PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF JUVENILE QUAHOGS, *MERCENARIA MERCENARIA*, TO LOW TEMPERATURES: POTENTIAL FOR MITIGATION OF OVERWINTERING MORTALITIES

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ABSTRACT

Bricelj, M.V., C. Ouellette, M. Anderson, N.T. Brun, F. Pernet, N. W. Ross, T. Landry. 2005. Physiological and biochemical responses of juvenile quahogs, *Mercenaria mercenaria*, to low temperatures: potential for mitigation of overwintering mortalities. Can. Tech. Rep. Fish. Aqua. Sci. 2739: vii + 44 p.

The northern quahog, *Mercenaria mercenaria*, is a temperate bivalve that experiences site-specific and variable losses when deployed for growout at small sizes (less than about 20 mm in shell height) at field sites in Atlantic Canada and the US. This study determined the long-term effects of low temperatures (1, 7 and 12^{o}C) on survival, psysiological rates (oxygen consumption, clearance rates and growth rates), and biochemical responses (gross proximate composition) of quahog juveniles held in a land-based nursery system supplied with a moderate diet of cultured algae. Quahog seed experienced progressive and variable mortality rates both at 7 and 1^{o}C, but not at 12^{o}C. Feeding rates were suppressed at 1^{o}C, leading to progressive, linear tissue weight loss at this temperature. Organic weight loss could be entirely accounted for by the utilization of carbohydrate reserves, as there was no measurable utilization of either protein or lipid reserves. During simulated spring conditions following overwintering, quahogs showed reduced growth rates when subjected to both a temperature increase and reduced salinity (2 wks at 17 ‰), than those that only experienced an increase in temperature but remained at constant salinity (30 ‰), suggesting that the spring freshet may aggravate overwintering stress. Notata seed showed significantly higher mortalities during simulated overwintering at 1^{o}C than native seed from the same hatchery source. Preliminary data suggest that pre-conditioning at higher temperature (18 and 23^{o}C) increased the survival of juvenile quahogs overwintered at 1^{o}C. Acute cold shock (reduction in temperature from 12 to 1^{o}C) induced the expression of heat shock or stress proteins HSP70 and HSP40 in juvenile quahogs, but this effect was more prolonged and greater in magnitude when the cold shock lasted 12 h vs. 3 h. Thus this study documents for the first time the timing and magnitude of overwintering losses of *M. mercenaria* seed and suggests several potential strategies for the mitigation of winter losses.

RÉSUMÉ

Bricelj, M.V., C. Ouellette, M. Anderson, N.T. Brun, F. Pernet, N. Ross, T. Landry. 2005. Physiological and biochemical responses of juvenile quahogs, *Mercenaria mercenaria*, to low temperatures: potential for mitigation of overwintering mortalities. Can. Tech. Rep. Fish. Aqua. Sci. 2739: vii + 44 p.

Des pertes localisées ont été observées dans le Nord-Est de l'Atlantique chez les palourdes américaines (*Mercenaria mercenaria*) juvéniles au cours de la période de croissance. Ces pertes minent le développement de l'aquaculture. Nous avons isolé les effets à long terme des températures de 1, 7 et 12 ^{o}C sur la survie, le métabolisme, l'alimentation, la croissance et la composition biochimique de palourdes américaines juvéniles dans des nurseries terrestres. Les mortalités ne se sont produites qu'aux deux températures les plus basses. À 1 ^{o}C, l'alimentation a été suspendue; la perte de poids s'explique par l'utilisation des glucides. Quand on a provoqué l'augmentation de la température et la réduction des taux de salinité, simulant la crue printanière, on a constaté une aggravation du stress lié à l'hivernation. À 1 ^{o}C, le taux de mortalité était plus élevé chez les palourdes de la variété notata que chez les semences de variétés indigènes. Le préconditionnement à des températures plus élevées (de 18 à 23 ^{o}C) semble accroître les chances de survie lorsque la température chute à 1 ^{o}C. Un choc thermique froid important (de 12 à 1 ^{o}C) a provoqué l'expression de protéines de stress. Soulignons que l'induction était plus prolongée et plus prononcée lorsque le traitement durait 12 heures que lorsqu'il durait 3 heures. Les résultats de ces recherches servent à formuler des stratégies d'atténuation afin de compenser les pertes liées à l'hivernation.

INTRODUCTION

The northern quahog (hard clam), *Mercenaria mercenaria*, is a temperate bivalve species occurring in Atlantic Canada at the limit of its northern distribution. This native species has recognized potential for culture in the region, i.e. high value and market demand in North America, and well established hatchery practices. Natural quahog populations occur in the southern Gulf of St.-Lawrence, Sam Orr's Pond, NB and St. Mary's Bay, NS (LeBlanc et al., 2005), the latter an area that sustains a valuable commercial fishery. *M. mercenaria* has undergone extensive aquaculture development and population enhancement led by the provincial Fisheries and Aquaculture Department of Prince Edward Island (PEI), New Brunswick (NB) and Nova Scotia (NS) (Fig.1) as well as DFO in recent years with mixed success (AquaInfo 2003 a and b).

Growth rates of field planted seed (≤ 20 mm), most notably of the notata variety (a genetic shell marker rare in nature, used to distinguish cultured from native (wild) stock (Chanley 1961; Humphrey and Walker 1982; see Fig. 2) have been promising (market size attained in 4 yrs.). However, unexplained, highly variable, site-specific winter mortalities are considered the principal obstacle for the development of aquaculture of this species. Field studies have not been able to determine whether mortalities occur during the winter or following spring, as seed deployed in the fall cannot be recovered until the spring. These losses create an unknown risk for growers and a deterrent for industry expansion until enhanced, more predictable winter survival is achieved. Unpredictable and variable overwintering mortalities of seed are also a concern in mid-Atlantic US waters (Zarnoch and Scheibman 2005; J. Kraeuter, Rutgers University, NJ, unpublished data). Recent problems faced by the mussel industry in Canada, including saturation of culture sites and tunicate invasion, have stimulated the need for species diversification to achieve sustainability and maximize productivity of the shellfish industry, thus spurring an interest in determining the culture potential of *M. mercenaria*.

Little is known about the physiological and biochemical responses to low temperature of temperate bivalves such as *M. mercenaria*, especially of juvenile stages, as most prior studies have focused on growth optimization at higher temperatures, and on the physiology and biochemistry of adults (Grizzle et al 2001), or were conducted on other bivalve species (Laing and Child 1996; Child and Laing 1998; Bricelj et al. 2000). Juveniles often present a bottleneck for winter survival, as they are generally more vulnerable to thermal and nutritional stress than conspecific adults. In Manila clam (*Tapes philippinarum*) seed, mortalities at low temperatures were found to be temperature, food and time-dependent. Thus, Manila clams showed 100% survival when held at 9^{o}C for 11 wks (fed or unfed), but at 3^{o}C survival ranged from 58 to 0% in fed and unfed clams respectively after 11 wks (Laing and Child 1996). Thus, synergism between environmental factors (food levels, temperatures and salinity) may influence mortality rates in the natural environment, e.g. during the spring freshet when bivalves already stressed from the winter, and experience increasing temperatures and low salinities. Rearing methods (stocking densities and type of culture) also influence mortality rates (C. Gionet and A. Mallet 1999, 2000 and 2001 Hatchery, nursery and field trials, unpublished reports).

