

Condition Index and Neutral Red Assay Response of Cultured *Mytilus edulis* L. Stored in a Wet Holding Facility during Winter and Spring in North-eastern Newfoundland

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Abstract

In order to determine the effects of extended wet-holding on cultured blue mussels under ambient conditions during the winter and spring in Newfoundland, we investigated physiological stress response using the Neutral Red Assay, change in dry and wet weight and in overall condition based on the dry tissue weight/dry shell weight ratio and the dry tissue weight/wet tissue weight ratio of animals held for up to three months in a commercial holding facility and compared with field controls. During the winter season, dry weight and condition of mussels in the holding facility increased significantly over the three month period when compared with samples from the field. Spring-held mussels were observed to lose dry weight and condition in holding as compared with field controls. The Neutral Red Assay indicated an observed but not statistically significant change in physiological stress response for mussels held during the winter season. No observed change was noted for field controls. During the spring no overall change was noted in Neutral Red response for held or field control samples. Dry weight and condition analysis indicated that the winter season was the most stable for long term holding of harvested mussels. Overall analysis of lysosomal neutral red retention time did not reveal a statistically significant response for mussels held during winter or spring suggesting that low temperatures may affect the haemocyte lysosomal response to Neutral Red retention. Based on the observed change in condition and dry weight across season, we recommend that mussels can be held for at least two months during the winter season and at a limit of one month for spring (especially late spring i.e. May and June) before a significant loss in condition and potential quality is observed.

Keywords: *Mytilus edulis*; Live holding; Stress response; Condition index; Neutral red assay

Introduction

Longline culture of the blue mussel (*Mytilus edulis* L. 1758) is increasing in Newfoundland and Labrador (NL) with the expansion of existing leases, the creation of new culture sites and a new organic designation. Since 2001 production has increased from 1,452 t to 4,400 t in 2012 [1]. As market demand and production increase, product is being shipped to local, national, and international markets. However occasionally there can be delays in shipping freshly harvested product due to weather or other factors such as ice conditions on farms. Such issues can postpone transport to market, resulting in a need to store product in holding facilities for extended periods of time. The maximum duration for holding bivalves in wet storage under ambient water conditions is dependent on the environmental variability in the facilities. Recently it was found that harvested farmed mussels in Newfoundland could only be held for periods up to one week during summer and up to one month during the declining temperatures in the fall before changes in stress response and condition were noted to become significant [2]. Determining the maximum time spent in holding, especially with respect to season and before there is a significant decrease in meat yield and shelf life, is crucial to the industry.

Several factors can affect condition and physiological stress response in mussels, especially during long-term holding; these include temperature, population density, reproductive effort and food availability [3,4]. Short-term storage in ambient water results in the lowest degree of stress response compared to storage on ice or in chilled air [5]. Handa et al. [6] indicated that mussels could be stored for longer periods at water temperatures below 70C as compared to 140C if they were not fed. Additionally, as noted in Wyatt et al. [2],

extended storage during warm water seasons can increase the stress response and decrease condition. This can lead to mortality and thus loss of product.

Studies have shown low winter growth and metabolic rates in mussels compared to warmer water seasons. These differences were thought to be due to a combination of low food concentration, low temperature regime, reproductive effort and partly due to the physiological cost of pumping water with increased viscosity at low temperatures [7-9]. Typically, enzyme levels appear to be at their lowest during the winter, likely as a result of a shift in metabolic requirements and an increase in lipid peroxidation [8]. Levels of antioxidant enzymes follow seasonal cycles that are governed mainly by changes in temperature and nutrient levels [10-13].

Several studies have evaluated the impact of seasonality and environmental stressors on mussel physiological stress [5,7,12-18]. Some of these studies have also noted that the changes in the expression of genes associated with oxidative stress and innate immunity (i.e.,

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Mytilin, Defensin, SOD, catalase, and lysozyme) could be correlated with temperature, influx of fresh water, and chlorophyll levels [17,18]. Similarly, stressors including post-harvest live holding, processing and handling, air exposure, and changes in temperature also have the potential to impact the physiology of mussels and cause an increase in stress response. Using the Neutral Red Assay as a measure of physiological stress response, Harding et al. [5] found that Neutral Red dye retention time in haemocyte lysosomes was increased in cultured mussels during the winter months, gradually decreasing throughout late spring/early summer, suggesting that cold temperatures may reduce the impact or response to physiological stressors. Harding et al. [5] suggested that mussel response to stressors could also be modulated by the physiological condition of the animals.

