, Young 1985, Hauton *et al.* 1998).

Water temperatures in Newfoundland for the 2001 / 2002 season were highest in August 2001 and lowest in February 2002. Food availability was lowest in June 2001. Previous studies have shown that mussels unable to cope with elevated temperatures may be a factor in summer mortalilty, particularly when coupled with periods of low food

availability and following spawning (Sephton *et al.* 1993, Myrand and Gaudreault 1995). Mortality does not appear to be a factor in winter, when water temperatures are below 0°C. However other environmental conditions, such as ice cover and algae drape over mussels may aid in heat retention lessening the impact of subzero conditions (Williams 1970, Freeman and Dickie 1979, Hatcher *et al.* 1997). It is possible that even with the minor spawning in October and November that lowered NRR significantly, lower water temperatures reduced the compounding effect of stressors on the mussel, leading to a higher survival and longer shelf life.

In Newfoundland, total particulate matter (TPM) is highest in summer in open areas, but higher in autumn and spring in small, semi-enclosed, inlets, such as Charles Arm, the sampling site for this experiment (Thompson 1984a, Penney *et al.* 2001, Nichols *et al.* 2002). Recovery of mussels following spawning and associated shelf life may reflect reduced stress with increased food availability and low temperatures in autumn.

In general, the combination of thermal, nutritive, and reproductive stress influences the outcome of stress response as assessed by NRA, shelf life, and meat yield of mussels.

4.4 Rapid water temperature fluctuations

Oceanographic events, such as tides, winds, and currents can rapidly alter water temperatures, particularly in enclosed inlets where water column stratification is enhanced. The results of experiments simulating the effects of rapid temperature

fluctuations on cultured mussels that may be associated with natural oceanographic events showed there was a significant change in NRR following only one 6 h change in only one of three trials. However there was no subsequent change in stress response during a 24 h cycle, with a temperature change every 6 hours, once the highest stress response was observed.

The effects of events such as rapid, tidally-induced thermal inversions previously reported on mussels suggest it is more likely that air exposure causes greater alterations in physiological conditions than rapid water temperature changes (Baird and Drinnan 1957, Widdows 1976, Tremblay and Pellerin-Massicotte 1997). Moore (1976) determined that a change in water temperature of at least 13°C was required to induce significant, irreversible physiological changes in wild mussels. The present results suggest that rapid alternating of thermal changes may induce a major stress response in cultured mussels, but further work is needed to verify this hypothesis.

4.5 Air exposures

Air temperatures, such as those likely to be found on a barge deck during harvesting, influence subcellular stress responses in mussels, even those within 13°C of ambient water temperatures, a thermal change reported to alter mussel physiological processes (Moore 1976). Overall, the results of my experiments showed that air temperatures equivalent to seasonal ambient water temperatures are less detrimental to mussels, followed by those below ambient water temperatures or within 5°C of ambient. The exception was air temperatures at subzero. In general, air temperatures within a 5°C

of ambient seawater temperatures have little effect on stress response, but greater thermal changes affect the response (NRR) proportionally. Air temperatures that exceeded 15°C induced the highest stress response in mussels. Air temperatures vary throughout the year and may harm mussels if exposed to such conditions for extended periods (up to 5 hours of acute exposure) (Widdows and Shick 1985). Moore *et al.* (1979) demonstrated that in both *Mytilus edulis* and *Cerastoderma edule* an air temperature exposure of 10°C for 8 h induced no change in lysosomal activity, but did at 20°C. This is consistent with the present findings. As well, the effects of subzero air temperatures previously described in a study by Williams (1970) showed that *Mytilus edulis* can tolerate freezing up to about −10°C, but that tissues become injured when 64% of the cellular water is converted to ice. Other studies have shown that there is a high incidence of mortality in mussels harvested during subzero temperatures, especially if measures are not taken to shield the mussels from the elements during harvest (Slabyj and Hinkle 1976, Slabyj 1980).

Long-term exposure to air also leads to desiccation, and a reduced metabolic rate that can lead to eventual death of mussels (Eertman *et al.* 1993). Experiments on Newfoundland cultured mussels revealed that extended exposure to air (≥ 4 h), no matter what the temperature, increased stress response in mussels during every sampling season. Various studies on wild mussels support this finding as air exposure has been shown to cause physiological changes where lysosomal stability was altered in *Mytilus edulis* and *Mya arenaria*, ammonia accumulated in plasma, and the number of hemocytes increased in mussels (Thompson *et al.* 1978, Tremblay and Pellerin-Massicotte 1997).

Understanding the influence of air exposure is important during harvesting on barge decks so as to minimize the impact on the mussels.

4.6 Processing activities

There are clear benefits and harm caused by processing activities, such as washing and declumping. Generally these processes are undertaken to meet hygienic standards and supply consumers with a clean product (Brooks and Harvie 1981). During certain times of the year (e.g., recovery and overwintering period), the action of washing and declumping had a minimal effect on the mussel stress response, whereas during other times the response was increased, particularly in the prespawning period. Some studies have reported that washing and declumping improves the condition of mussels, as it removes contaminants such as fouling organisms, mud, and silt (Warwick 1984). In the present study, it is possible that during the summer and autumn when this activity reduced stress response in mussels, that there were more contaminants present than at other times of the year and their removal had a greater effect on stress response than the activity of washing and declumping in itself. Alternatively, other studies have found that washing and declumping is harmful to mussels, as sorting increases damage to shells, tissue damage results from byssal thread removal, and liquor (mantle fluids) lost, all leading to an increase in mortality rate (Dare 1974, Slabyj and Hinkle 1976, Slabyj 1980, Warwick 1984).

The activity of debyssing induced a high stress response in mussels in all seasons. Slabyj (1980) and Slabyj and Hinkle (1976) reported that byssal damage was a

contributing factor to reduced quality and shelf life of wild mussels, and the present study support this finding for cultured mussels.

The process of reimmersion mussels for 24 h following any of the other previous processing activities reduced the initial stress response level caused by that activity. Previous research supports this finding, where survival of mussels increased by over 30% with a 12 h reimmersion period and 50% after a 48 h reimmersion period (Slabyj 1980, Prochazka and Griffiths 1991). It has been found that the condition and quality of mussels improves with reimmersion as liquor is recovered, byssal damage is repaired, and trapped mud can be filtered out by the mussels (Slabyj and Hinkle 1976).

From the present results, some processing activities were observed to be both harmful and beneficial, depending on the time of year, such as washing and declumping (harmful in spring mussels, but beneficial in summer and autumn mussels). Other processing activities were clearly harmful (i.e., debyssing) or beneficial (i.e., reimmersion) at all times. Reimmersion mussels for 24 h following a processing activity clearly reduced stress response, therefore would be recommended for all mussel processors to ensure higher quality product and prolonged shelf life.

4.7 Post-harvest storage conditions

Wet storage of mussels in ambient temperature water generally resulted in the lowest stress response, longest shelf life, and slowest mortality rate, and using ice was beneficial during dry air storage of mussels. This study was consistent with Tremblett (2001) who found the same results using the NRA technique in the winter, and with the

findings of Boyd and Wilson (1978) and Warwick (1984) where melting ice over mussels increased shelf life. Although many studies have not examined the advantages of long term wet storage, some producers sometimes find this practice is not economically feasible. Equipment and labour required for extended wet storage facilities can be very costly. As well, byssal thread production by mussels can be very abundant with extended holding in wet storage, making the product clump and become unappealing to customers, especially in North America. Furthermore the return value for the mussels can end up being very low, if costs to maintain the mussels are excessively high. However, wet storage for periods up to 7–10 days produce beneficial effects that should offset the costs and be short term enough to avoid excessive byssal formation.

The use of ice increases the average shelf life of the green-lipped mussel, *Perna canaliculus*, and the blue mussel, *Mytilus edulis* in comparison to mussels held in ambient air temperatures without ice or held in chilled rooms without ice (Slabyj and Hinkle 1976, Boyd and Wilson 1978, Brooks and Harvie 1981). As well, holding mussels under melting ice, as opposed to just in or on ice, can further increase shelf life by more than five days (Boyd and Wilson 1978, Brooks and Harvie 1981, Warwick 1984).

4.8 Spatial comparison

The seasonal and environmental conditions at a site influences the stress response levels and condition index of the mussels as shown by experimental evaluation of various sites in Newfoundland where condition indices appear to be site specific. Studies in the

past have shown that the geographic layout, and environmental conditions of a site can influence the condition, reproduction strategies, growth, and mortality of mussels. Numerous studies have shown also that mussels placed in different sites, or from different sites have varying rates of growth and mortality (Freeman and Dickie 1979, Incze *et al.* 1980, Dickie *et al.* 1984, Mallet *et al.* 1987, Smaal *et al.* 1991, Sephton *et al.* 1993, Tremblay *et al.* 1998a, b).

Water temperatures have been reported as a major contributing factor influencing summer mortality in mussels at different sites (Incze *et al.* 1980, Sephton *et al.* 1993, Tremblay *et al.* 1998a). Food availability is also an obvious indicator of how well mussels will grow (Incze *et al.* 1980, Thompson 1984a, Smaal *et al.* 1991, Penney *et al.* 2001). Other limitations of a site have been related to flushing rates and winter ice cover. Charles Arm is a small semi-enclosed inlet with restricted flushing and winter ice cover, both leading to low food availability during parts of the year (Penney *et al.* 2001). Areas that are more open and / or are ice-free have been observed to have higher tissue growth and more consistent food supply (Thompson 1984a, b, Mallet *et al.* 1987, Penney *et al.* 2001), and this helps explain the differential in patterns among the sites in the present study.