Cold adaptation of temperate, marine invertebrates encompasses mechanisms from the molecular and cellular level to that of the whole organism (Pörtner 2002). In temperate bivalves, low temperatures usually lead to reduced pumping and feeding and to a lower rate of oxygen consumption (VO_2). However, the relative magnitude of these reductions is species-specific and has not been determined for juvenile *M. mercenaria*. Loss of reserves and negative scope for growth may occur if the feeding rates are too low to meet the metabolic demand, potentially leading to mortalities if weight drops below a critical threshold. At the molecular level, adaptation to temperature extremes may involve changes in membrane lipid composition to maintain membrane function (Hazel 1995; Gillis and Ballantyne 1999; Hochachka and Somero 2002) or induction of stress or heat shock proteins (HSPs) in response to temperature elevation.

Heat shock proteins (HSPs) are ubiquitous, highly conserved, and have been shown to increase upon exposure to various stressors. More specifically, HSP70 plays important roles in the protection and survival of normal cellular activities resulting from exposure to various types of stressors, including elevations in temperature (Feder and Hofmann 1999). HSP70 expression has been well characterized in mussels (Smerdon et al. 1995, Chapple et al. 1997); however, limited information is available for other bivalves, and none is available for quahogs. In addition, no work has been published on HSP expression in response to cold shock in bivalve mollusks. It has been demonstrated that artificial induction of sublethal temperature shock (increase in temperature), leading to an increase in HSP levels, may promote increased thermotolerance of oysters, *Crassostrea gigas*, to subsequent stressors (Clegg et al. 1998) and thus have a protective value of potential practical value for shellfish growers.

The present study provides the first results of physiological responses (feeding and metabolic rates) and biochemical responses (changes in proximate composition and HSP response) of juvenile *M. mercenaria* at low temperatures ($\leq 12^{o}$C), as physiological work to date was undertaken at temperatures $\geq 12^{o}$C that support growth of clams (Grizzle et al. 2001).

OBJECTIVES

The main objectives of this project were to determine for juvenile quahogs (hard clams), *Mercenaria mercenaria,* in a land-based, experimental nursery:

- The timing and rate of mortalities experienced at 1^{o}C (simulated overwintering) compared to those at 7^{o}C, and 12^{o}C (control treatment at which growth is known to occur). A treatment at 7^{o}C was included as preliminary experiments (see methods) suggested that this temperature might be particularly stressful for clams;

- The aerobic metabolic rate (oxygen consumption rate) and feeding rate (clearance rate) at 1^{o}C, 7^{o}C and 12^{o}C;

- The rate of change in tissue weight and in energy reserves (protein, total lipids and carbohydrates) at these temperatures;

- The magnitude and duration of expression of stress or heat shock proteins in response to acute, heat or cold shock. This will allow assessment of whether HSP induction could be applied towards developing increased tolerance of clams to extremes of environmental conditions and thus mitigate winter losses of seed under natural conditions. It may also allow for the management of stress during transfer of seed from the hatchery to the field for grow-out, and during harvesting and post-harvesting practices of adults.

Additional objectives of the present study were:

- To conduct a preliminary experiment using survivors of overwintering at 1°C, to determine whether the effects of increasing temperatures alone or in combination with low salinity (spring simulation) contributed to clam mortalities and weight loss in the spring;

- To compare the performance (survival) of notata and native *M. mercenaria* varieties exposed to low temperature stress (long-term holding at 1 and 7°C);

- To determine whether the temperature of pre-conditioning or acclimation (12, 18 and 23°C) could influence overwintering mortalities of notata and native clams held at 1°C.

Ultimately, the goal of this study was to develop a first-order understanding of the causes and factors (environmental parameters, and endogenous factors related to stock condition and genetic makeup) responsible for losses of seed during and following the winter, in order to suggest possible mitigation strategies to the aquaculture industry and identify promising avenues of future research.

MATERIALS AND METHODS

EXPERIMENTAL SETUP AND SOURCE OF CLAMS

Representative seasonal temperature records at hard clam field growout sites are shown in Figs. 3 and 4. These were used to establish ecologically relevant conditions in land-based experiments (e.g. holding temperature and duration of overwintering). All experiments were conducted at IMB's Marine Research Station (MRS), Sambro, NS, in closed, aerated, recirculating experimental systems.

A first experimental batch of *M. mercenaria* seed (notata variety produced at the Ellerslie Shellfish Hatchery, PEI; see Fig.1) was received at MRS in December 2002. This batch had been held in Malagash, NS, by a commercial grower under suboptimal conditions, i.e. relatively high stocking densities in an outdoor nursery supplied with pumped, ambient seawater. These clams were culled on site prior to transport to MRS, and likely suffered from exposure to low air temperatures. At MRS they were held in recirculating, passive downwellers at 7°C and fed continuously with cultured algae (see below) until they could be used for experimental trials.

Mortalities were determined by visual inspection at approximately weekly intervals and dead clams removed. Due to the high mortalities experienced by this batch during acclimation at 7°C, these clams were not used for subsequent low temperature trials. However, they provided useful preliminary information in conducting subsequent experiments described in the present study. They showed that accurate mortality measurements for clams ≤ 6 mm required prying open of individual clams (thus only mortalities for the 11 mm size class are reported in Fig. 5), and also suggested that a 7°C long-term holding temperature might be stressful for juvenile clams. Finally, they indicated that husbandry/initial condition of clams is critical for determining subsequent survival rates at low temperatures, although genetic differences may also be a contributing factor (see subsequent comparison of notata and native clams). This effect is reflected by the much higher mortalities obtained with this batch (averaging 3.1% day^{-1}) than those recorded with a subsequent batch obtained from a Massachusetts hatchery (averaging 0.7% day^{-1}) (see below) when held under identical conditions, despite the fact that the latter were smaller in initial size (mean = 6.7 mm compared to 11.4 mm for Malagash clams).

OVERWINTERING EXPERIMENTS

A second batch of clam seed was thus obtained from the Aquaculture Research Corporation (ARC), Dennis, Massachusetts, USA, as no other source of local seed was available in Atlantic Canada at this time of the year. At the ARC clams were raised from ambient temperatures to ~ 18°C and fed cultured algae to ensure that they were actively growing and thus in good condition prior to air shipping to Halifax. Clams were received at MRS on February 26, 2003, and were comprised of 35% notata and 65% native clams. A schematic of their pre-conditioning history at MRS and of the experimental design are shown in Fig. 6. Clams were divided equally between three experimental temperature treatments: one group was maintained at 12°C, and two groups experienced a gradual temperature decrease (at a rate of 1.5°C day^{-1}) to 7 and 1°C respectively. Clams were held in passive upwellers (3 per treatment) within recirculating, aerated nurseries (capacity = 1300 L) (Fig. 7) maintained in walk-in, temperature-controlled chambers. The 1°C nursery system was held in a separate chamber at ~4°C air temperature and seawater was recirculated through an external chiller (Aquanetics, ½ HP) to maintain the required temperature. The 7 and 12°C nurseries were held in a second walk-in held at an air-temperature of 12°C; an external chiller maintained the 7°C temperature in the corresponding nursery. Once attained, the experimental temperatures were maintained constant for 8.6 wks for the 7°C treatment and for an additional 4 wks for 1 and 12°C treatments, and monitored continuously via submersible automatic temperature loggers (Optic StowAway Temp™, Onset Computer Corp.).

