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Research Article

Investigating mitigation of juvenile European green crab *Carcinus maenas* from seed mussels to prevent transfer during Newfoundland mussel aquaculture operations

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Abstract

The mussel aquaculture industry has raised concerns following the discovery of green crab *Carcinus maenas* in Placentia Bay Newfoundland in August 2007. Post-larval green crabs have been found in feral mussel beds in high densities in Europe. If this is true for other green crab populations, mussel seed transfers from Placentia Bay could provide a vector for post-larval juvenile crab transfer to other areas like Notre Dame Bay where provincial mussel aquaculture is concentrated. Green crab is currently not found in this area of Newfoundland. Newly settled green crab juveniles were collected and used in a series of lab scale mitigation trials. Crab and seed mussels were exposed to thermal shocks applicable and feasible for mussel seed management in Placentia Bay. Crab mortality was measured in the treatments and seed mussels were monitored for stress response using the lysosomal destabilization assay. Exposure to heated salt water to 45, 50 and 55°C was effective in culling juvenile green crab while causing minimal stress to mussel seed. The method can be employed in mussel seed management and transfer operations where there are concerns related to potential introductions of hitch-hiking green crab.

Key words: removal, juvenile *C. maenas*, Neutral Red Assay, mussel stress, green crab, Northwest Atlantic, invasive species

Introduction

Newfoundland is the second largest producing jurisdiction of blue mussels in North America and produced 4,400 metric tonnes valued at $14 million CAD in 2012 (DFA 2013). It is a rapidly expanding industry, requiring a good seed supply for its continued growth. The majority of mussel farming takes place in Notre Dame Bay on the Northeast coast of the province with a few farms on the West and South coasts and in Placentia Bay. Most of these areas collect mussel seed for grow out within the same area as the farm, but with industry expansion in recent years, seed shortages are occurring on farm sites. To resolve this problem seed is being collected, harvested, and shipped to different farms. Some of the seed collection is occurring in Placentia Bay with hopes to transfer the mussel seed to farms in Notre Dame Bay.

European green crab, *Carcinus maenas* (Linnaeus, 1758), was found to have an established population in Newfoundland waters in 2007 in Placentia Bay. It is a euryhaline portunid crab with a native range from Iceland to Norway south along the Atlantic coast to Mauritania (Yamada 2001). It is indigenous to European and Northern Africa coasts and estuaries and has high tolerances for temperature extremes, air exposure, and desiccation. They have a relatively high fecundity and long planktonic larval duration, are voracious omnivores and aggressive competitors. This makes them an ideal global invader (Cohen et al. 1995; Roman and Palumbi 2004). *Carcinus maenas* is ranked as one of the 100 worst invasive species in the world (Lowe et al. 2000). In all areas where they have invaded, its potential for significant impacts on fisheries, aquaculture, and the ecosystem has caused concern (Klassen and Locke 2007).

C. maenas have had a strong influence on the abundance of natural bivalve populations by virtue of their very aggressive predation patterns (Grosholz and Ruiz 1995). Bivalves are their preferred prey based on stomach contents analysis (Elner 1978). They not only cause a decrease in prey abundance but have also been found to change molluscan defense characteristics like shell thickness. In areas of high predation, *C. maenas* has induced changes in *Mytilus edulis* including thicker shells; relatively more shell mass, and the mussels were more tightly attached to the substrate (Leonard et al. 1999). In the situation with mussels, the increased shell thickness results in decreased meat yields, and higher losses incurred during processing with an overall lower quality product. Miron et al. (2005) found that adult green crab prefer blue mussels and soft shelled clams over oysters and quahogs in a population in New Brunswick, Canada. Pickering and Quijon (2011) found that *C. maenas* prefer soft shelled clams, and blue mussels than oysters in order of preference as the abundance of each decreased in natural beds in Prince Edward Island, Canada.