Although sites may be geographically close together, the basal stress response level of mussels may still vary between the sites, and appears to be a factor of seasonal and environmental conditions, both of which may influence reproductive strategies, and be reflected in the NRA stress response measurement. No attempt was made to determine species composition at the various sites so the role of *Mytilus edulis* versus

Mytilus trossulus ratios in relation to stress response has yet to be determined. Hence, location specific conditions and genetics affecting stress response and performance of mussels may need to be examined further before general conclusions can be made.

5. Conclusions and Recommendations

The neutral red assay (NRA) shows promise as a rapid test that can be used to determine if a stress response is induced by various conditions to which cultured mussels are exposed. The NRA measure of subcellular changes in the hemocyte lysosomes provides an early indicator for impending whole animal stress response changes (possibly irreversible). It was clearly shown how stress response could be altered or different in mussels based on seasonal, environmental and reproductive conditions, post-harvest processing, handling and storage, and on spatial comparisons.

By examining stress responses with the NRA, late autumn (December) through to spring (May) appears to be the times of the year when mussels are in the best physiological condition, and can best cope with handling, processing, and storage stressors.

Examinations of stress response associated with air exposure suggests that harvesting on barge decks causes least stress when air temperatures are close to being equivalent to ambient water temperatures. Such harvesting should be avoided for prolonged periods when air temperatures are below 0°C. If air temperatures exceed 5°C in difference from the water temperature, mussels should be covered to avoid stress response changes and reduction in shelf life. As well, extended holding of mussels on deck should be minimized to less than 4 h during any season.

The differential in stress responses also indicates that the accepted method of debyssing mussels should be more closely examined. Mechanical pulling of the byssal

threads is a significant stress response inducing procedure. Other debyssing methods, such as removing the byssal threads by cutting them at the hinge of the shells, as well as consumer education about the problems associated with debyssing whereby byssal threads are torn from the mussels, should be a priority for examination in areas where such a method is practiced (i.e., North America). It should also be a priority to incorporate reimmersion measures at processing plants following debyssing, as it has been shown to aid in mussel recovery to a level that could improve the overall condition and quality of the mussels.

The NRA, along with shelf life data, provided evidence that wet storage is a great benefit to prolonging the quality and condition of mussels. Producers should at least use ice as part of their storage practice to increase the shelf life and extend the value of their product.

Although the NRA has provided a great deal of information about culture conditions and their effects on mussels, there are a few questions left unanswered. One critical question, which is partially answered with the shelf life data, is how these identified stress responses would be implemented at the whole animal level. From the point of harvesting to storage of market-sized mussels, shelf life is the key issue, thus experiments to examine shelf life after just harvesting and processing activities would be of great benefit to growers. The answer to this question is more important when examining stress response in hemocyte lysosomes during grow-out situations. The results of the NRA that examined seasonal variations need to be correlated with growth and mortality data while mussels are still in the water. Even under periods of expected

high stress conditions as assessed by the NRA (i.e., spawning, low food), shelf life results could be extremely good (i.e., autumn 2001), and thus are not a good index to determine effects of stress in mussels during growout stages. Other studies could examine the effects of grading and socking on juvenile mussels, whereby initial NRA results could be later correlated to growth and mortality data. It would be expected that mussels that undergo more rigorous and high stress conditions during grading and socking (i.e., extended air exposure, longer washing and declumping, greater mechanical agitation) would have lower growth rates and higher mortality rates. Also it needs to be understood how the duration of a stress condition can impede the response at the whole animal level. It needs to be determined what short-term and long-term conditions are most detrimental to mussels.

A second critical question is how do other physiological changes influence lysosomal stress response changes. The lysosome has been stated as the site of earliest "detectable" change, and knowing that the role of lysosomes is largely immunological, it is conceivable that stress response, such as changes in protein concentrations, O_2 consumption, ammonia levels, and hemocyte activity may be a trigger of lysosomal changes. Alternatively, it is possible that the lysosomal changes trigger the other physiological conditions to change. There is a need to develop understanding of the integration of all these processes and find out if the lysosomal changes are primary or secondary response, and under what stress conditions they are induced.

Thirdly, the question of genetics and stress response needs to be addressed. Different species (*Mytilus edulis* vs. *M. trossulus*) may have different adaptive abilities

and thresholds of tolerance to a variety of conditions. Studies are needed to quantify stress response in mussels of different genetic makeup.

Fourthly, one of the major concerns within the shellfish culture industry is infection and disease, and it is important to find out how susceptible "stressed" mussels may be to pathogens, as opposed to "unstressed" mussels. The breakdown of the lysosomal system (a primary defence mechanism) due to non-pathogenic conditions, such as sub-lethal stresses, may increase susceptibility of mussels to disease and may lead to high levels of mortality or a low quality product. Research on other bivalve species, especially the Eastern oyster, *Crassostrea virginica*, has shown that disease has been more pronounced, in both adults and juveniles, when the animals were under various stressors including high temperature, high salinity, nutritional imbalances, extrinsic physical agents, and genetic defects (Fisher and Newell 1986, Bricelj *et al.* 1992, Ford and Tripp 1996, Shumway 1996). Also, the work of Hauton *et al.* (2001) showed that the course of bacterial infection in the various bivalve species European flat oyster (*Ostrea edulis*), Pacific oyster (*Crassostrea gigas*), and scallop (*Pecten maximus*), was dependant on water temperature.

Lastly, the NRA needs to be further developed and refined to address the question of its farm-based practical use. As the assay is performed at present it is not applicable for routine use in the field or on farm sites due to the various chemicals and equipment, light conditions, and length of time required. Advancement of this test and other chemical indices (i.e., enzymes) could lead to a cost-effective, reliable field-based test that growers and researchers could use to assess the effects of various conditions. The

present work has provided evidence that manipulating or altering standard culture practices can improve mussel quality and production, and in the end increase profits for farmers; however, further evaluation of factors inducing stress response as well as field validation of these experiments are required.

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Table

Table 1. Air temperature exposures during of early summer (June 2001), summer (August 2001), early winter (January 2002), and spring (May 2002).

Season	Below ambient water temperature (°C)	Ambient water temperature (°C)	Above ambient water temperature (°C)
Early summer	2	7	17
Summer	6	16	21
Early winter	–3	2	12
Spring	0	5	15

Figures

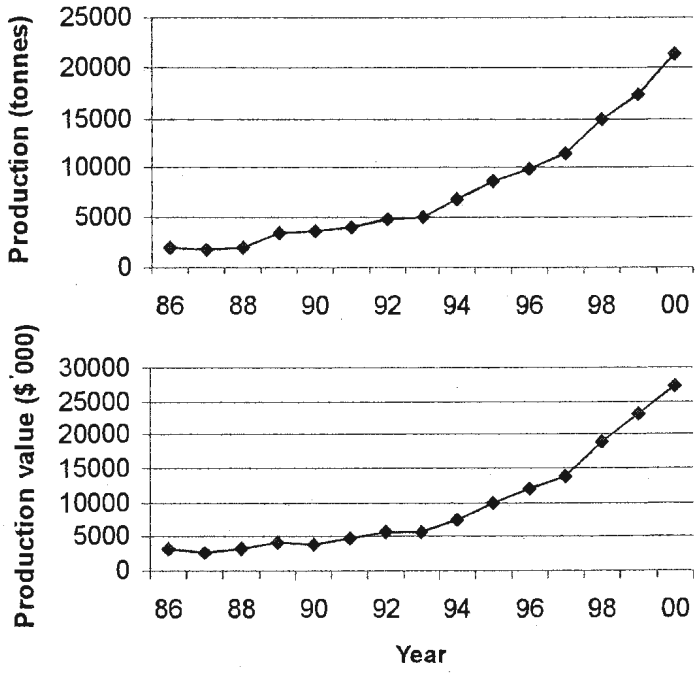

Figure 1. Canadian mussel production statistics (DFO 2001).

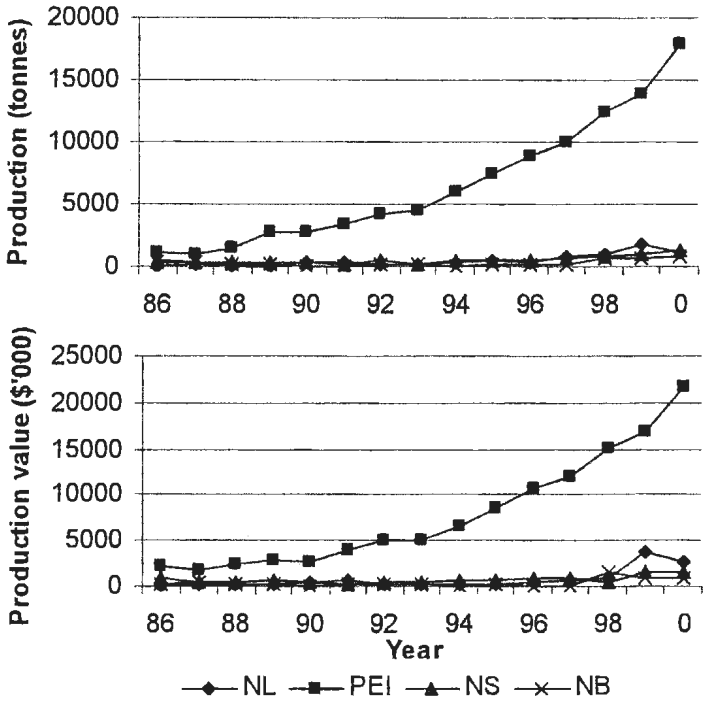

Figure 2. Atlantic Canada mussel production statistics (DFO 2001).