Wyatt et al. [2] investigated the physiological and immune stress responses of cultured mussels held in a commercial processing facility for extended periods during the warmest water seasons in Newfoundland (i.e., summer/fall) and found a significant decline in condition and an increase in physiological stress response (as measured using the neutral red assay) compared to field controls. While the reproductive condition and post reproductive condition also seems to play a role, temperature appeared to be a major contributing factor in this case [5].

During winter and early spring in Newfoundland, coastal seawater temperatures can drop to as low -1.5°C [7]. Temporary holding and wet storage facilities for harvested cultured mussels in this region use a combination of deep water, cold temperature conditions year round and/or hold them in chilled seawater. To date no studies have specifically evaluated the effects of maintaining mussels under actual commercial holding conditions during winter and early spring water temperatures in this part of the Province.

The main objective of the study was to test the hypothesis that extended wet-holding under ambient conditions during the coldest water seasons (winter and spring) in Newfoundland affects overall condition and physiological stress response in cultured mussels when compared to field controls. This will be accomplished by analysing two calculated condition indices and the haemocyte lysosomal retention time of Neutral Red Dye (Neutral Red Assay).

Background

Blue mussel aquaculture is increasing in Newfoundland and Labrador. As production increases, product is shipped to local, national, and international markets. Unforeseen delays can prevent the shipment of fresh product. Such delays result in a need for storage in holding facilities. Determining the maximum holding period before there is a significant decrease in product quality is crucial. It has been shown that extended commercial storage during the warmest water seasons in Newfoundland (summer and fall) can increase the stress response and decrease condition.

Purpose

To date no studies have evaluated the specific effects of maintaining mussels under commercial holding conditions during winter and early spring. The present study evaluated whether extended wet-holding under ambient conditions during the coldest water seasons in Newfoundland would affect condition and the physiological stress response based on two calculated condition indices and the haemocyte lysosomal retention time of Neutral Red Dye (Neutral Red Assay).

Methods

Experimental set-up

The experimental setup and sample collection followed that were described in Wyatt et al. [2]. Briefly, *M. edulis* (2008 year class) was cultured using the traditional longline system and collected from Bulley's Cove, near Pleasant view in Notre Dame Bay, Newfoundland, Canada. Mussels were harvested in the winter and spring of 2010 using standard commercial protocols and transported to the processing facility where they were separated and held unprocessed in two replicate unstacked plastic D332 800 litre "cod tubs" modified with false bottoms to avoid dead water spaces (Inside dimensions 112 cm long, 95 cm wide, 78 cm high). Each tank contained 10 to 12 socks and the total density, as recommended by the industry, was calculated to be approximately 0.362 kg/L for a total weight of 289 kg per container.

Animals were maintained in holding under ambient water conditions using a continuous flow-through (~ 60 L/min), non-aerated system with unfiltered water pumped from the bay from a distance of 145 m and an approximate depth of 13 m from the surface. Temperature was measured using an electronic thermometer approximately six times per day by facility staff and data were summarized and reported as daily averages. Daily temperature checks at the main intake showed that there was no difference between this and the water in the tanks, so for the purpose of the present experiment only those temperatures in tanks were recorded and summarized. Water temperatures were not measured at the farm field sites.

Mussels were sampled at the beginning of each season (winter and spring) followed by one week in holding, one month in holding, and a final sample at three months (winter) and two months in holding (spring). At each sample time mussels were also collected from the original grow-out site to serve as a field control.

Condition analysis

At each time point 150 mussels were subsampled randomly from those collected from both the holding facility (2 tank replicates) and field site and transported in coolers on ice back to St. John's, NL for analysis. For logistical reasons sampled mussels were stored at 4°C for approximately eight hours prior to sampling for wet weight, dry weight, dry shell weight and condition. Total live weight was recorded using a laboratory scale, after which, the meat was carefully dissected away from the shell and dried at 80°C for 48 to 72 hours (modified from Lutz et al. [19]). All weights were measured to the nearest 0.001 g. Condition index was calculated as the ratio of dry tissue weight to dry shell weight and the ratio of dry tissue weight to wet tissue weight based on the models described in Lucas and Beninger [20].