Not only are blue mussels one of the preferred prey species for green crab, they are also a preferred protective habitat for early life stage *C. maenas*. When pre-settlement megalopae were given habitat choices in the lab they chose mussel substrate over eelgrass and macroalgae for their initial settlement (Hedvall et al. 1998). After megalopae settle onto their preferred habitat on the shore, they remain in that protected area throughout the juvenile stages.

It is evident that green crab effectively uses mussels as a settlement substrate, protective habitat and prey species in native and invaded areas of their distribution. This raises concern for native Newfoundland bivalve populations; the mussel aquaculture industry being one. With the recent discovery of green crab in Placentia Bay, Newfoundland, there is a risk of transferring early life stage *C. maenas* with mussel seed to high value mussel growing areas in the province like Notre Dame Bay, or introducing the crab to a new locale for invasion. Therefore, the development of methods to mitigate mussel seed transport as an Aquatic Invasive Species (AIS) vector, while maintaining a healthy supply of high quality mussel seed, will be critical to the growth and prosperity of the mussel industry in Atlantic Canada (Vickerson 2009; Vickerson et al. 2011).

Mussel seed transfer is the main vector investigated in this study and is the greatest threat to the Newfoundland mussel industry for dispersal of juvenile green crab. Other human mediated vectors can threaten unestablished areas like ballast water, sea chests, shipment of commercial shellfish and aquaculture products, marine construction equipment, movement of sediment/sand and accidental release from research facilities (Cohen et al. 1995; Grosholz and Ruiz 2002). These vectors have been responsible for the transfer of invasive species other than green crab and can be prevented with education to those sectors. Natural dispersal is also a threat as green crab larvae have been documented to transfer in currents up to 50 km per day on the West coast of North America in 1998 (Yamada and Becklund 2004). In Canada there is also a threat to larval transfer via currents but not from this site of interest in Placentia Bay to Notre Dame Bay in Newfoundland, therefore human mediated transfer is the main vector.

There are no specific mitigation techniques aimed at green crab on aquaculture sites used by industry anywhere in the world that we are aware of. Treatment of oysters for the removal of barnacles, other bivalve spat and seaweed has been established internationally and solitary and colonial tunicates have been the AIS focus in the Maritime Provinces. The techniques already used to treat for these species include treating mussels with fresh water, vinegar, lime (Denny 2008; Vickerson et al. 2011), air drying and high pressure water blasting (Forrest and Blakemore 2006).

Adding a small step to the harvesting and grading of seed can add significant time and cost to the operation before seed transfer, so the more simple and cost effective a mitigation method the better. The most simple and available technique considered for this study was immersion in heated salt water. Other methods of pest removal such as heated freshwater, lime, acetic acid treatments used for some soft bodied invasive species are not appropriate for use on a farm in the marine environment, and would not likely meet regulatory approvals for use in Canada on the water. The seawater thermal shock technique has been used on oyster species to remove other pests. The Center for the Experimentation and Development of Marine Aquaculture removed undesired mussel spat from Pacific oysters *Crassostrea gigas* by immersion in water at 80 and 85°C for 2 and 3 second durations and then slowly cooled to ambient temperature (CREAA 2004). Park et al. (1998) removed a variety of fouling organisms from *C. gigas* by immersion in 60°C water for 10–15 seconds in Korea and Arakawa (1980) did the same in Japan. Forrest

and Blakemore (2006) experimented with removal of the kelp *Undaria* from green lipped mussels *Perna canaliculus* using immersion in water heated to 35, 45 and 55°C for a range of exposure times. They found 55°C for approximately 5 seconds to be the most appropriate and least harmful to the green lipped mussel seed. Smith and MacNair (2000) suggested that blue mussels cultivated in suspended culture have thinner shells than those of oysters and they may be more sensitive to high temperature baths. Therefore the techniques used for removing fouling from oysters may be more harmful to blue mussels. Increased body temperatures can cause short term problems, like stronger byssal thread attachment and shell gaping, and long, term effects of high stress levels from temperature shock that can decrease growth rate and overall mussel health during grow out.