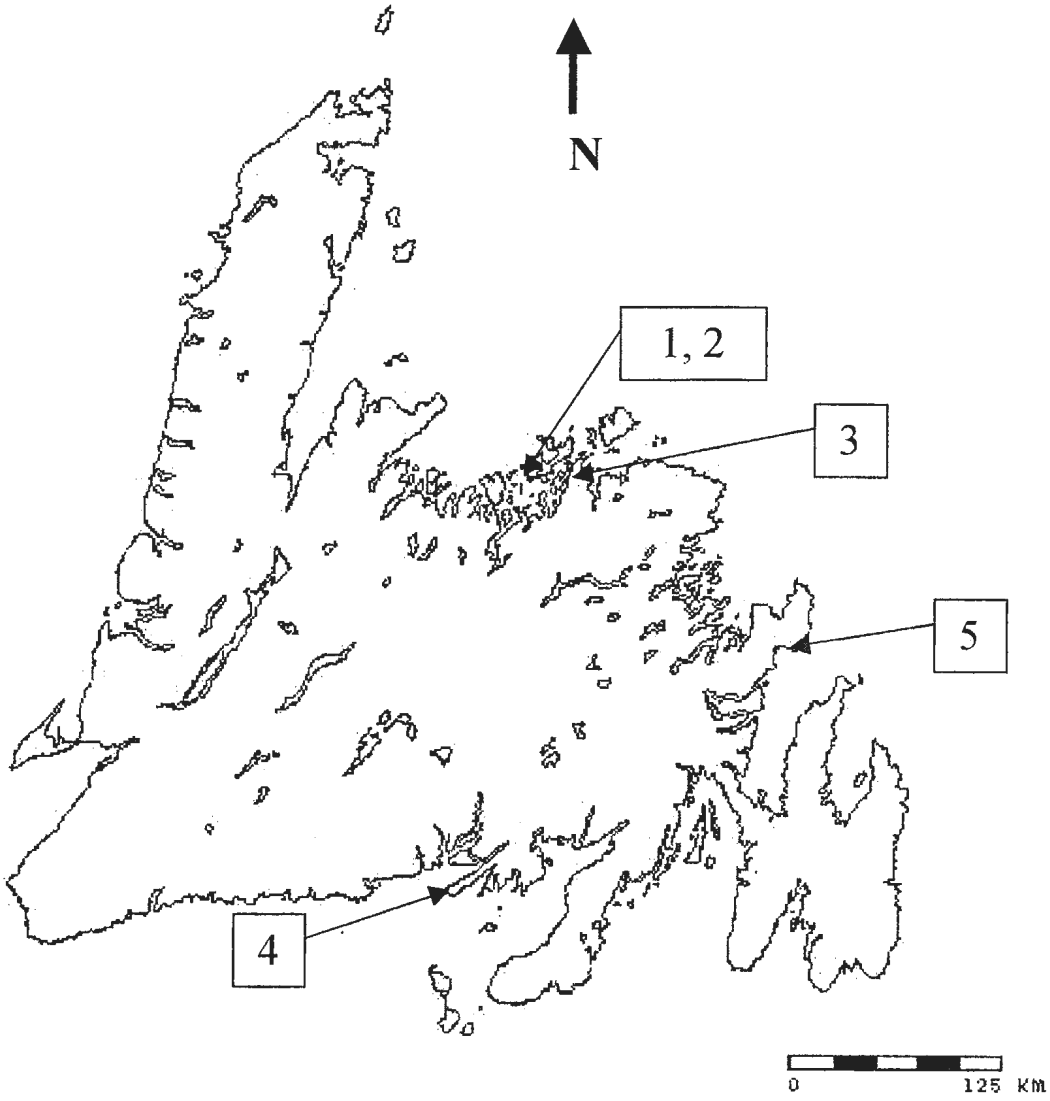

Figure 3. Newfoundland map indicating sampling sites. 1. Charles Arm, Notre Dame Bay (49.34 N, 55.28 W) 2. Bulley's Cove Notre Dame Bay (49.35 N, 55.35 W), 3. Reach Run, Notre Dame Bay (49.42 N, 54.69 W) 4. Salmonier Cove, Connaigre Bay (47.59 N, 55.78 W) 5. Cap Cove, Trinity Bay (48.40 N, 53.37 W)

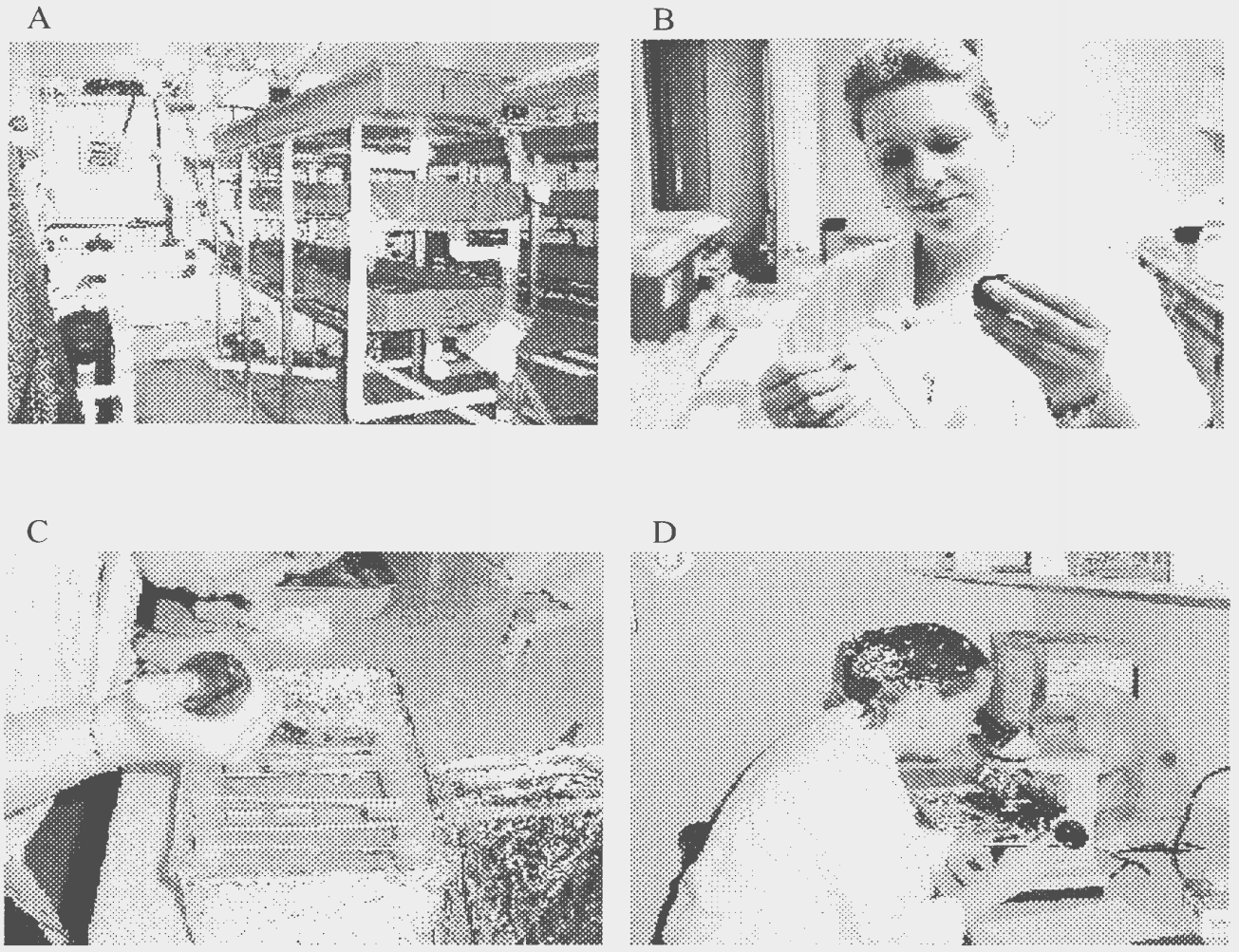

Figure 4. Holding and handling of mussels and hemolymph / hemocytes samples for the neutral red assay (NRA). (A) maintenance of mussels in seawater raceway system, (B) hemolymph collection from adductor muscle, (C) addition of neutral red dye to hemocyte layer on slides held in light proof chamber, (D) examination of lysosomal changes in hemocytes stained with neutral red dye. Photos courtesy of C. Couturier.