Neutral red assay

The neutral red assay procedure followed that described in Wyatt et al. [2]. Briefly, 0.1 mL of hemolymph was extracted from the posterior adductor muscle of 12 individual animals using a 22.5 gauge needle containing 0.1 mL of physiological saline. The mixture was placed in a siliconized Eppendorf® tube and gently mixed, then pipetted onto a poly-L lysine coated slide and incubated for 15 minutes. Neutral red working solution (20 μL of stock solution in 5 mL of mussel physiological saline) was applied to the cell mixture and incubated for 15 minutes in a light proof humidity chamber before a coverslip was added. Initial observations were followed by 15 minute intervals up to the first 30 minutes followed by 30 minute intervals up to 180 minutes.

Observations were made on a compound microscope (VWR Scientific Products) using the 40x objective under low light.

Observations were recorded by assigning a four point numerical score (1–4) to 25 cells per field of view. The scoring was assigned as:

1: low stress indicated by the appearance of tiny pink dots (lysosomes) in the cytosol.

2: moderate stress as indicated by an increase in lysosome size.

3: represented moderate high stress as indicated by leakage of dye from lysosomes to the cytosol.

4: represented high stress response indicated by increased membrane degradation, lysosomal vacuolation, or rounding up of cells. Once 50% of the cells showed a high degree of stress response the slide was terminated and the retention time was recorded.

Statistical analysis

Neutral Red and condition data were analyzed using Sigmaplot 12.3.0 statistical and graphical software (Systat software). Data were tested for normality and the means (\pm SE) were calculated. Two-way Analysis of Variance (ANOVA) was conducted using time and treatment (holding vs field) as factors. Significance was set at $\alpha=0.05$. Since the assumptions of equivalence and normality (Shapiro-Wilk Test) were not met for condition data and an interaction was reported between factors, a pairwise comparison (time within treatment) was performed using the Holm-Sidak method at a significance level of $\alpha=0.05$. The Neutral Red data gave no interaction after two-way ANOVA analysis but no significant differences were noted in the data ($\alpha=0.05$).

Results

The average daily water temperatures in holding during the winter experiment (January to March 2011) and the spring experiment (April to June 2011) were -0.81°C and 5.19°C , respectively (Figure 1). Over the course of the study, the maximum and minimum recorded temperature during the winter trial was 1.37°C and -1.59°C , respectively. The maximum and the minimum recorded temperature during the spring trial were 11.39°C and 0.38°C , respectively.

Condition and dry weight analysis

A summary of wet weight, dry weight and condition index for field control mussels and those under holding conditions (winter and spring) are shown in Tables 1 and 2, respectively. Changes in tissue weight (wet and dry) and condition index for holding and field control mussels during both winter and spring are shown in Figures 2 and 3, respectively.

Mean wet tissue weight (Figure 2a; Table 1) decreased significantly for mussels during the winter holding trial (from 18.18 g initially to 15.30 g at the final sample ($p<0.05$)). In contrast there was a significant increase in wet tissue weight (Figure 2a; Table 2) during the spring trial (from 10.56 g to 13.39 g). Wet tissue weight also decreased significantly in field control animals during the winter trial but increased over the course of the spring period (Figure 2b, Tables 1 and 2).

Mean dry tissue weight for mussels during the winter holding experiment (Figure 2c; Table 1) showed a slight but not significant increase over the course of the trial (from 1.30g to 1.45g), however, mussels held in the spring did show a significant decrease in dry weight (0.87g to 0.67g) (Figure 2c, Tables 1 and 2). In comparison, winter field

controls showed a significant decrease ($p<0.05$) in dry tissue weight over the course of the experiment whereas spring controls showed a dramatic increase ($p<0.05$) in dry weight between initial and final samples (Figure 2d, Tables 1 and 2).