The nurseries were filled with 1 μm-cartridge filtered, incoming seawater (salinity = 30 ‰) at the desired temperatures. Cleaning and complete replacement of seawater and algae in the suspension was conducted twice a week. All wastewater was chlorinated and dechlorinated with 1 M sodium thiosulphate prior to release into the environment. Clams (initial mean shell length, SL = 6.73 ± standard error, SE = 0.06 mm) were held at an initial stocking density of 4800 per downweller (diameter = 30.5 cm) on a 1 mm Nitex square mesh. No density-dependent effects are expected at this low clam density. The flow rate through each downweller was ~ 14 L min^{-1}.

In all treatments, clams were maintained at a constant algal concentration of 30 *Isochrysis galbana* (clone T-Iso) cell volume equivalents μl^{-1}. They were initially offered a mixed suspension (50:50 by volume) of *Chaetoceros muelleri* (clone CHGRA, mean equivalent spherical diameter, ESD = 5.5 μm) and T-Iso (mean ESD = 4.4 μm); starting on April 4, *C. muelleri* was replaced by *Pavlova pinguis* (ESD = 3.7 μm) due to difficulties experienced with mass culture of *C. muelleri*. Although *M. mercenaria* juveniles grow well on a unialgal diet of T-Iso at higher temperatures (Wikfors et al. 1992), they were fed this mixture to ensure an adequate complement of the essential polyunsaturated fatty acids (PUFA) 22:6n-3 (DHA) and 20:5n-3 (EPA), as these are known to play an important role in membrane adaptation to low temperatures (e.g. Hall et al. 2002). Algal concentrations in the nurseries were monitored daily with a Beckman-Coulter electronic particle counter using a 100 μm aperture tube, and algae were batch or continuously added via a peristaltic pump from a concentrated stock as required. Algae were batch-cultured in 200 L photobioreactors filled with pasteurized seawater in f/2 medium, under continuous light, at 21 ± 1°C and a pH of ~7.9, controlled by pulsed CO_2 addition. Algae were harvested in the late exponential growth stage.

DETERMINATION OF CLAM SURVIVAL, GROWTH AND PROXIMATE COMPOSITION

Clams (50 clams per upweller) were randomly sampled from each treatment for determination of weekly mortality rates. Individuals were pried open with a scalpel to ensure that reliable counts of live and dead animals were obtained. Dead clams other than those sampled with live ones for various analyses were not removed from the systems to allow calculation of cumulative mortality rates.

Every 2 wks, 3 subsamples of clams were removed for determination of individual shell length and whole body biomass (n = 20 per upweller). For proximate analysis, 3 samples of 10 pooled clams per upweller were used for carbohydrate analysis, and the same number was used for lipids and for total protein. Dry weight was determined after oven-drying for 24h at 80°C and ash weight after combustion for 24 h at 480°C, to allow calculation of ash-free dry weight (AFDW). Whole individuals were analyzed as they were too small for reliable dissection of soft tissues.

For lipid analysis clams were placed in pre-washed, glass tubes with Teflon-lined caps, containing 3 ml of chloroform, stripped of oxygen with nitrogen gas, immediately frozen and stored at -80°C until analysis. Lipids were extracted following the method of Folch et al. (1957) as modified by Parrish (1999). Briefly, samples were homogenized in 4 ml $CHCl_3$-MeOH (2:1 v/v) with a glass pestle in a 7 mL Tenbroeck tissue grinder. The grinder was rinsed twice with 4 mL $CHCl_3$-MeOH (2:1 v/v) and solutions were pooled together. The solution was mixed thoroughly with 3 mL of distilled water and centrifuged at 800g for 2 min at 4°C. Lipids were recovered from the lower organic layer and solvent was evaporated under nitrogen. Extracts were concentrated to 1 ml and 4 μL of each was applied to Chromarods-SIII for separation of lipid classes by thin layer chromatography. A short development in acetone was used to concentrate samples into a narrow band on the Chromarods. After drying the rods, lipid classes were separated in a stepwise procedure using developing solvents of increasing polarity (Parrish, 1999). This method separates aliphatic hydrocarbons (HC), sterol and wax esters (SE-WE),

ketones (KET), triacylglycerols (TAG), free fatty acids (FFA), free fatty alcohol (ALC), free sterols (ST), diacylglycerols (DG), acetone mobile polar lipids (AMPL) and phospholipids (PL). Total lipid was obtained by summation of lipid classes and the mean AFDW for the corresponding sampling date was used to calculate total lipid per AFDW. Chromatograms were analyzed using integration software (Peak Simple version 3.2, SRI Inc).

For protein and carbohydrate analysis, clams were lyophilized, and pulverized in a mortar and pestle. Protein was determined from organic nitrogen analysis with a CHN Analyzer using acetanilide as standard, and a nitrogen to protein conversion factor of 5.8 (Gnaiger and Bitterlich 1984). Carbohydrate was determined in triplicate for each sample homogenate prepared with a Polytron homogenizer, by the phenol-sulfuric method of Dubois et al. (1956) using glucose as standard. The lyophilized weight of pooled clams in each sample tube was determined to express carbohydrate content on a per weight as well as individual basis. Differences in mean (n = 3 upwellers) percent content and weight between initial and final total lipid, carbohydrate and proteins were compared by an ANOVA; the arcsin transformation was used for percentages).

DETERMINATION OF PHYSIOLOGICAL RATES

The rate of aerobic metabolism (oxygen consumption, VO_2) and the clearance rate (CR, volume cleared of particles per unit time) were determined two to four times in each treatment over the course of the experiment. A subsample of clams (n = 20 to 65 per chamber depending on the temperature) was removed from the nurseries and maintained in five 365 ml acrylic experimental chambers in which the suspension was mixed via stirring bars placed on the top of the chamber (Fig. 8) and rotated by magnets driven by a slow-speed motor (modified from MacQuarrie 2002). This setup prevented resuspension of biodeposits but ensured mixing of algae in the suspension. The clams were acclimated overnight (~18 h) under flow-through/fed conditions and CR determined using the method of Coughlan (1969). Incubations lasted between 2-41 hr, 10-35 min and 3-15 min in 1, 7 and 12^{o}C trials respectively to ensure that percent algal depletion in the chamber, determined with an electronic particle counter (in triplicate) did not exceed ~ 30-35%. A 6th chamber with no clams was used as a control for algal settlement. Following re-acclimation to flow-through conditions for ~ 1 hr, VO_2 was measured for the same clams using a Strathkelvin multi-channel respirometer. These incubations lasted 14-41 hr, 7-12 hr and 2.4-3.3 hr for 1, 7 and 12^{o}C treatments respectively. All incubations were conducted in dim light. At the end of each trial the volume of seawater in each chamber was corrected for clam displacement and % mortality was determined. A subsample of 20-40 live clams per chamber was frozen for determination of SL and AFDW used to calculate weight-standardized physiological rates (based on the number of live clams). VO_2 values were corrected for any O_2 consumption/production detected in the control chamber containing the algal suspension. This averaged ~14% at 12^{o}C and 7^{o}C and from 19 to 30% at 1^{o}C. Visual observations were also made on May 10 and May 14-15 to determine the proportion of the clam test population with visibly open shells, extended mantle and/or siphons, presumed to be capable of feeding or at least indicative of activity.