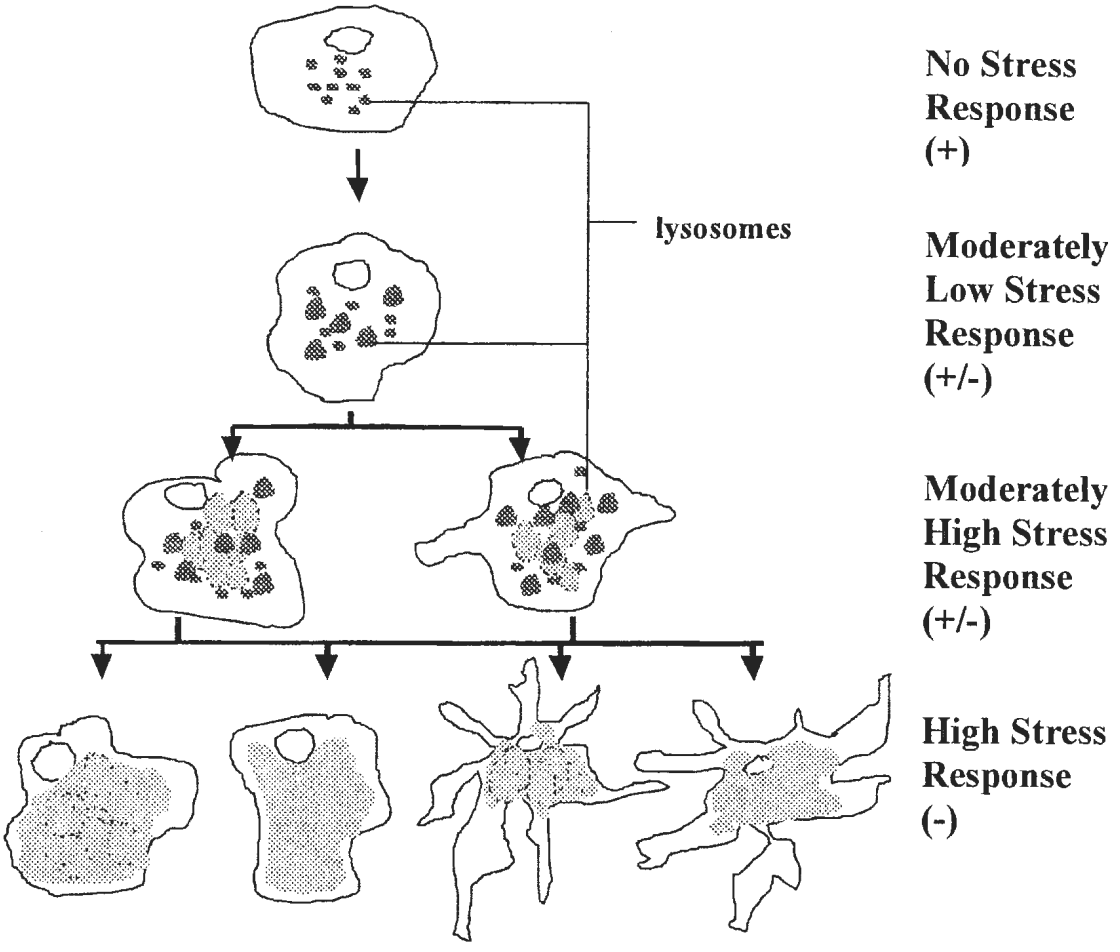

Figure 5. Schematic representation of the lysosomal and hemocyte characteristics during the neutral red assay (NRA). **No stress response** = characterized by appearance of tiny pink dots, which are lysosomes containing neutral red dye particles. **Moderately low stress response** = beginnings of stress response apparent with increase in the size of lysosomes due to lysosomal membrane fusion. **Moderately high stress response** = lysosomes appear larger and more faint in colour due to fusion of lysosomal membranes and leakage of neutral red dye into the cytosol. **High stress response** = high stress response characterized by cytosol having become totally tinged pink due to leakage of neutral red dye out of the lysosomes, and only remnants of the membranes may be still visible. Once 50% of hemocytes from a mussel reach this level the assay is terminated for that mussel. Hemocytes requiring more than 60 minutes to attain the high stress response appearance are from animals considered to be under low stress conditions. The hemocytes in the schematic diagram are approximately 5 – 10 μm in actual size.

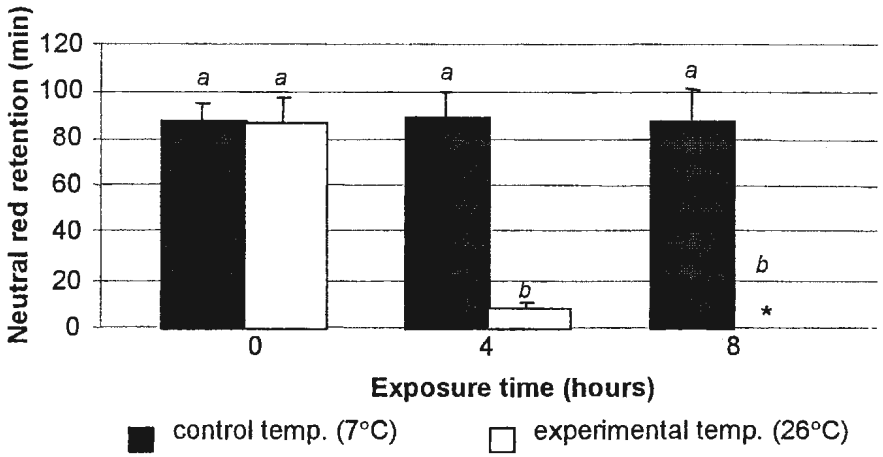

Figure 6. Comparison of neutral red retention in lysosomes of *Mytilus* spp. exposed to a temperature shock of 19°C (from 7°C to 26°C) over an 8 hour period. Mussels were collected in April 2002 and were in an overwintered condition. Bars represent the mean ± S.E., n = 12 mussels. Common letters denote no significant difference among all treatments (Tukey's b, $p>0.05$). (* indicates mean = 0)

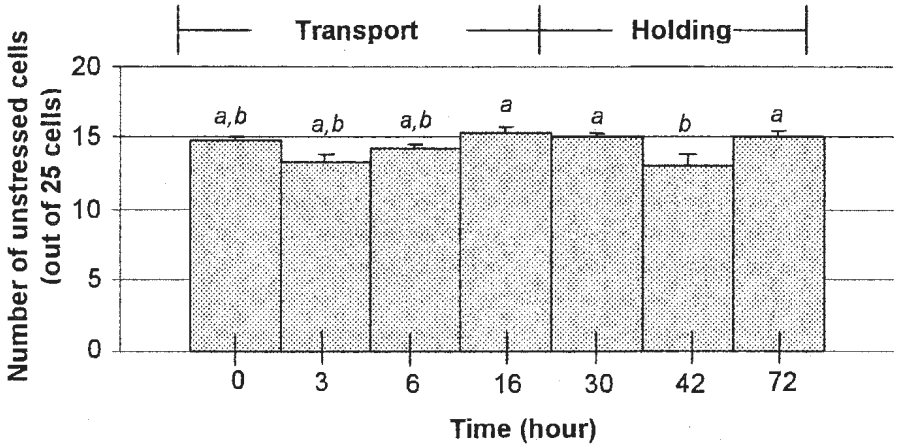

Figure 7. Progression of neutral red retention in lysosomes of *Mytilus* spp. exposed to initial transport and laboratory holding conditions. Mussels were held on ice for the first 30 hours and were then placed in ambient seawater in holding tanks for the remainder of the period. Bars represent the mean number of unstressed cells (out of 25 cells) ± S.E., n = 10 animals. Common letters denote no significant difference among treatments (Tukey's b, $p>0.05$).

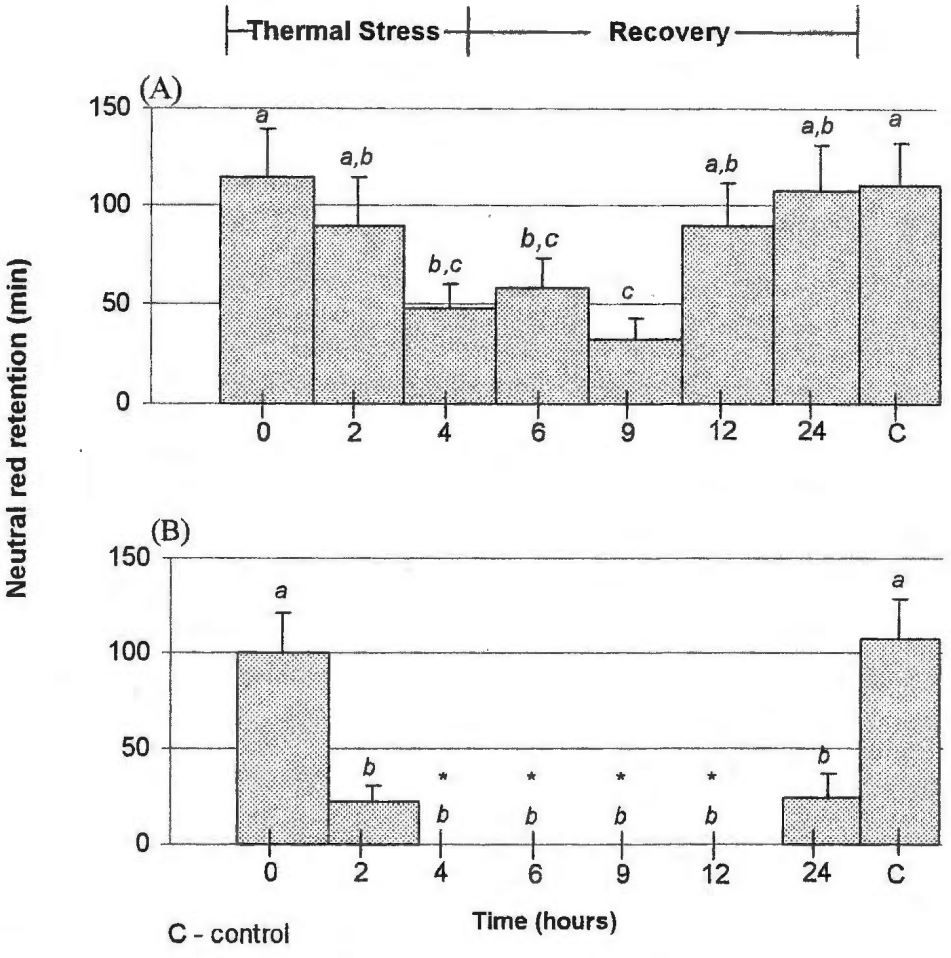

Figure 8. Comparison of neutral red retention in lysosomes of *Mytilus* spp. exposed to different temperature shocks for 4 h duration. Bars represent the mean ± S.E., n = 6 mussels. Mussels were collected in May 2002 and were in early prespawning condition. Common letters denote no significant difference among treatments (Tukey's b, $p>0.05$). At time 4 hours, mussels were placed back into ambient temperature water (recovery) for remainder of the experiment. (A) Temperature shock of 10°C (from 5°C to 15°C), (B) Temperature shock of 20°C (from 5°C to 25°C). (* indicates mean = 0). Note, control samples (5°C) were taken at hour 24 for each temperature shock.