The Neutral Red Assay has been proven to successfully determine if conditions where cultured mussels are exposed will induce a stress response at the subcellular level (Harding et al. 2004; Vickerson et al. 2011). Stress level is a direct measure of bivalve health and can predict future growth success. In unstressed cells lysosomes will accumulate and retain the neutral red dye for an extended period of time. Once destabilized (or stressed) the dye will leak into the cytosol of the cell through the damaged membranes (Moore 1980). Because Neutral red is a weak cytotoxic compound, it too will act as a secondary stressor (Lowe et al. 1995) and all cells will become stressed eventually. The longer the retention time the lower the stress level of the cells and the individual that haemolymph was extracted from.

The *C. maenas* temperature tolerance information found in the literature relates to adult crab and slower exposure times and behavior changes. At temperatures above 25°C we can assume that the health of an adult green crab will start to diminish (McGaw and Whiteley 2012), and even more so for a more vulnerable and thinner shelled juveniles. Taylor and Wheatly (1979) found that *C. maenas* adults will migrate to an area above water once water temperatures in the intertidal reach 28°C so they can actively decrease their body temperature. Kelley et al. (2011) found that crab could live in water temperatures up to 36.2°C starting at 23°C and increasing the temperature 1 degree an hour. We hypothesize that juvenile green crab from Placentia Bay will not tolerate short exposure times to moderate to large thermal shocks.

The goal of this study was to investigate the effectiveness of the environmentally friendly heated salt water immersion technique for preventing *C. maenas* juveniles from being transported with Newfoundland blue mussel seed. We hypothesized that there will be little to no stress response from blue mussels treated with these techniques.

Materials and methods

Green crab collection

Juvenile green crabs (carapace width (CW) 2.5-16mm, $\bar{x}$ = 9.7, SD=3.7, n=190) were collected by hand from the intertidal zone at low tide between August and October 2012 from two sites (North Harbour and Southern Harbour) in the Northern area of Placentia Bay, Newfoundland (Figure 1). Each individual was held live in open 20 mL glass vials with water from the collection site. Animals were transported to the laboratory and kept at room temperature (~20°C) for less than 12 hours before experiments were conducted.

Green crab mitigation trials

Groups of 60 juveniles (triplicates of 10, plus corresponding control animals, 720 total) were subjected to one of 12 treatments (Table 1). Juveniles were individually poured into salt water soaked tea bags and then subjected to either room temperature (control) or heated (experimental) salt water baths for either 5 seconds or 1 minute durations using a Fisher Scientific model 2332 water bath. Each animal was then returned to ambient holding vials with clean salt water of the same salinity they were collected from in the field. After 10 minutes of recovery the activity level and number of limbs lost for each animal were observed and assigned a number (Table 2). Overall average for each treatment was calculated and direct comparisons were made for duration times within each temperature and then among all treatments and the controls.

Neutral red assay

The lysosomal destabilization assay using neutral red has been shown to be a good predictor of thermal stress in a variety of marine invertebrates including blue mussels (Harding et al. 2004; Lowe et al. 1995; Vickerson 2009). Neutral red assays were conducted on mussels exposed to mitigation techniques identical to those employed on juvenile green crab, to determine if these treatments would cause stress and potential reduced growth in the mussels. Blue mussels, *Mytilus edulis,* with shell