Mean condition index calculated both as a ratio of dry tissue weight to dry shell weight and wet tissue weight (Figure 3; Tables 1 and 2) increased significantly for winter held mussels but was shown to decrease for spring held mussels (Figure 3a-c; Tables 1 and 2). Field control samples showed a small but significant increase in mean condition index during the first week of the winter trial but then showed a decline to the final sampling period. Similarly spring controls showed a significant and dramatic increase following the first week but then also showed a decline to the final sample (Figure 3b and 3d; Tables 1 and 2). Change in condition index between initial and final samples was significantly higher in winter held mussels than winter field controls ($p<0.05$). During the spring experiment field controls gave the highest change over time ($p<0.05$) as compared to held mussels.

Neutral red assay

Observed neutral red retention time decreased over the winter season but was not found to be significant ($p>0.05$) (Figure 4a). Winter field control samples also did not show any significant change over the course of the experiment ($p>0.05$) (Figure 4a). Similarly, during the spring there was no notable change detected in neutral red retention time over the course of the season for either the mussels in holding or the field controls ($p>0.05$) (Figure 4b).

Discussion

Winter and early spring in Newfoundland bays and inlets are characterized by low water temperatures and the potential for ice cover.

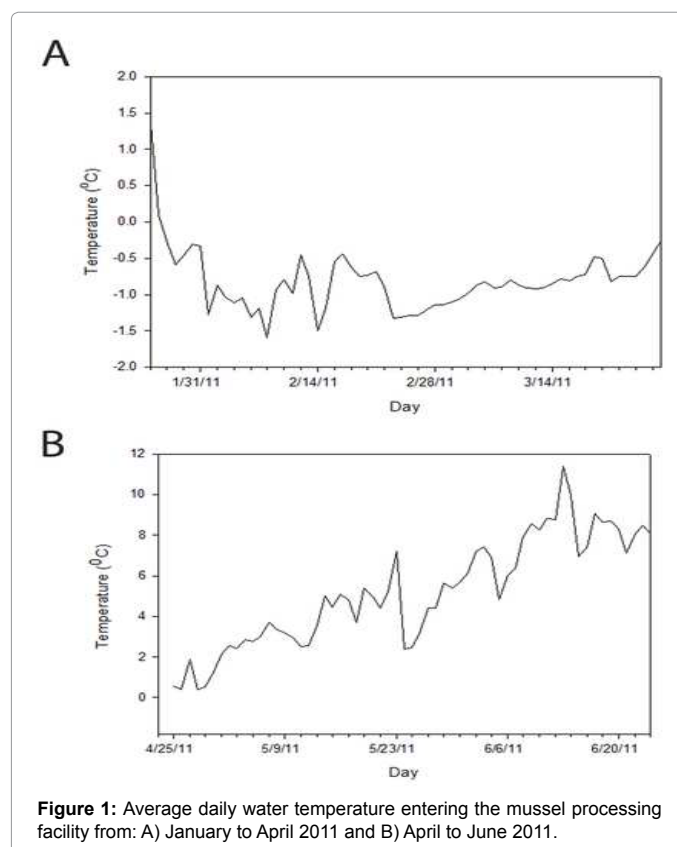


Figure 1: Average daily water temperature entering the mussel processing facility from: A) January to April 2011 and B) April to June 2011.