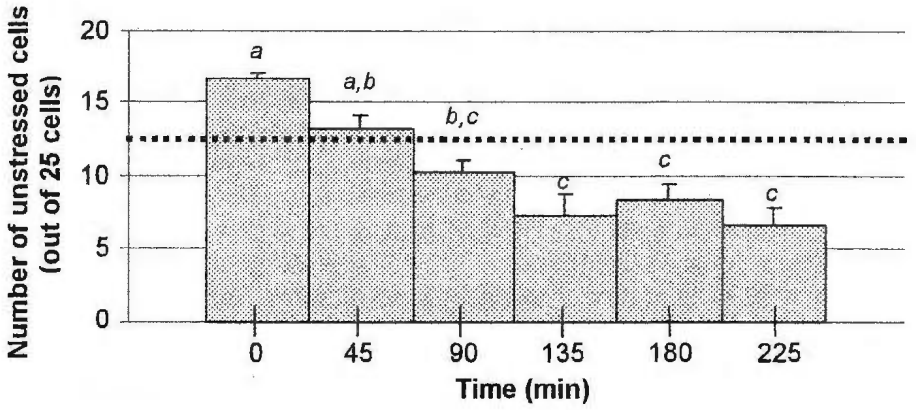

Figure 9. Progression of neutral red retention in lysosomes of *Mytilus* spp. (as experienced by the number of unstressed cells at time of sampling out of 25 cells) during a four-hour temperature shock of 20°C (from 5°C to 25°C). Bars represent the mean number of unstressed cells out of 25 ± S.E., n = 12 mussels. Common letters denote no significant difference among treatments (Tukey's b, $p>0.05$). Dotted line denotes the number of unstressed cells required for a neutral red retention final score to be greater than 0 minutes.

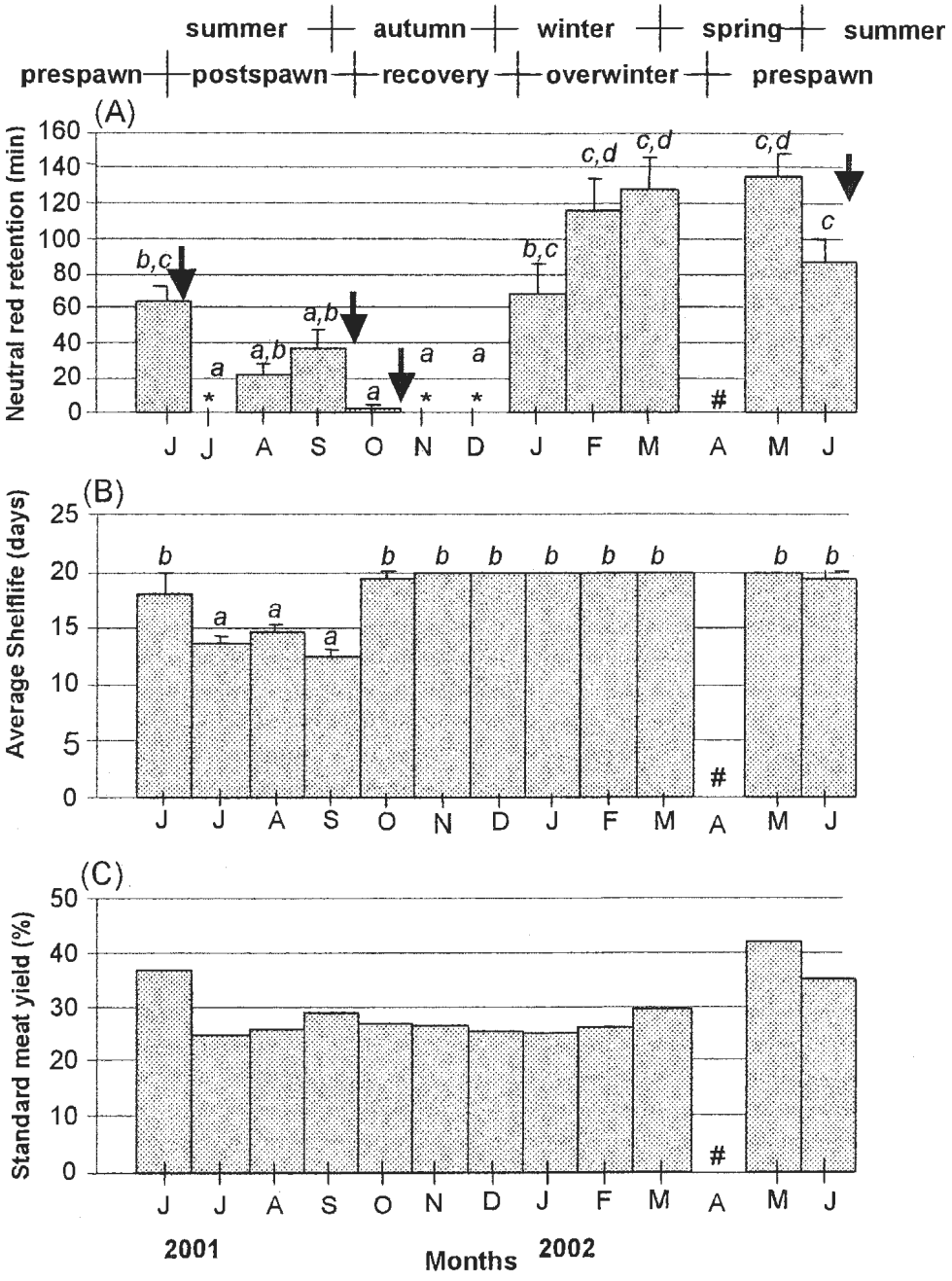

Figure 10. Seasonal variation of NRR, average shelf life, and standard meat yield in *Mytilus* spp. Common letters denote no significant difference among treatments (Tukey's b, $p>0.05$). (A) Neutral red retention in lysosomes during summer, autumn, winter and spring conditions. Bars represent the mean ± S.E., n = 12 mussels (* indicates mean = 0), (B) Average number of days for 5% mortality to be reached. Each bar represents the mean + S.E., n = 3 groups of 50 mussels, (C) Calculated meat yields. Arrows indicate spawning events. Note: no samples were collected for the month of April (#).

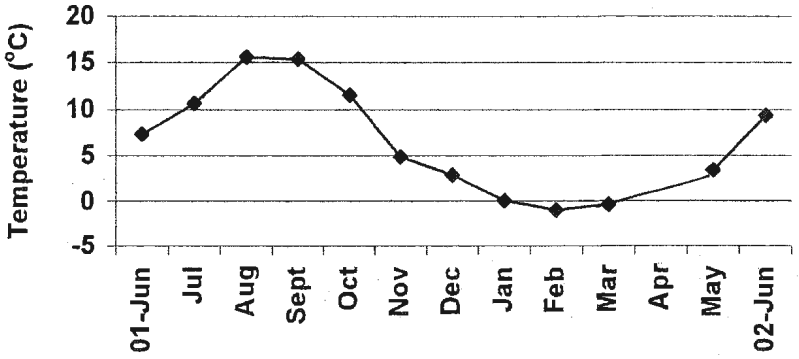

Figure 11. Seasonal water temperatures at Charles Arm for year of June 2001 to June 2002. Data collected at same time as mussel samples.