SPRING SIMULATION

Survivors of the overwintering (1°C) treatment were further subjected to the following environmental conditions for 2 wks: (a) increase in temperature at a rate of 1°C day^{-1}, and b) the same increase in temperature but at a constant low salinity = 17 ‰, to simulate spring conditions experienced at field growout sites (see schematic in Fig. 6). Performance of these two groups was compared to a control treatment (extended overwintering) that remained at a constant temperature and salinity (1°C, 30‰). A 1°C day increase in temperature was considered representative of field conditions in the spring, based on an increase of 0.6°C day^{-1} calculated from temperature records at Shemogue, New Brunswick (Fig. 4b). The acute salinity reduction from 30 to 17‰ simulated a spring freshet as experienced at some sites in Atlantic Canada (e.g. Malagash, Kevin LeBlanc, DFO, Moncton, pers. comm.). A salinity of 17‰ was obtained at MRS by treating 30‰ ambient seawater through a reverse osmosis system which reduced the salinity to 1 ‰ by removal of NaCl. A 1:1 mixture of 1 ‰ and 30 ‰ water yielded 17‰ seawater.

Following the 2 wks spring simulation, when clams attained 12°C, they were held at this temperature and 30 ‰ for an additional 2 wk-recovery period from May 28 to June 11. Differences in percent mean (n = 3 upwellers) mortalities (arcsin-transformed) and AFDW between the control and two spring treatments were compared by ANOVAs followed by Bonferroni multiple comparison tests.

EXPRESSION OF HEAT SHOCK PROTEINS

Heat and cold shock experiments were conducted using the ARC clams in the same recirculating nurseries described earlier. Controls that were handled identically to temperature-shocked clams except for the change in temperature, were run in parallel to the treatments. Whole animal and/or soft tissue dissected from individual clams were collected and immediately frozen in liquid nitrogen and/or dry ice. Samples were homogenized, centrifuged, and aliquots of the supernatant were stored at –80°C until analysis. HSP70 levels of individual whole clams or clam mantle tissues were determined using an Enzyme-linked Immunosorbent Assay (ELISA), validated with Western Blotting and immunodetection methods. HSP data were analyzed using factorial ANOVAs, followed by Tukey's multiple comparisons analysis (SPSS, version 12.0). Samples from treatments 1 to 4 (see below) were assayed for HSP40 levels (data not shown), and those from treatment 4 were also analyzed for protease activity using zymography (Hassel et al. 1996). Proteases have been previously associated with immune response in vertebrates and invertebrates (Hjelmeland et al. 1983; McKerrow 1989). Specific treatments included:

1) 3 h acute heat shock: 1°C to 12°C, sampling times: T_0-28d.
2) 3 h acute cold shock: 12°C to 1°C, sampling times: T_0-28d.
3) 12 h acute cold shock: 12°C to 1°C, sampling times: T_0-28d.
4) 3h acute cold shock: 20°C to 3°C, sampling times: T_0-28d (only protease data are shown in results).

COMPARATIVE SURVIVAL OF NOTATA AND NATIVE CLAMS AT DIFFERENT TEMPERATURES

Native and notata clam varieties were obtained from the Ellerslie Shellfish Hatchery, PEI, where notata broodstock from ARC and native broodstock from a population held in PEI waters for several generations were spawned. A first batch of clams (both varieties) was received at MRS on June 12, 2003. A schematic of the acclimation and experimental history of this batch of clams is shown in Fig. 9. The native batch initially suffered 19% mortality but mortalities rapidly stabilized. However, due to this reduction in numbers an additional batch of native clams was obtained from Ellerslie on July 9. This group experienced no mortalities and was merged with survivors of the first batch. All experiments with native and notata clams were conducted in nursery systems consisting of a 1000 L insulated xactic tank containing active upwellers, i.e. a submersible pump attached to the inflow of each upweller delivered a flow rate of ~ 12.6 L min^{-1} through each unit. Use of these smaller units allowed tank replication and easier maintenance. Notata and native clams were held at a stocking density of 3750 native and 3940 notata clams per upweller respectively. Throughout the experiment (including the acclimation period) clams were fed a 50:50 mixture by volume of *Pavlova pinguis* and *Isochrysis galbana* (T-Iso), at a total, constant cell density of ~ 42 T-Iso cell volume equivalents μl^{-1}.

Native and notata clams were pre-conditioned to three constant temperatures: 12, 18 and 23°C for a minimum of 3 mo., then experienced a gradual decrease in temperature at a rate of 1.5°C day^{-1} until the three groups attained 1°C on Nov. 25 and were held at this overwintering temperature for ~2.3 mo and 30‰ (Fig. 9). For the overwintering trial, clams were divided between two replicate xactic tanks [one upweller of each clam variety per tank] except for the clams pre-conditioned at 18°C, for which only notata clams were used. Notata clams held at 18°C, that were subjected to heat shock (i.e., control vs. shocked) experiments (see methods above), were held in one upweller per treatment in both replicate tanks. Mortalities in each tank were determined at weekly intervals as described above for ARC clams (3 subsamples of 30 clams from each of two upwellers). In all overwintering experiments, initial mortalities of native and notata clams were subtracted to allow comparison of strain-specific mortalities. These ranged from undetectable for notata to ≤ 13% for natives. Clams experienced no mortalities during the extended acclimation period at 12°C.

Weekly survival of native and notata clams was also followed during long-term holding of clams at 7°C [one upweller per variety held in one tank (Fig. 9)] as these clams were available for other studies and could be readily monitored. Although a strict comparison between these clams and the ones held at 1°C is not possible, these data provide additional useful information on the relative survival of the two varieties at this intermediate temperature (7°C), since native and notata clams were held together in the same tank under identical conditions of temperature and food.

RESULTS

OVERWINTERING EXPERIMENT WITH ARC CLAMS

GROWTH, SURVIVAL AND BIOCHEMICAL COMPOSITION

Temperatures achieved in the three treatments averaged 1.8 ± 0.4°C, 7.1 ± 0.3°C and 12.9 ± 0.2°C over the experimental period. A 1-2°C experimental temperature adequately simulated winter temperatures in the region, as those recorded at clam growout sites in NB and NS range from ~ +5°C to -2°C (Figs. 3 and 4). Juvenile (6 mm) clams held at a constant food concentration and temperature for ~ 2 months experienced increasing, time-dependent cumulative mortalities at 7 and 1°C (simulated winter) but not at 12°C (Fig. 10). ARC clams held at 1°C attained 28% and ~68% mortalities by the end of 2 and 3 mo. respectively. Mortalities remained low, below 10%, for the first month of overwintering. Thus, winter mortality is not a mass episodic event, but mortalities increase progressively over time.