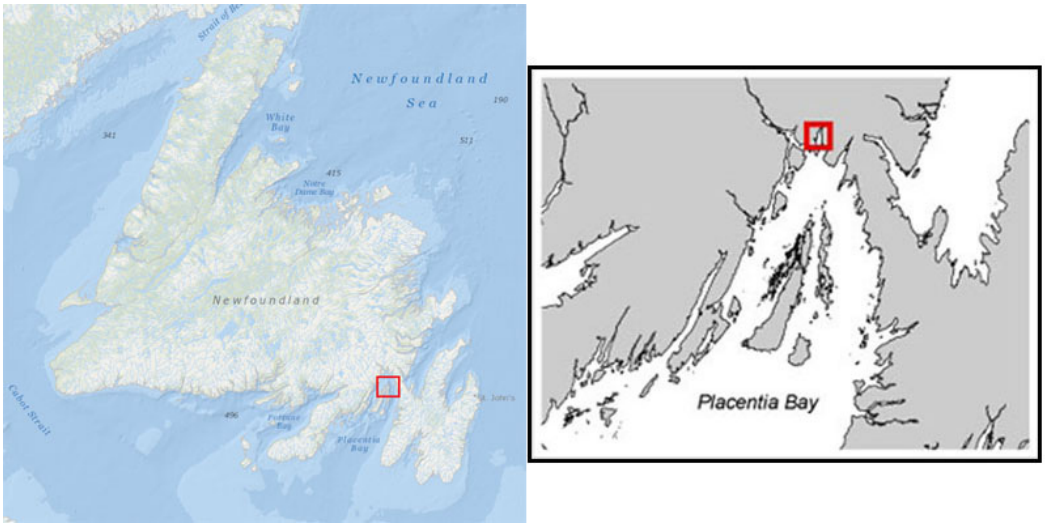

Figure 1. Map of Newfoundland, Placentia Bay and North Harbour, primary juvenile green crab collection site (Source C. McKenzie).

Table 1. Mitigation treatments for juvenile green crab. D – dip for 2 seconds.

No.	Water Temperature (°C)	Duration (sec)	Treatment name
1	20	5	20D
2	25	5	25D
3	30	5	30D
4	35	5	35D
5	40	5	40D
6*	40	60	40 1min
7	45	5	45 D
8*	45	60	45 1min
9*	50	5	50D
10*	50	60	50 1min
11*	55	5	55D
12*	55	60	55 1min

*Treatments used on both juvenile crab mitigation trials and mussel NRA.

lengths 45–55 mm (mussel seed socked in Newfoundland is between 25 and 45 mm and anything under 55mm is below market size) from South Arm, Bay of Exploits Newfoundland were cleaned and washed and donated to this experiment by Norlantic Processors and held in a Fukui trap suspended in a flow through tank at the Northwest Atlantic Fisheries Center (DFO St. John's, Newfoundland). After a minimum 1 week acclimation in tanks, at ambient temperatures and salinities individual mussels were subjected to either room temperature (control) or heated (experimental) salt water treatments (see Table 1) using the same methods as for juvenile crab.

The 5 most lethal treatments of the 12 used on juvenile green crab were applied to seed mussels to test stress levels. Blood samples were taken from the adductor muscle of each mussel immediately after each thermal shock treatment and neutral red assays were performed based on methods from (Wyatt et al. 2013) with some modification to the duration of reaction. For each treatment group six mussels were randomly selected and removed using scissors to detach byssal threads from the Fukui trap and treatments were administered using a Fisher Scientific model 2332 water bath. Six mussels per treatment were chosen for the stress index in this experiment as mussels during the late summer and early fall typically have low variability in stress response (Harding 2003).

Approximately 0.1 mL of haemolymph was extracted from the posterior adductor muscle using a 1 mL syringe and 21 gauge needle filled with 0.3 mL of physiological saline corrected to pH 7.2. The needle was removed after extraction to reduce cell damage while removing the solution from the syringe and into an Eppendorf ® tube, inverted and held on ice. 40 µL of the haemolymph solution was pipetted onto slides pretreated with poly-L-lysine (20 µL PLL in 100 µL distilled water) and cells were left to incubate and adhere for 15 minutes on slides in individual humidity chambers. After adhesion, 40 µL of working neutral

Table 2. Juvenile crab responsiveness scale modified from Forrest and Blackmore (2006).

1	Dead, no antennule activity
2	Critically weak, partial recovery, lethargic movement, unable to right themselves, sporadic antennule activity
3	Unaffected, makes a full recovery, alert posture, consistent antennule activity

Table 3. Haemocyte rating system for stress quantification in blue mussels (based on Wyatt et al. 2013).