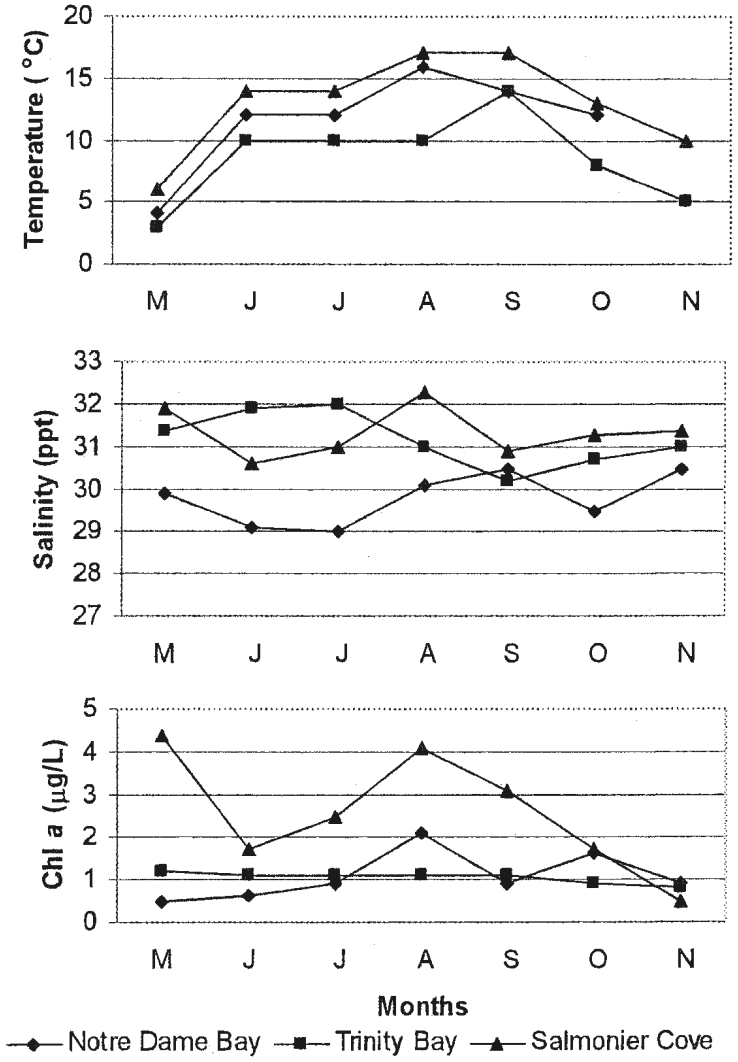

Figure 12. Environmental data for mussel aquaculture sites in Notre Dame Bay, Trinity Bay, and Salmonier Cove for months of May 2001 to November 2001. Data collected with SBE 25 and provided courtesy of Nichols *et al.* (2002).

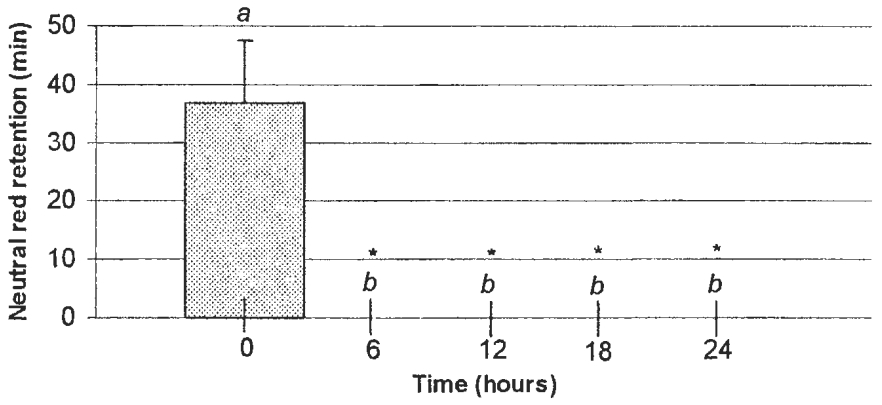

Figure 13. Neutral red retention in lysosomes of *Mytilus* spp. exposed to temperature changes (10°C) associated with rapid water fluctuations. Data presented are for mussels collected in September 2001 that were in late postspawning / early recovery condition. Bars represent the mean ± S.E., n = 12 mussels. Common letters denote no significant difference among treatments (Tukey's b, $p>0.05$). (* indicates mean = 0). Note: NRR scores for mussels collected in June and August were all 0 minutes and are not presented.

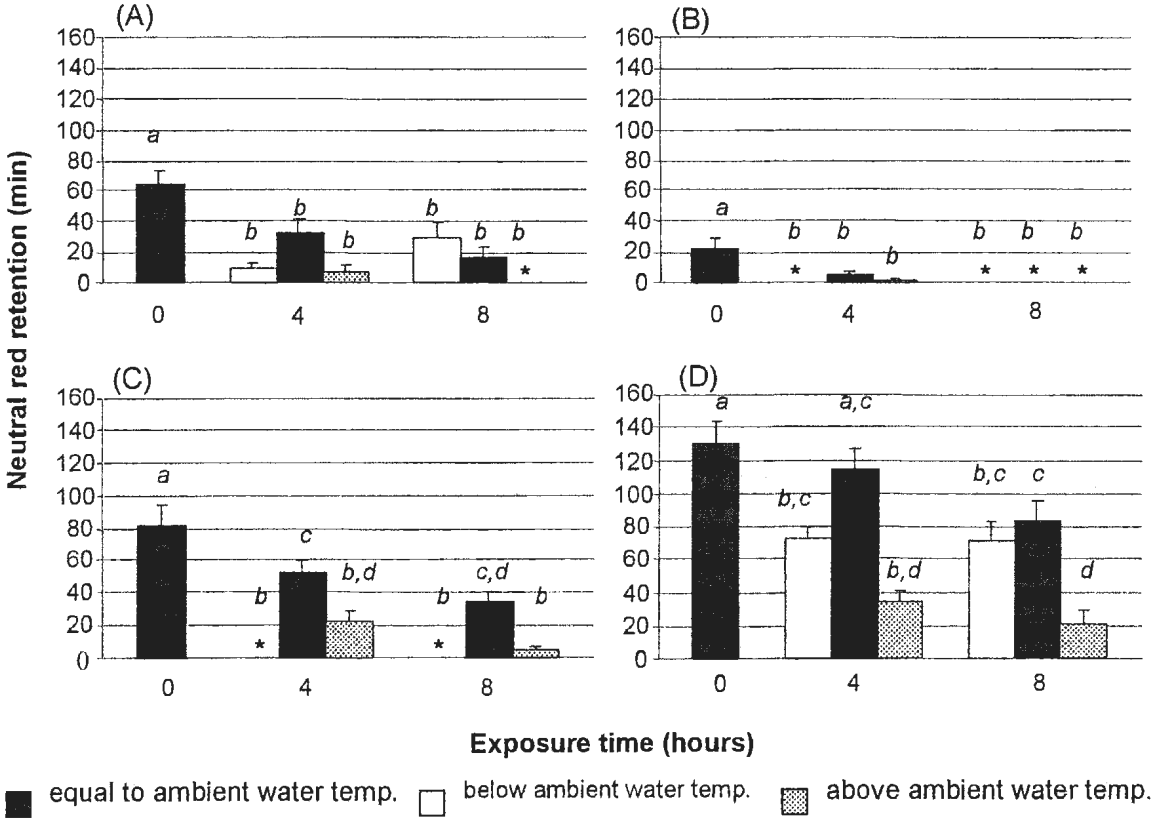

Figure 14. Neutral red retention in lysosomes of *Mytilus* spp. exposed to various air temperatures as may be found on barge deck during harvesting. Bars represent the mean ± S.E., n = 12 mussels (* denotes mean = 0). Common letters denote no significant difference among treatments within each season (Tukey's b, $p>0.05$). (A) Early summer mussels (June 2001, prespawned), (B) Summer mussels (August 2001, postspawned), (C) Early winter mussels (January 2002, recovered), (D) Spring mussels (May 2002, overwintered). See Table 1 for temperature conditions.

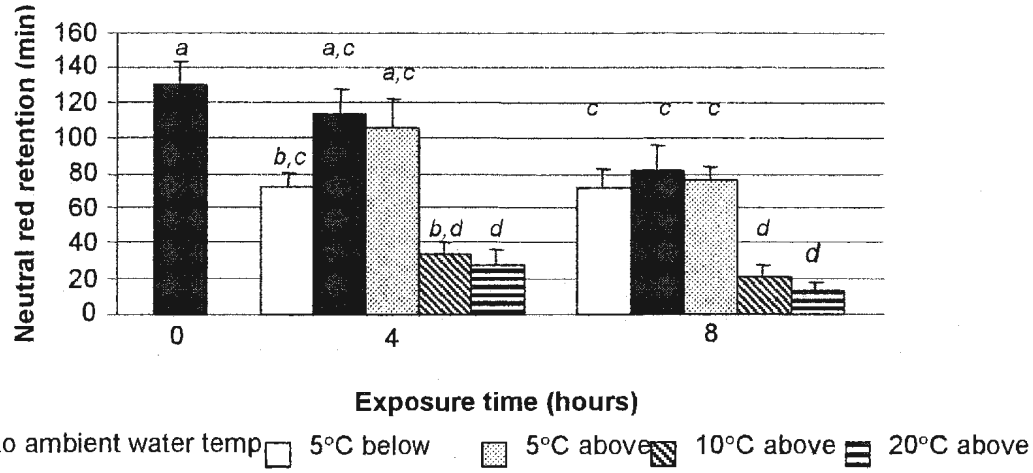

Figure 15. Neutral red retention in lysosomes of *Mytilus* spp. exposed to various air temperatures typical for harvesting in May. Bars represent the mean ± S.E., n = 12 mussels. Common letters denote no significant difference among treatments (Tukey's b, $p>0.05$). Ambient water temperature during this period was 5°C.