In addition to high survival, clams at 12°C (controls) showed positive tissue growth. Ash-free dry weight showed a highly significant linear increase over time, with approximately a doubling in AFDW by the end 8 wks (May 9, Table 1). Clams at 7°C showed a statistically significant increase in AFDW, but only a 1.2-fold increase in AFDW over the same experimental period, indicating that these animals were maintained very close to their maintenance ration at this temperature, i.e. food uptake was sufficient or slightly exceeded that required to balance the energy demand. In contrast, clams that overwintered at 1°C experienced a slow, linear decline in AFDW over time of ~ 0.1% per day. This reduction in AFDW was not statistically detectable by May 9 but was highly significant ($p<0.001$) by the last sampling date of that treatment (12.7 wks on Jun 11) (Table 1).

The initial and final absolute concentration (in mg $clam^{-1}$) and % composition of total lipid (TL), protein and carbohydrates are shown in Fig. 11 (time course not shown). Protein concentration and % relative content increased significantly by the end of 8 wks only at 12°C, and showed no significant change at the two low temperatures. Total lipid (TL) concentration increased at all three holding temperatures, but % lipid content only increased significantly at the low temperatures. However, % lipid showed no clear pattern (either increase or decrease) in any of the treatments when the whole time course was examined. No utilization of TL or of protein was detected at 1°C; carbohydrate was the only substrate that showed significant depletion during overwintering and is thus the main energy reserve contributing to the decline in AFDW at this temperature.

Lipid classes detected in juvenile hard clams in this study were triglycerides (TAG), sterols (ST), acetone mobile polar lipids (AMPL) and phospholipids (PL). Minor amounts of free fatty acids ≤ 0.5% were occasionally detected and not reported hereafter. In all cases, phospholipids comprised the bulk of total lipids (e.g. 77-78% on May 8, the last date sampled). Therefore, the temporal patterns in total lipids per clam reflect those of phospholipids (only TL is shown in Fig. 11). Triglycerides (TAG), generally considered the main energetic source among lipid classes, contributed ≤ 10% to total lipids in clam tissues and showed no consistent pattern over time in the various treatments (data not shown). Sterol also contributed a small fraction to total lipids (5.2 and 5.5% at 1 and 7°C respectively, and 7.5% at 12°C), but sterol levels showed a significant increase over time at 12°C (from 14.4 to 44.9 μg $clam^{-1}$) and little change over time at 7 and 1°C (significant time and temperature interaction, $P<0.001$). AMPL, a lipid class which includes

photosynthetic pigments, glycolipids and monoacylglycerol, contributed ~7% of the total lipids in clam tissues and showed no consistent pattern over time in the various treatments (data not shown). It is noteworthy that phospholipids, which are important components of membranes, made up the largest fraction of clam lipids at these sizes.

The ratio of PL to ST, generally considered as an indicator of membrane fluidity, varied as a function of time and temperature (Fig. 12). On one hand, the PL to ST ratio of juvenile hard clams decreased during the period of study (P<0.001) (Fig. 12A). On the other hand, *M. mercenaria* held at 12°C were characterized by a decrease in their PL to ST ratio compared to those maintained at 1°C (P=0.031) (Fig. 12B), thus suggesting a first level of homeoviscous adaptation in membranes.

PHYSIOLOGICAL RATES

Weight-standardized CR and VO_2 were strongly dependent on acclimation temperature (Fig. 13A). Feeding rates were essentially nil at 1°C (average between trials < 0.03 ml min^{-1} for a clam 1 g in AFDW) and confirmed via long incubations. This was supported by behavioral observations indicating that 100% of the clams at this temperature maintained closed shells (Table 2). In contrast, clams exhibited measurable feeding activity (CR) at 7°C, although there was a progressive decline in CR over time at this temperature (Fig. 13A), suggesting that acclimation of feeding rates is more prolonged at low temperatures, and may take as long as 6 wks. It is also possible that lower physiological rates over time reflected the clams' increasingly poor condition, given that 54% and 43% cumulative mortalities were attained at 7°C and 1°C respectively by the end of 9 wks and/or the effects of selective mortalities. Clearance rate was also highly variable over time at 12°C despite correction for weight differences (from 6.2 to 18.6 ml min^{-1} for a clam 1g in AFDW), with an overall increase in weight-standardized CR over time. It is noteworthy that clam visual observations conducted in May, showed that only 46 to 70% of clams were open at any given time at 12°C, indicating that clams feed intermittently at this temperature. This supports observations of clams made directly in the experimental chambers at 12°C during the first feeding trial which showed that approximately half of the clams (mean 49 ± 2 %; range = 15-60%; n = 5 chambers of pooled clams) had extended siphons. The percentage of open clams was reduced to less <1% at 7°C, although these observations were made after long-term holding at this temperature (Table 2) and were likely higher earlier on.

Aerobic metabolic rate was also a positive function of temperature (Fig. 13B). Overall, the reduction in CR with decreasing temperature over the range tested, and especially between 7 and 1°C, was much greater than the reduction in VO_2, resulting in the observed energy imbalance (tissue weight loss) observed at 1°C.

SPRING SIMULATION

Cumulative mortalities during the 4 wks of spring simulation were lowest for the controls maintained at 1°C, intermediate for clams that experience only a temperature increase and highest for those that underwent both a temperature increase and low salinity treatment (42%, 53% and 62% respectively) (Fig. 14A), although these differences were not statistically

significant (ANOVA, $p<0.091$). These results indicate that mortalities do not cease during the early spring period simulated by the experiment, but continue for some time during the period of rising temperatures, and may be exacerbated by low salinities. Growth differences over the 4 wks among the three treatments were marginally significant (ANOVA, $p<0.056$). Thus, while clams maintained at 1°C continued to lose weight, positive growth resumed in both spring simulation treatments, but was slower in clams subjected to both an increase in temperature and low salinity (Fig. 14B).

Since there were no significant differences in clam AFDW among upwellers within a treatment/date, it was possible to pool all clams for ANOVAs that compared initial and final (4 wks) AFDW within each treatment and thus increase the power of the statistical analysis. Thus, controls showed significant weight loss over 4 wks ($p<0.01$), clams subjected to an increase in temperature showed highly significant weight gain ($p<0.001$), and those subjected to reduced salinity showed no significant change in AFDW over 4 wks ($p>0.29$). Examination of these data only for the first 2 wks (without including the recovery period) indicated that clams exposed to the low salinity treatment lost considerably more weight [(26.1% reduction over the first 2 wks compared to 6.8% in clams that only experienced an increase in temperature (data not shown)]. Overall, these results indicate that survivors of overwintering have the potential to resume growth during the spring under suitable temperature and food conditions, but that low salinity representative of spring freshet conditions may reduce growth.