1	Low stress, lysosomes clearly defined in the cytosol
2	Moderate stress, increase in lysosome size
3	Moderate/high stress, leakage of dye from lysosomes to cytosol
4	High stress, increased membrane degradation and cell lysis

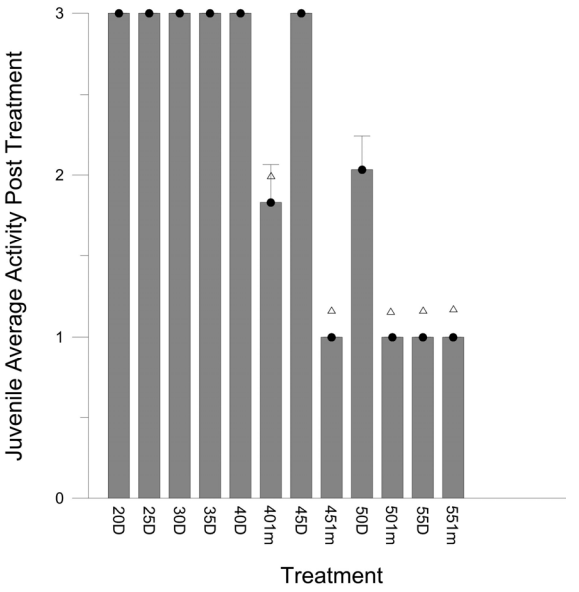

Figure 2. Juvenile crab average activity results post treatment for 12 treatments. (1=dead, no antennule activity, 2=critically weak, partial recovery, lethargic movement, unable to right themselves, sporadic antennule activity, 3= =unaffected, makes a full recovery, alert posture, consistent antennule activity). Bars represent average response for n= 30 crabs/treatment. Significant treatments from the control indicated with triangles ($p<0.05$). There were no significant differences among treatments for 25, 30, 35, 40, 45 °C dip and control. When grouped together there was a significant difference between 40 °C 1 min ($\bar{x}$=1.83, SD= 0.55), 45 °C 1 min ($\bar{x}$=1.00, SD=0.00), 50 °C Dip ($\bar{x}$=2.03, SD=0.41, 50 °C 1 min ($\bar{x}$=1.00, SD=0.00), 55 °C Dip ($\bar{x}$=1.00, SD=0.00) and 55 °C 1 min ($\bar{x}$=1.00, SD=0.00).

red dye solution was pipetted onto sides and further incubated for 15 minutes. After the dye has had time to enter haemocytes a cover slip was placed and the first reading was made. By using a compound microscope under 40x magnification, 50 haemocytes were assessed and assigned a score (Table 3). Readings were made 15 minutes after the initial reading and then every 30 minutes for up to 120 minutes. Readings continued until 50% of the cells were stressed (level 2 and higher) or there was no change in stress level for two consecutive readings.

Statistical analysis

Data were analyzed using IBM SPSS Statistics 19. One-way analysis of variance was used to determine significant treatments for green crab mitigation and paired t test was used to compare neutral red retention times of control and treatment mussels.

Results

Green crab mitigation trials

The results of the 12 mitigation treatments are shown in Figure 2. The treatments were found to be significantly different (ANOVA; $F_{(11,\ 348)} = 830.186$, $p < .05$). Tukey post hoc analysis revealed that several treatments differed from one another. Treatments that had no difference from the control and from each other 25, 30, 35, 40 and 45 °C Dip were significantly different from 40 °C 1 min ($\bar{x}$= 1.83, SD= 0.46), 45 °C 1 min ($\bar{x}$= 1.00, SD=0.00), 50°C Dip ($\bar{x}$= 2.03, SD=0.41), 50°C 1 min ($\bar{x}$= 1.00, SD=0.00), 55°C Dip ($\bar{x}$= 1.00, SD=0.00) and 55°C 1 min ($\bar{x}$= 1.00, SD=0.00).