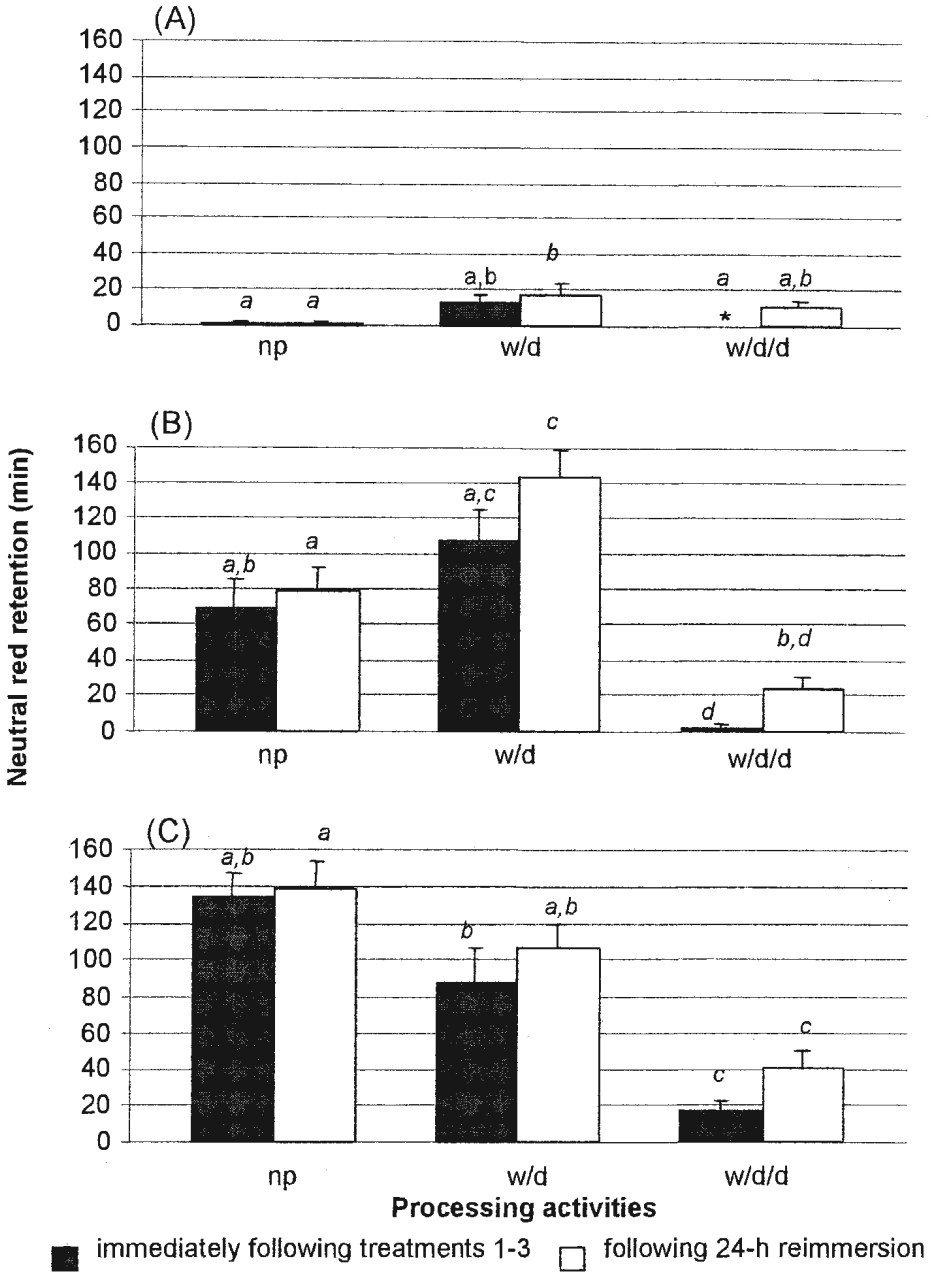

Figure 16. Comparison of neutral red retention in lysosomes of *Mytilus* spp. exposed to various processing activities: unprocessed (np), washed and declumped (w/d), washed, declumped and debyssed (w/d/d), and 24-hour recovery period in ambient seawater of mussels from each of previous 3 treatments. Bars represent the mean + S.E., n = 12 mussels (* denotes mean = 0). Common letters denote no significant difference among treatments within each season (Tukey's b, $p>0.05$). (A) Summer mussels (August 2001, postspawned), (B) Early winter mussels (January 2002, recovered), (C) Spring mussels (May 2002, overwintered). Note, scores for early summer mussels were all zero and are not presented.

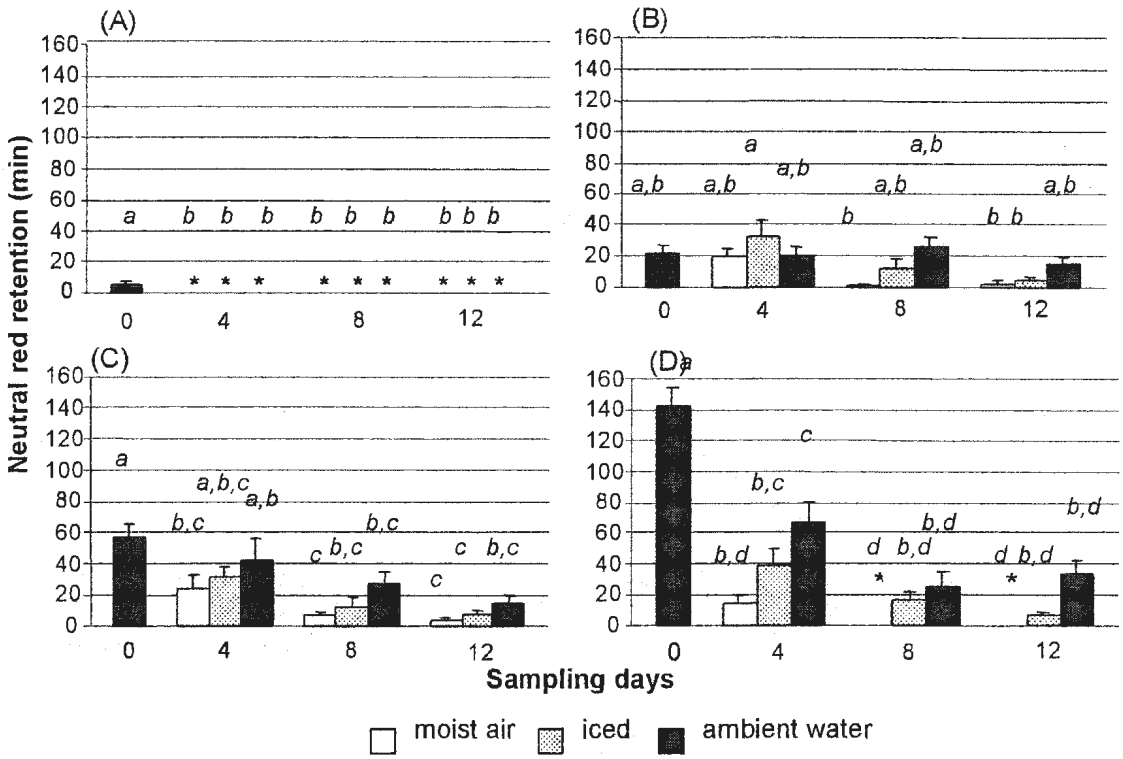

Figure 17. Comparison of neutral red retention in lysosomes of *Mytilus* spp. held under various post-harvest storage conditions: ambient water (2°C to 15°C), chilled moist air (2°C to 4°C), and on ice (0°C). Bars represent the mean ± S.E., n = 12 mussels (* denotes mean = 0). Common letters denote no significant difference among treatments within each season (Tukey's b, $p>0.05$). (A) Early summer mussels (June 2001, prespawned), (B) Summer mussels (August 2001, postspawned, (C) Late autumn mussels (November 2001, recovered), (D) Spring mussels (May 2002, overwintered).