COMPARATIVE PERFORMANCE OF NOTATA AND NATIVE CLAMS

Direct comparison of mortalities of native and notata clams from the same hatchery source indicated that in general, mortalities of native clams were lower than those of notata clams, and that the difference in viability among the two genetic strains at low temperatures became more pronounced over time and was greater at 1°C than at 7°C (Figs. 15 and 16). Although the experiments at 7 and 1°C were not conducted simultaneously, their overlay shows that, in contrast to the experiment with ARC clams, mortalities were lower at 7°C than at 1°C. This reversal in ranking with ARC clams (see Fig. 10) could be due to tank variability (tanks were not replicated given the large scale of that experiment), or be associated with brief spiking in temperature recorded in the 7°C nursery due to transient chiller failure [on March 21 this nursery experienced a 5°C overnight (16 hr) increase in temperature]. It is important to note that in the experiments with native and notata varieties, mortalities appeared to level off rather than continuing to rise linearly over time.

STRESS PROTEINS

No significant differences in HSP70 levels were found between the handled controls and the undisturbed control animals that remained in the tanks. Therefore, only the former are shown in the figures. For all experiments, HSP40 expression patterns were comparable to those obtained for HSP70 (HSP 40 data not shown). Our initial experiments showed that acute heat shock (1°C to 12°C for 3 h) of juvenile quahogs was ineffective in inducing statistically significant elevation in HSP70 levels (Fig. 17). However, acute cold shock induced expression of

HSP70 (Figs. 17 and 18) and HSP40 (not shown). This is the first time that HSP70 expression has been characterized in *Mercenaria mercenaria*, and the first study showing HSP response to cold shock in any bivalve.

The duration of the cold shock affected both the magnitude and duration of the HSP response. When a 3 h cold shock (12^{o}C to 1^{o}C) was applied, HSP70 levels in cold-shocked clams did not differ significantly from control animals after 7d of recovery (Fig. 18). However, when a more extended cold shock was applied (same temperature increment but duration of 12 h) highly significant elevation in HSP levels relative to controls ($p<0.001$) was maintained for at least 28 days (Fig. 19).

Samples from a different cold shock experiment (20^{o}C to 3^{o}C for 3 h), analyzed using zymography, showed the appearance of a 70-80 kDa single band, which was further characterized as a metalloprotease. However, no changes in the activity of this enzyme were observed over time between cold-shocked and control groups (Fig. 20). Thus, cold shock did not modulate protease activity levels.

DISCUSSION: RECOMMENDATIONS AND FUTURE RESEARCH DIRECTIONS

This study demonstrates that prolonged holding (> ~3 wks) of small hard clam seed (~ 6-10 mm SL) at winter temperatures (1^{o}C) can lead to significant and time-dependent mortalities, although the magnitude of mortalities was found to vary considerably depending on the prior holding history and husbandry as well as the genetic makeup of seed. Long-term holding of small seed at 7^{o}C, a temperature at which feeding activity is shown to be severely reduced, is also stressful for seed clams at these sizes and leads to mortalities. In contrast, clam seed can be kept for long periods of time (≥ 3 mo) at 12^{o}C without detectable mortalities, and this temperature allows them to maintain slow but positive growth if sufficient food is available. Thus 12^{o}C is recommended as a suitable temperature for long-term holding of juvenile clams, at a land-based facility, as it leads to no detectable stress or loss of condition, and only moderate food consumption, and thus relatively low cost. Similarly, small juveniles of related clam *Tapes* spp. (ranging in initial AFDW from 0.3 to 3.1 mg) showed 100% survival and positive growth when held for 11 wks at 9^{o}C, but experienced high mortalities at 3^{o}C even when fed (Laing and Child 1996). At 11 wks, these ranged from 42% to 100% in Manila clams, *Tapes philippinarum*, and *Tapes decussatus* respectively.

Changes in AFDW of ARC clams did not reflect differences in mortality rate at the two low temperatures and appear to be a poor predictor of stress levels. Changes in AFDW at 1^{o}C were relatively small, and the rate of organic weight loss was comparable to that of fed Manila clam seed held at 3^{o}C (Laing and Child 1996). Manila clams were also able to maintain or slightly increase their AFDW at 6^{o}C, as was observed for hard clams in the present study at 7^{o}C.

Although this study showed that utilization of bulk protein or lipid reserves do not appear to be important under low temperature stress, further evaluation of the composition of PUFAs may provide critical information on overwintering biochemical responses, given that PUFAs are

responsible for maintenance of membrane function (membrane fluidity) at low temperature (Pernet and Bricelj, in prep.). Carbohydrates were found to be the main energy reserve used to fuel metabolism during overwintering at 1°C, when feeding was entirely suppressed. Clams at 7°C experienced time-dependent mortalities even though they suffered no significant loss in AFDW or in carbohydrate reserves. This suggests that other mechanisms than weight loss or depletion of reserves may have contributed to mortalities of small seed at low temperatures (≤7°C). Since this study tracks the average biochemical composition of survivors in the population, results may also be influenced by selective mortality.

Mortalities of notata seed during overwintering at 1°C were significantly greater than those of native seed of the same size produced at the same hatchery using the same culture methods and held under the same experimental conditions at MRS. The basis for these strain-specific differences remains to be determined and biochemical analysis of the clams used in these experiments as well as comparative electrophoretic analysis of 8 polymorphic loci between the initial population and survivors may shed additional light on this question. This is an important finding given that notata seed have been recommended in the past as the variety of choice for quahog aquaculture in Atlantic Canada, based on their superior growth rates determined from long-term field studies. Our results indicate that this recommendation may need to be revised unless pre-conditioning methods for notata seed that enhance overwintering survival can be further developed or a critical size threshold can be identified via future research that minimizes winter losses of notata seed. It is especially encouraging in this regard, that the magnitude of overwintering mortalities (at 1°C) was inversely related to the pre-conditioning temperature in both clam varieties (Fig. 15), as this suggests a potential mitigation strategy to enhance winter survival. Differences in tolerance to low temperatures have also been found among the introduced *T. philippinarum* and native European clam, *T. decussatus*. Manila clams grew faster at 9°C, and also exhibited an order of magnitude lower reduction in AFDW and higher survival at 3°C than the native clam species (Laing and Child 1996).

It is also interesting to note that overwintering mortalities (at 1°C) of ARC clams were intermediate between those determined for PEI notata and PEI native clams (~45%, 75% and 30% respectively, after ~2.3 mo (70 d) of overwintering. This is consistent with the fact that ARC was a mixed population (~35% notata and 65% native).

Results of the spring simulation experiment indicated that although the major mortalities occur during the winter, and are a function of the length of time that clams remain at low temperatures that suppress (1°C) or severely reduce feeding (7°C), additional mortalities may follow during the spring period of rising temperatures and potentially low salinities. This supports results of Zarnoch and Schreibman (2005), who found that food availability during the spring, ie., the timing of the spring bloom, was important in controlling mortalities following overwintering in hard clams (notata variety) deployed in Jamaica Bay, New York. Thus a very slow rise in temperature during the spring (as evidenced in Fig. 3B in which it took ~ 2 mo. for temperatures to rise between 0 and 12°C), especially if coincident with low food levels, is expected to further stress seed clams coming out of the winter.