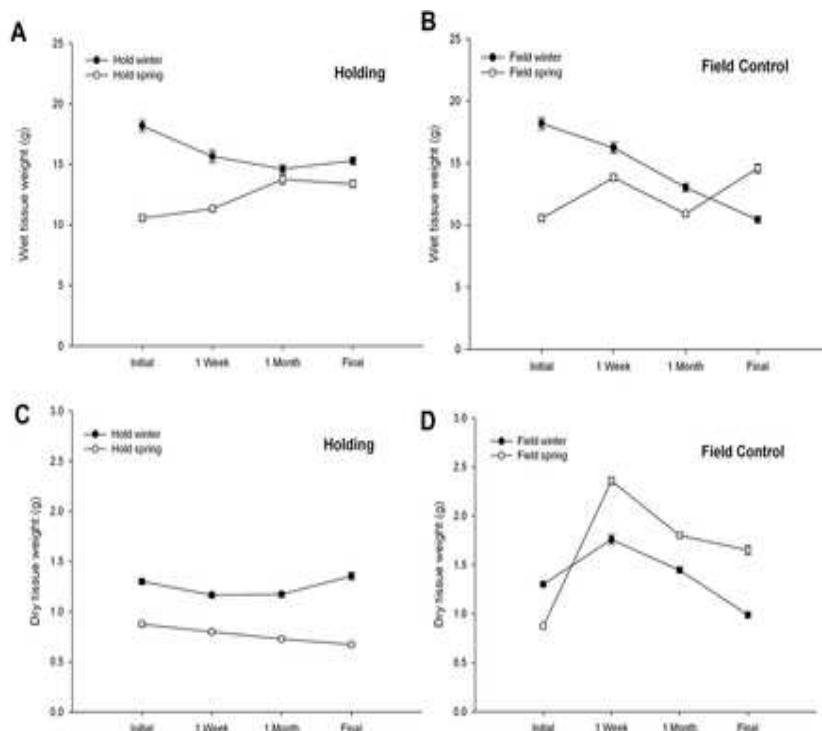


Figure 2: Change in mean wet tissue weight (A) and mean dry tissue weight (C) for cultured mussels held in ambient wet storage during winter (January 2011 to April 2011) and spring (April 2011 to June 2011) in north-eastern Newfoundland, compared to change in mean wet tissue weight (B) and mean dry tissue weight (D) for field control samples taken during the same period (+/- SE; n=150).

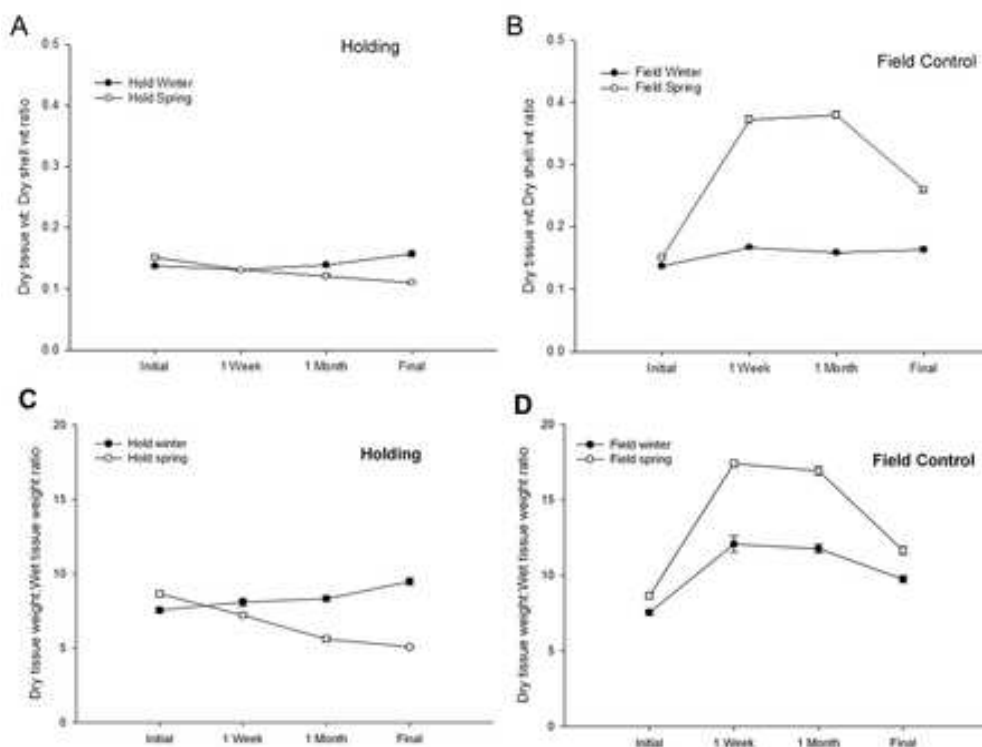


Figure 3: Change in mean dry tissue weight: dry shell weight ratio (A) and mean dry tissue weight: wet tissue weight ratio (C) for cultured mussels held in ambient storage during winter (January 2011 to April 2011) and spring (April 2011 to June 2011) in north-eastern Newfoundland, compared to change in mean dry tissue weight: dry shell weight ratio (B) and mean dry tissue weight: wet tissue weight ratio (D) for field control samples taken during the same period (+/- SE; n=150).