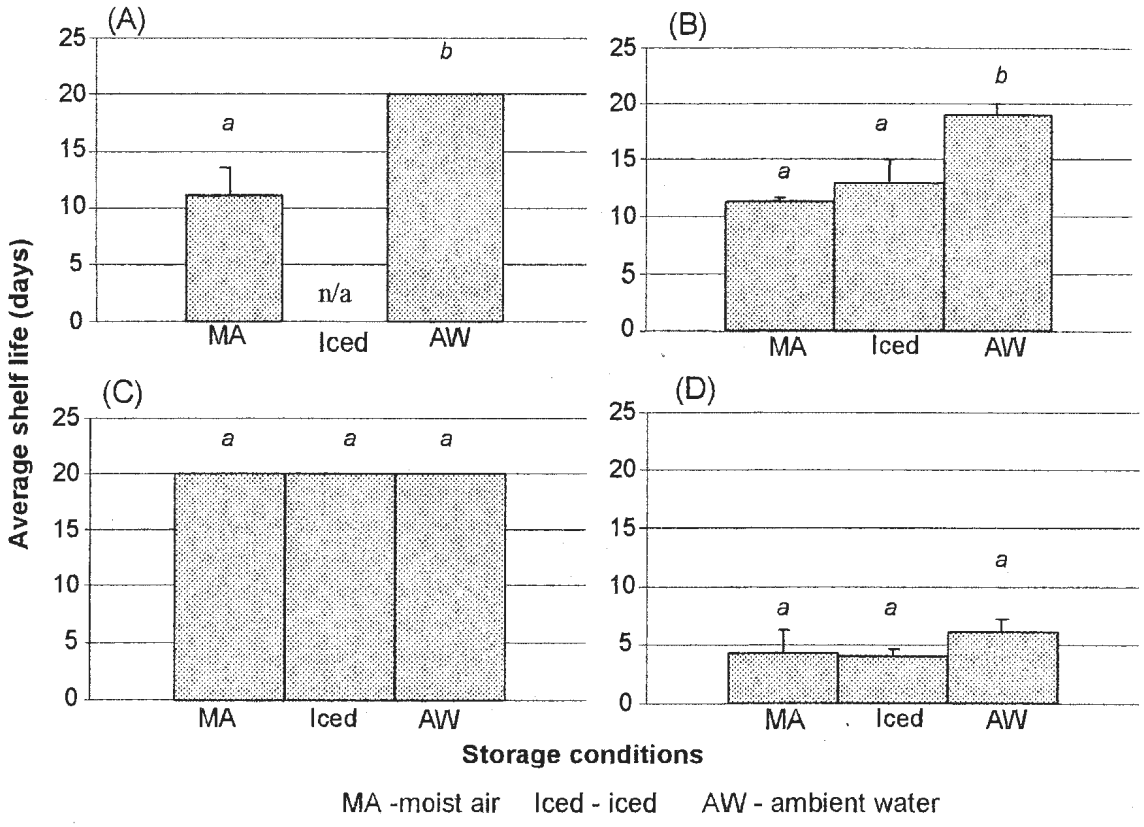

Figure 18. Average number of days for *Mytilus* spp. to reach 5% mortality held under various post-harvest storage conditions: AW - ambient water (2°C to 15°C), MA - moist air (2°C to 4°C), and iced (0°C). Bars represent the mean ± S.E., n = 3 groups 50 mussels. Common letters denote no significant difference among treatments within each season (Tukey's b, $p>0.05$). (A) Early summer mussels (June 2001, prespawned), (B) Summer mussels (August 2001, postspawned), (C) Late autumn mussels (November 2001, recovered), (D) Spring mussels (May 2002, overwintered).

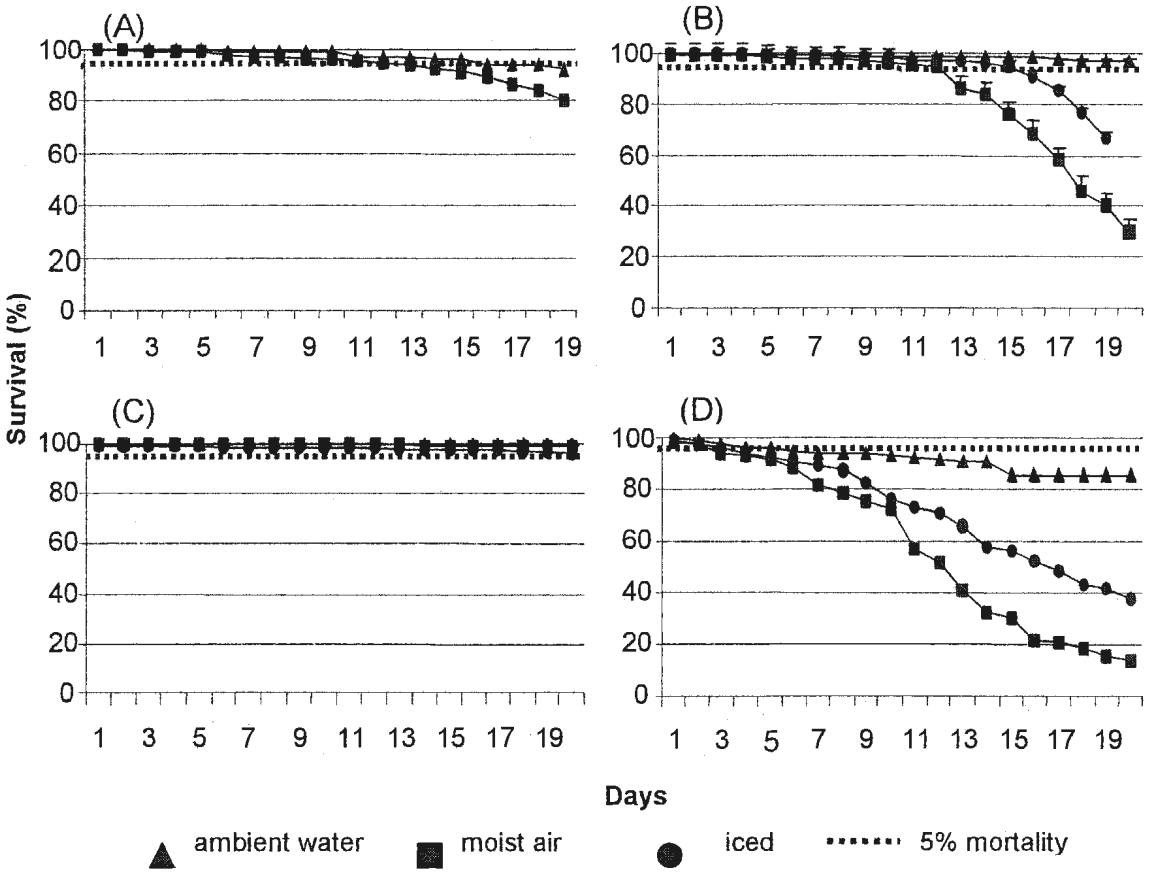

Figure 19. Percent survival for *Mytilus* spp. held under various post-harvest storage conditions: ambient water (2°C to 15°C), moist air (2°C to 4°C) and on ice (0°C). Each point represents the mean of 3 replicates. (A) Early summer mussels (June 2001, prespawned), (B) Summer mussels (August 2001, postspawned), (C) Late autumn mussels (November 2001, recovered), (D) Spring mussels (May 2002, overwintered).

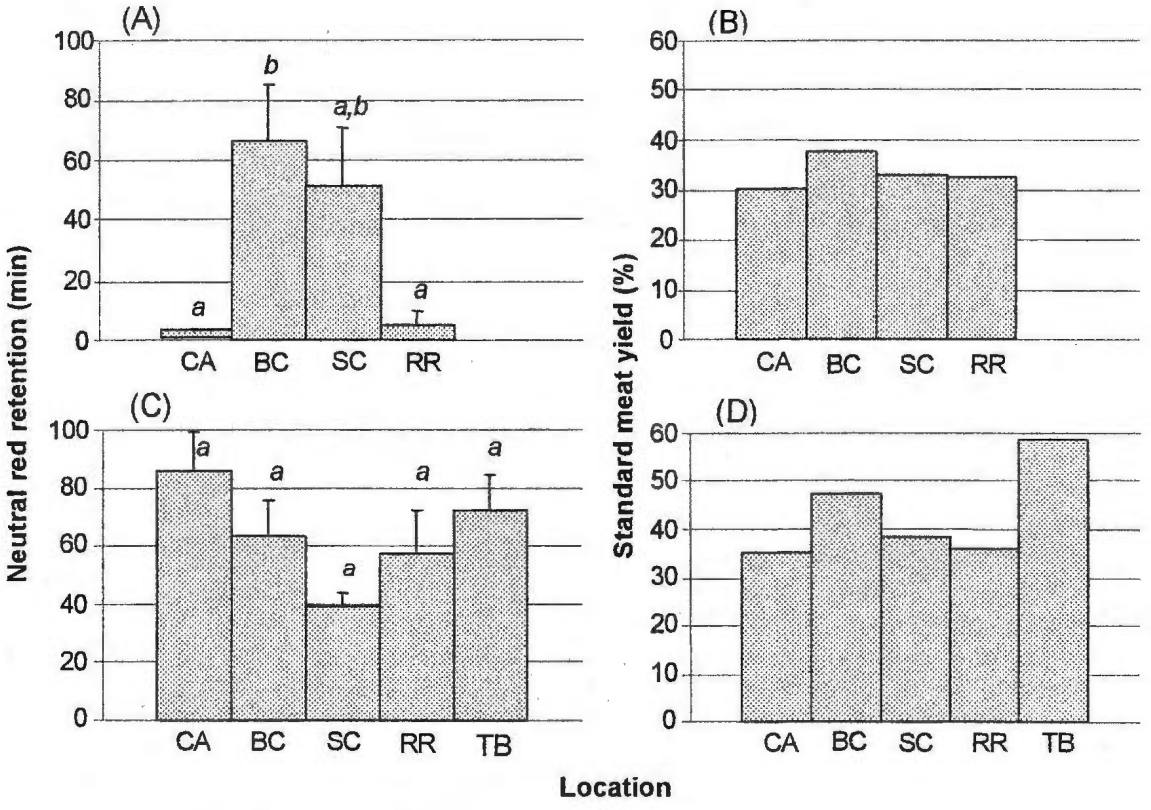

Figure 20. Comparison of neutral red retention in lysosomes and standard meat yields of *Mytilus* spp. from different geographical locations in Newfoundland: Charles Arm (CA), Bulley's Cove (BC), Salmonier Cove (SC), Reach Run (RR), and Trinity Bay (TB). Bars for neutral red retention represent the mean + S.E., n = 12 mussels. ANOVA: $p<0.005$. Common letters denote no significant difference among treatments (Tukey's b, $p<0.05$). (A) Autumn sampling for NRR (November 2001, recovered mussels), (B) Autumn sampling for standard meat yield (November 2001, recovered mussels), (C) Spring sampling for NRR (May 2002, overwintered mussels), (D) Spring sampling for standard meat yield (May 2002, overwintered mussels).