The underlying basis for the differences in mortalities at low temperatures remains to be determined and appears to be more complex than a simple loss of organic weight or carbohydrate reserves below a critical threshold. However, our results to date suggest that mitigation strategies to enhance winter survival, (especially genetic stock selection and pre-conditioning during the fall) have great potential and should be further explored. Additional work is also needed to determine the basis for the differences in low temperature survival of notata and native varieties, as well as to determine the effect of seed size and temperature fluctuations on winter survival.

This study showed for the first time that cold shock could induce elevation of HSP70 and HSP40 levels in juvenile *M. mercenaria*. Surprisingly, an equivalent heat shock did not elicit this response. A longer cold shock (12 h vs 3 h application) was shown to greatly increase the magnitude and duration of the HSP response, and could potentially provide protection from stress up to a period of ~ 1 mo. A higher temperature differential and/or repeated applications may be necessary to maintain elevated HSP levels and enhance winter survival of seed at sites where temperatures remain below the feeding threshold for 2 to 3 mo (Figs. 2 and 3). Alternatively, the induction of stress proteins may have greater potential as a protective strategy under conditions of fluctuating temperature, as might occur in the intertidal zone. These daily fluctuations may provide continual upregulation of HSP levels necessary to confer increased survival of the clams during the winter. Additional work is required to examine these hypotheses, but our results to date demonstrate that this approach holds great promise to mitigate the effects of extremes of environmental stress.

Small *M. mercenaria* may be particularly susceptible to winter losses compared to other temperate bivalves such as oysters. For example, no mortalities of European oyster (*Ostrea edulis*) seed were found by holding these in the same nursery system used for ARC clams for 84 d at constant low temperatures (2.5 and 7^{o}C) and a mixed algal suspension of *P. lutherii* and *Thalassiosira weissflogii* (20,000 T-iso volume equivalents ml^{-1}), or incoming ambient seawater (~1 to 3^{o}C) (Bricelj et al. 2000). The oyster seed were slightly larger, however, (mean SL = 14 mm), than the hard clams used in the present study (mean SL = 6 to 8 mm).

CONCLUSIONS

- A temperature of 12^{o}C and moderate diet equivalent in volume to 30,000 *I. galbana* cells ml^{-1} are recommended for long-term, low-cost, land-based holding of juvenile hard clams, as these conditions lead to high survival (~100%), with a relatively low algal food requirement and relatively low heating cost. Clearance rate data obtained in this study can be used to determine the economic feasibility of this strategy at various latitudes. Temperatures between 7 and 12^{o}C may also be effective but remain to be tested before they can be implemented; however, prolonged holding of seed at 7^{o}C also induces mortalities.

- The magnitude of overwintering mortalities at 1^{o}C depends on the duration of holding at this temperature, as significant mortalities (>10%) did not occur until after ~3 wks of maintenance at 1^{o}C, and increased progressively thereafter.

- Survival of native *M. mercenaria* seed at a size of 6-8 mm at winter temperatures (1°C) was significantly greater than that of notata seed. Therefore, successful overwintering of notata aquaculture stock may require use of larger seed, or other mitigation strategies. Determination of a minimum size threshold for survival is worthy of further investigation, as well as understanding of the genetic basis for the differential survival of native and notata seed. Our study indicates that selection of stock more tolerant to low temperatures has great potential.

- Juvenile hard clams utilize only carbohydrate reserves during overwintering. Therefore carbohydrate levels provide a more sensitive index of the nutritional condition of animals by the end of the winter that lipid or protein levels, or organic weight of soft tissues.

- Our results suggest that the combined action of rising temperatures and low salinities (17‰) during the spring can aggravate mortalities initiated during the winter. Therefore, it is recommended that sites for spring growout avoid areas exposed to low salinities. Additional work is required to determine the synergism between salinity and temperature in controlling growth and survival of juvenile hard clams and the role of fluctuating temperatures during the winter-spring.

- Our results also suggest that the nutritional condition of clam seed prior to entering the winter is an important factor influencing their subsequent survival during the winter. Diet manipulation in the fall as a pre-conditioning strategy is also an area that merits additional research.

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Table 1. *Mercenaria mercenaria (ARC source).* Initial and final mean (±SE) total body dry weight (DW), ash-free dry weight (ADFW) and shell length (SL) of juvenile quahogs held at three experimental temperatures (T). Linear regression equations were fitted to a time series of AFDW (in mg) vs. time (in days). Results of ANOVAs comparing initial vs.final parameters (n=3 upwellers) are shown; ns = non-significant; *** = $p \leq 0.001$; ** = $0.001 < p \leq 0.01$; * = $p < 0.05$. [1]Extended overwintering.

T (°C)	Date	SL (mm)	DW (mg)	AFDW (mg)	Regression Equation	ANOVA for AFDW
12°C	March 14 (initial)	6.52 ± 0.03	58.88 ± 1.96	4.50 ± 0.02	$y = 0.077x + 4.512$ $r^2 = 0.998$	$F_{(1,4)} = 24.646$**
	May 9 (final)	8.35 ± 0.29	106.52 ± 8.44	8.85 ± 0.88		
7°C	March 14 (initial)	6.37 ± 0.07	57.23 ± 1.58	4.25 ± 0.20	$y = 0.018x + 4.033$ $r^2 = 0.818$	$F_{(1,4)} = 9.954$*
	May 9 (final)	6.53 ± 0.06	63.36 ± 1.80	5.16 ± 0.21		
1°C	March 14 (initial)	6.39 ± 0.05	51.16 ± 0.19	4.03 ± 0.05	$y = -0.004x + 3.949$ $r^2 = 0.376$	$F_{(1,4)} = 5.335^{ns}$
	May 9 (final)	6.42 ± 0.07	51.93 ± 1.16	3.84 ± 0.07		
	[1]June 11	6.18 ± 0.01	46.74 ± 1.11	3.33 ± 0.03	$y = -0.005x + 3.967$ $r^2 = 0.515$	$F_{(1,4)} = 141.113$***

Table 2. Mean percent of clams with extended and fully or partially dilated siphons at different temperature treatments. Visual observations of clams during replicate trials were made after 18h of acclimation (May 10), and after 20h and 44h (May 14-15). Visual observations were taken during a 4h period every hour (repeated observations of n = 20 clams/replicate).