Neutral red assay

The results of the 5 NRA treatments are shown in Figure 3 and Table 4. There was no significant difference in neutral red retention time among the treatment and the control mussels. None of the thermal stressed mussels reached level 4 stress levels or higher, even after the allotted amount of time (120 minutes).

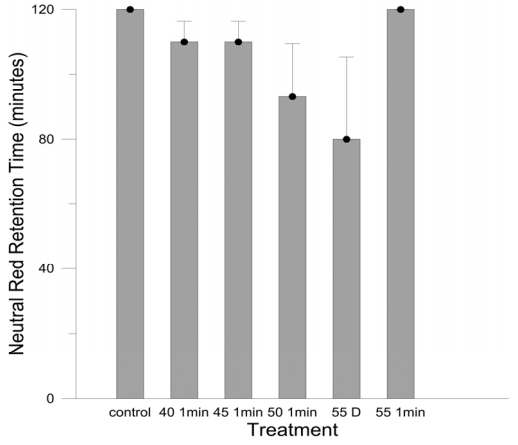

Figure 3. Neutral red retention time for control (20D) and five treatments and the highest stress level reached during mussel thermal exposure trials. Bars represent the mean +/- S.D. of n=6 mussels for each treatment.

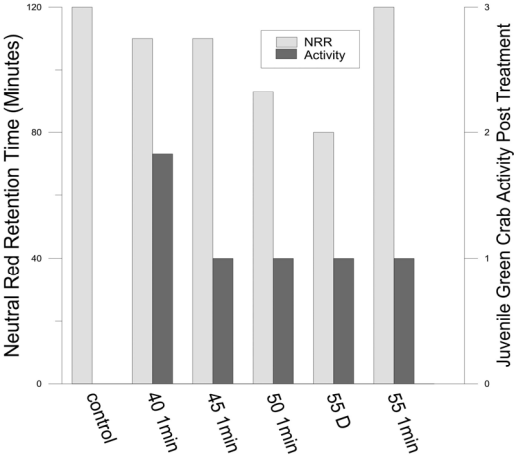

Figure 4. Juvenile green crab average activity post effective treatments and corresponding mussel neutral red retention times. (1=dead, no antennule activity, 2=critically weak, partial recovery, lethargic movement, unable to right themselves, sporadic antennule activity, 3= unaffected, makes a full recovery, alert posture, consistent antennule activity). Here we can see that 45 1 minute has the most effective crab culling capacity while causing the least amount of stress in mussels.

Table 4. Results of paired t-tests to evaluate the difference from the control (salt water at room temperature or 20Dip) on neutral red retention time (control neutral retention time NRR = 120 min) in seed mussels exposed to a variety of thermal shocks.

Treatment	NRR time	t	df	p
40 1 min	110	1.581	5	0.175
45 1 min	110	1.581	5	0.175
50 1 min	93	1.620	5	0.166
55 Dip	80	-	-	-
55 1 min*	120	-	-	-

* Treatment NRR time = control NRR time.

Combined results

The final result from this study is the choice of the most suitable treatment while combining the two factors investigated. This is the combination of the juvenile green crab mitigation trials and then their applicability with regards to stress response on mussel seed that would also be exposed to these treatments in practice when treating seed on a mussel aquaculture site. From section 3.2 we can see that all five effective mitigation techniques for juvenile crab culling did not cause significant stress on mussels. The treatment that had the most effective culling while having the highest neutral red retention time and lowest stress response was 45°C 1 minute (Figure 4).

Discussion

Green crab temperature tolerances are between -1 and 31°C and the limits for feeding and efficient cardiorespiratory functioning are approximately 5–25°C (Camus et al. 2004). The animals for this study were collected at ambient temperatures in Placentia Bay ranging between 18 and 25°C, making the animals used here at the upper limit of regular metabolic functions. CTmax or critical thermal maximum increases after heat shock; the stress response was short term in the present study and so would contribute to the ability to withstand short term fluctuations in temperature (Hyde et al. 2012). Starting with a higher tolerance range at collection, crab treated with temperatures of 20, 25, 30, 35 and 40°C were not adversely affected in the present study as they can realistically handle this relatively small fluctuation.