Parameter	Initial	One week		One month		Final	
		Holding	Field	Holding	Field	Holding	Field
Wet tissue weight (g)	18.18 ± 0.45 ^a	15.65 ± 0.50 ^b	16.23 ± 0.43 ^b	14.62 ± 0.32 ^b	13.02 ± 0.33 ^b	15.30 ± 0.32 ^b	10.44 ± 0.23 ^c
Dry tissue weight (g)	1.30 ± 0.03 ^a	1.17 ± 0.02 ^b	1.76 ± 0.05 ^b	1.17 ± 0.02 ^b	1.45 ± 0.03 ^c	1.45 ± 0.03 ^a	0.99 ± 0.03 ^d
Dry shell weight (g)	9.55 ± 0.14 ^a	8.95 ± 0.14 ^b	10.45 ± 0.16 ^b	8.79 ± 0.13 ^b	9.12 ± 0.14 ^a	8.77 ± 0.15 ^b	6.14 ± 0.11 ^c
Dry weight: Wet weight (X100)	7.43 ± 0.16 ^a	8.08 ± 0.25 ^a	12.06 ± 0.54 ^b	8.31 ± 0.17 ^{ab}	11.76 ± 0.29 ^b	9.48 ± 0.23 ^b	9.75 ± 0.23 ^c
Dry Weight: Shell Weight	0.137 ± 0.002 ^a	0.131 ± 0.002 ^a	0.167 ± 0.003 ^b	0.138 ± 0.002 ^a	0.159 ± 0.002 ^b	0.157 ± 0.004 ^b	0.163 ± 0.004 ^b

2008 year class

Values represent mean ± SE (n=150)

Different letter superscript in the same row represents statistical significance for that group (two-way ANOVA, p<0.05)

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

Where a, b, c, d letters in the tables represent statistical significance. Those values with the same letter superscript are not found to be significantly different from each other.

Table 1: Weight and condition measurements of *Mytilus edulis* in extended holding and field control during the winter (January to April 2011).

Parameter	Initial	One week		One month		Final	
		Holding	Field	Holding	Field	Holding	Field
Wet tissue weight (g)	10.56 ± 0.27 ^a	11.33 ± 0.24 ^a	13.83 ± 0.25 ^b	13.75 ± 0.36 ^b	10.90 ± 0.20 ^a	13.39 ± 0.30 ^b	14.57 ± 0.34 ^b
Dry tissue weight (g)	0.87 ± 0.02 ^a	0.80 ± 0.02 ^a	2.36 ± 0.03 ^b	0.73 ± 0.02 ^b	1.80 ± 0.03 ^c	0.67 ± 0.02 ^b	1.65 ± 0.04 ^c
Dry shell weight (g)	5.82 ± 0.11 ^a	6.21 ± 0.12 ^a	6.42 ± 0.10 ^b	6.07 ± 0.13 ^a	4.80 ± 0.08 ^b	6.14 ± 0.13 ^a	6.39 ± 0.14 ^c
Dry weight: Wet weight (X100)	8.63 ± 0.20 ^a	7.21 ± 0.14 ^b	17.04 ± 0.22 ^b	5.61 ± 0.20 ^c	16.91 ± 0.28 ^b	5.07 ± 0.10 ^c	11.64 ± 0.27 ^c
Dry Weight: Shell Weight	0.151 ± 0.003 ^a	0.131 ± 0.003 ^b	0.373 ± 0.005 ^b	0.121 ± 0.002 ^c	0.380 ± 0.005 ^b	0.110 ± 0.002 ^d	0.260 ± 0.005 ^c

2008 year class

Values represent mean ± SE (n=150)

Different letter superscript in the same row represents statistical significance for that group (two-way ANOVA, p<0.05)

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

Where a, b, c, d letters in the tables represent statistical significance. Those values with the same letter superscript are not found to be significantly different from each other.

Table 2: Weight and condition measurements of *Mytilus edulis* in extended holding and field control during the spring (April to June).

Winter conditions can be stressful for bivalves with temperatures hovering around 0°C, resulting in a distinct decline in tissue weight [21]. Previous work has shown that generally during this season, phytoplankton concentration can be low and growth of mussels has been shown to be generally slowed leading to an overall loss of condition [22,23]. In contrast, as water temperatures increase and ice cover retreats during the spring, phytoplankton blooms can occur resulting in dramatic increases in growth and condition in relatively short periods of time [3].

Based on the above it is clear that mussel condition can be affected by factors such as water temperature, food availability, and reproductive status [21,24-26]. Adverse environmental conditions including increased density (crowding) and/or the accumulation of waste can also be responsible for low condition index. Barrento et al. [27] concluded from a study simulating a variety of post-harvest holding conditions for cultured mussels, that while ammonia excretion and accumulation were more variable than oxygen concentrations, the optimal environmental conditions in holding were dependent more so on oxygen needs and water temperature. In contrast to the simulated conditions evaluated in Barrento et al. [27], the present study set out to examine the effects of extended wet holding or storage on cultured mussels maintained in a commercial facility following harvest under ambient water temperatures. Specifically, the study focused on changes in condition and stress response of cultured blue mussels during the winter and early spring seasons in Newfoundland. It would be interesting to evaluate the above physiological parameters under the real time commercial conditions described in the present study.

Recent studies have shown that changes in some environmental factors (i.e. water temperature), corresponding with seasonality can be important and affect the stress response in cultured mussels, especially during extended holding conditions [2]. In particular, extended holding under ambient temperatures during summer and fall can

negatively affect the overall condition of animals when kept beyond one month [2]. In the present study, the distinct and significant differences in condition indices observed between held and field control animals during the winter and spring seasons indicated that the environment in the holding facility seems to be different from on the farm. During the winter holding trials, results showed a significant increase in dry weight and condition after three months however dry tissue weight was noted to be at its lowest point at this time for the winter field control sample. Similarly, condition index did not change or improve significantly over the same time period for field control mussels. Previous studies have shown low respiration rates and high ammonia excretion rates in mussels exposed to winter ice cover, an indication of starvation [7], which could account for the decline in tissue weight observed in the field animals however does not account for the increase in condition over time in holding. These observations suggest that in the present experiment when ambient temperatures were approximately 4°C and below, the holding environment may in fact be less stressful than the field environment, thus resulting in a more stable physiological condition overall. Handa et al. [6] found that when mussels were stored at less than 7°C and not fed they were able to maintain a significantly higher specific growth rate (dry weight) than mussels held at 14°C. This suggested that storage time can possibly be prolonged at low temperatures if the mussels were not fed or fed at low levels.

During the spring season, as water temperatures increased and ice cover retreated an opposite response to that observed during the winter season was noted during comparisons of condition in field control and held animals. While the farm sites and the holding facility basically use water from the same source a dramatic improvement in condition of field control animals was observed during the spring study period. This was not evident in animals in holding. These springtime observations were very similar to that observed for held mussels during the fall [2].

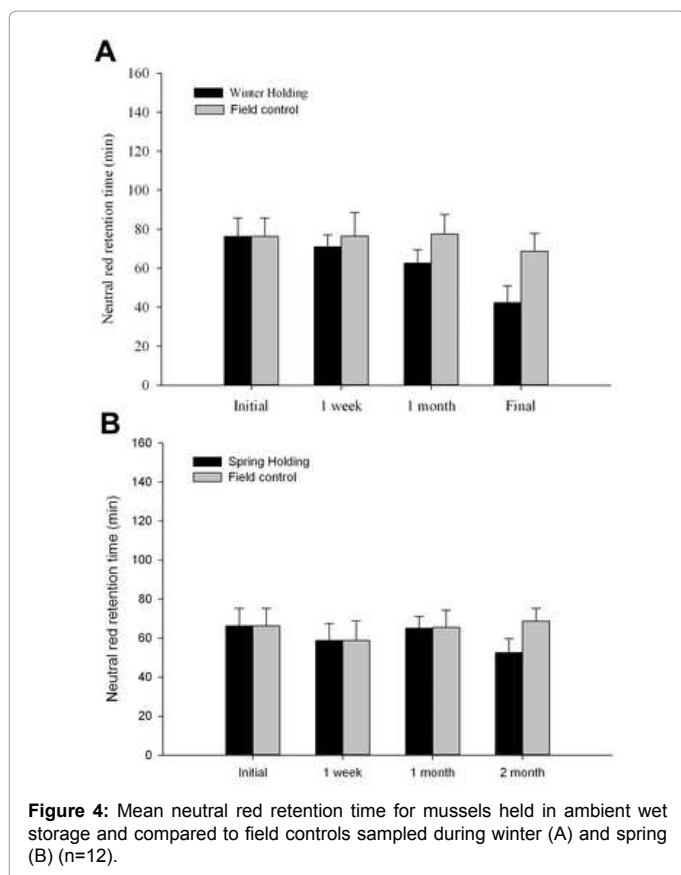


Figure 4: Mean neutral red retention time for mussels held in ambient wet storage and compared to field controls sampled during winter (A) and spring (B) (n=12).