Green crab inhabit intertidal areas where other less motile animals have reached summer high temperatures greater than 25°C e.g., limpets reaching 29°C and barnacles reaching 38°C (Taylor and Wheatly 1979). A crab that can tolerate a temperature maximum similar to these other intertidal organisms for a very short time period would not be exposed to a temperate change drastic enough to cause physiological damage and death. When there is a more drastic difference between temperature of acclimation and shock there is a lower CTmax temperature. In the case of crab acclimated to 6°C, CTmax was reached at 34.7°C (at a rate of 1°C /hour) a difference of 27.7 degrees warmer (Kelley et al. 2011). This degree difference is comparable to the degree difference between the present study's acclimation temperature for juveniles of approximately 20°C and the effective culling temperatures of 45, 50 and 55°C. This is the first study to examine short

term temperature shocks on *C. maenas* of this small size. The present study's thermal shock was much more rapid than in Kelley et al. (2011) which would explain why the CTmax here was higher than 34.7°C, at 45°C. This would also explain why trials using 20, 25, 30 and 35°C herein were not drastic enough to cull juvenile *C. maenas* in our experiments.

All organisms in this study trial elicited a similar behavioral response to increasing temperature, which was characterized by initial hyperactivity, then muscular spasms, followed by lack of movement and finally relaxed open abdomens also seen in Kelley et al. (2011). As treatment temperature increased it was observed that animals would immediately start to convulse as soon as they were immersed, no matter if the exposure time was 5 or 60 seconds. This observation is indicative of reaching CTmax (Kelley et al. 2011).

The mussels which were also subjected to these treatments were found to be unaffected with respect to stress levels measured by neutral red retention time. It was hypothesized that there would not be a significant stress response from mussels and, if any, would increase with temperature (decrease in neutral red retention time). This trend did occur for treatments 40°C 1 min, 45°C 1 min, 50°C 1 min and 55°C dip. However 55°C 1 min, the highest temperature and exposure time, had the same stress response as the control which was not exposed to any stressor treatment. This unexpected result may be due to large mean error in responses for individual mussels under this treatment, n = 6 were therefore insufficient to represent this treatment's response. But because all mussels were in the same condition to start we are confident in our findings. The size of seed used in this experiment was on the larger end of seed sizes used for grow out in Newfoundland (25–45mm) and there may be a difference in stress tolerance for smaller sizes than used here. Vickerson (2009) tested seed 30–40mm and also found low stress levels for mussels temperature shocked for 4 hours with temperatures 10 degrees above ambient. Considering the effectiveness of each treatment in mitigating or culling 100% of juvenile *C. maenas* and causing little to no stress on the mussels also exposed to the same treatments, water temperatures of 40, 45 and 50 and 55°C for the duration of 1 minute and 55°C dip were chosen as the most appropriate due to very high neutral red retention times, and low stress response. None of the Neutral Red Assay treatments were significantly different from the control but the treatment that caused the least amount of stress on the mussels while culling 100% of green crab juveniles was 45°C for 1 minute, so is the most appropriate treatment from a practical perspective.

Forrest and Blakemore (2006) found that green lipped mussels could withstand 55°C for 5 seconds when evaluating mussel health post treatment using gapping tests and byssus reattachment time. This is similar to the findings presented here for another mussel species, the blue mussel.

Further investigation is needed to see if this mitigation technique is effective when juvenile green crab and blue mussels are treated together in the lab as well as in the field, and to make adjustments to temperature and duration to maintain these parameters. It is also advisable that the feasibility of this technique be tested with mussel growers in Newfoundland with mussels of smaller size. Upon approval of the methods by industry for farm scale operations further design and engineering would be needed to create a system that could easily produce and maintain the mitigation conditions on the farm site for an entire seed harvest.

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