While the temperature profiles are similar between these seasons, it is also possible that there is variation in the food supply or availability between the farm site and the wet-storage site and these differences could at least partially explain the observed differences in condition.

While loss of dry weight and condition can be considered as some of the first signs of environmental and/or physiological stress in mussels, other methods may be used to support these observations. Lysosomes play a critical role in detoxification and defense in shellfish with specific association with haemocytes [28,29]. These processes are membrane dependent and thus the stability of the lysosomal membrane can be used to determine efficiency in performing these functions [24]. Techniques such as the neutral red assay can use this variation in membrane stability as a way of measuring environmental stress response in bivalves [2,5,30]. Wyatt et al. [2] found a significant decrease in neutral red retention time in mussels during extended holding in the summer and fall in Newfoundland. Interestingly, over the course of both the winter and spring seasons there was no significant change in neutral red retention time and thus stress response in mussels from either treatment. These results are similar to a previous studies by Harding et al. [5], where neutral red retention times for stored farmed *M. edulis* were highest during the winter and spring months with little change between February and May. Winter wet weight and dry weight from the present study showed a significant decrease for field control mussels over the period of the experiment. This was also indicated to some degree in the calculated condition indices. The same level of tissue weight loss was not observed in winter held mussels. These results suggest some level of additional physiological stress in field control mussels as compared to those in

holding. This response was not reflected in neutral red retention times for either treatment suggesting that in this case haemocyte lysosomal response may not directly correspond with tissue level changes in the animal. Environmental temperature can play a significant role in lysosomal membrane stability. Both high temperatures (25°C) and extreme low temperatures (0°C) have been shown to result in shorter neutral red retention times [31,32]. The highest and lowest average daily temperatures during the course of the present study were recorded as -1.59°C and 7.23°C. These are in the low range of seasonal exposure for *M. edulis* but did not appear to cause significant decreases in dye retention time. Further work will be necessary to elucidate the reasons behind the winter response of both held and field control mussels and should include measurements of food concentration between sites.

Taken from a seasonal perspective, the markers for stress chosen for this investigation suggest that holding under ambient conditions especially in winter may provide a more stable environment for *M. edulis* than the open water lease. The response to holding in the spring seems to compare to fall conditions [2]. Further work will be necessary to dissect the specific physiological details involved. From the perspective of the mussel industry, under the conditions evaluated in this study, we suggest that harvested product could be maintained in ambient storage during winter for at least two months without any significant physiological effects. As with the recommendation put forth by Wyatt et al. [2], we suggest that one month be a maximum for mussels stored during spring (especially May and June) in Newfoundland.

Summary

The observed change in condition and dry weight across season indicated that mussels can be held for at least 2 months during the winter season and for a limit of 1 month in late spring before a significant loss in condition and quality is observed.

Conclusion

Based on the observed change in condition and dry weight across season, we recommend that mussels can be held for at least two months during the winter season and at a limit of one month for spring (especially late spring i.e., May and June) before a significant loss in condition and potential quality is observed. Dry weight and condition in holding during winter increased significantly when compared to samples from the field. Spring held mussels were observed to lose dry weight and condition in holding as compared to field controls. The Neutral Red Assay indicated an observed but not statistically significant increase in physiological stress response for held mussels during the winter season. No observed change was noted for field controls. During the spring no overall change was noted in Neutral Red response for held or field control samples. Dry weight and condition analysis indicated that the winter season was the most stable for extended holding of mussels.

Competing Interest

The authors declare that they have no competing interest.

Authors Contributions

Wyatt conducted experiments and drafted the manuscript; Kenny assisted in field sampling and coordination; Mills constructed and maintained the holding facility; Marshall assisted in analysis and manuscript preparation; Murray designed and coordinated the experiment, assisted in analysis and manuscript preparation.

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