Temperature	Date	Replicate	Mean ± SE (%)	Range (%)
12˚C	May 10	1	49.0 ±3.0	40-55
		2	70.0 ± 4.0	60-70
	May 14-15	1	46.0 ± 6.0	15-60
		2	54.0 ±5.0	30-70
7˚C	May 10	1	0.1 ± 0	0.1-0.1
		2	0.2 ± 0	0.1-0.3
	May 14-15	1	0.8 ± 0	0-6
		2	0	0
1˚C	May 10	1	0	0
		2	0	0
	May 14-15	1	0	0
		2	0	0

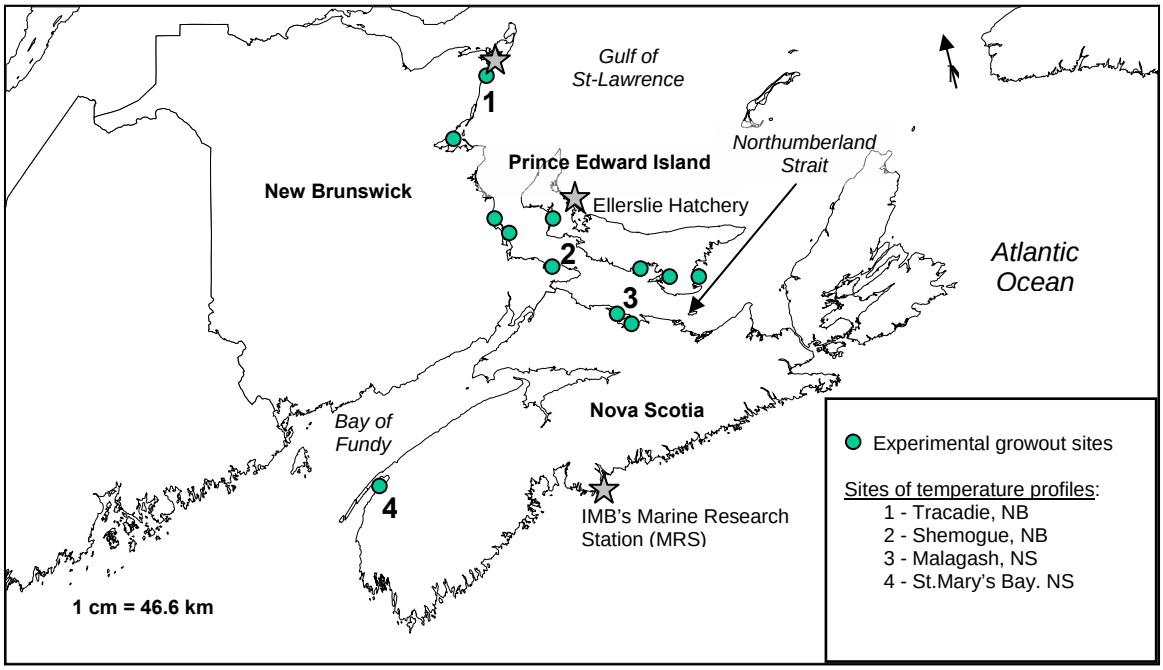

Figure 1. Map showing experimental growout sites of juvenile hard clams, *Mercenaria mercenaria*, in Atlantic Canada (source, DFO Moncton). Numbers indicate sites for which seasonal temperature profiles are reported in the present study (Figs. 2 & 3). The location of the Ellerslie hatchery, which provided clam seed for part of the study, and of the Institute for Marine Biosciences' Marine Research Station, where land-based overwintering experiments were conducted is also shown.

Figure 2. *Mercenaria mercenaria* varieties (juveniles ~ 6 mm in shell length). Left: notata homozygote with characteristic 2-stripe shell pattern; middle: notata heterozygote with characteristic zigzag pattern; right: native, no shell coloring.

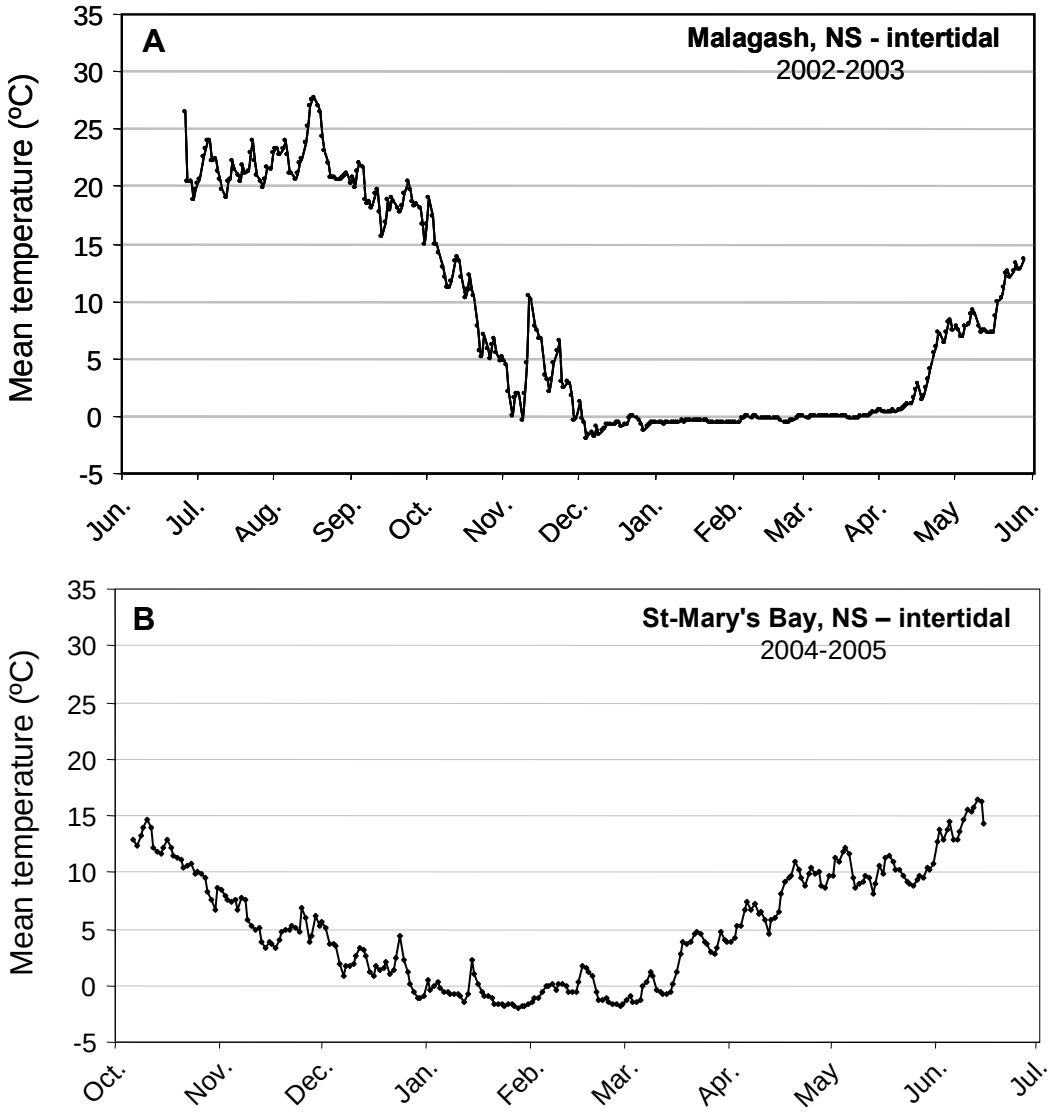

Figure 3. Seasonal temperature profiles at two intertidal growout sites: A) Malagash, NS and B) St.Mary's Bay, NS (Source, DFO Moncton).

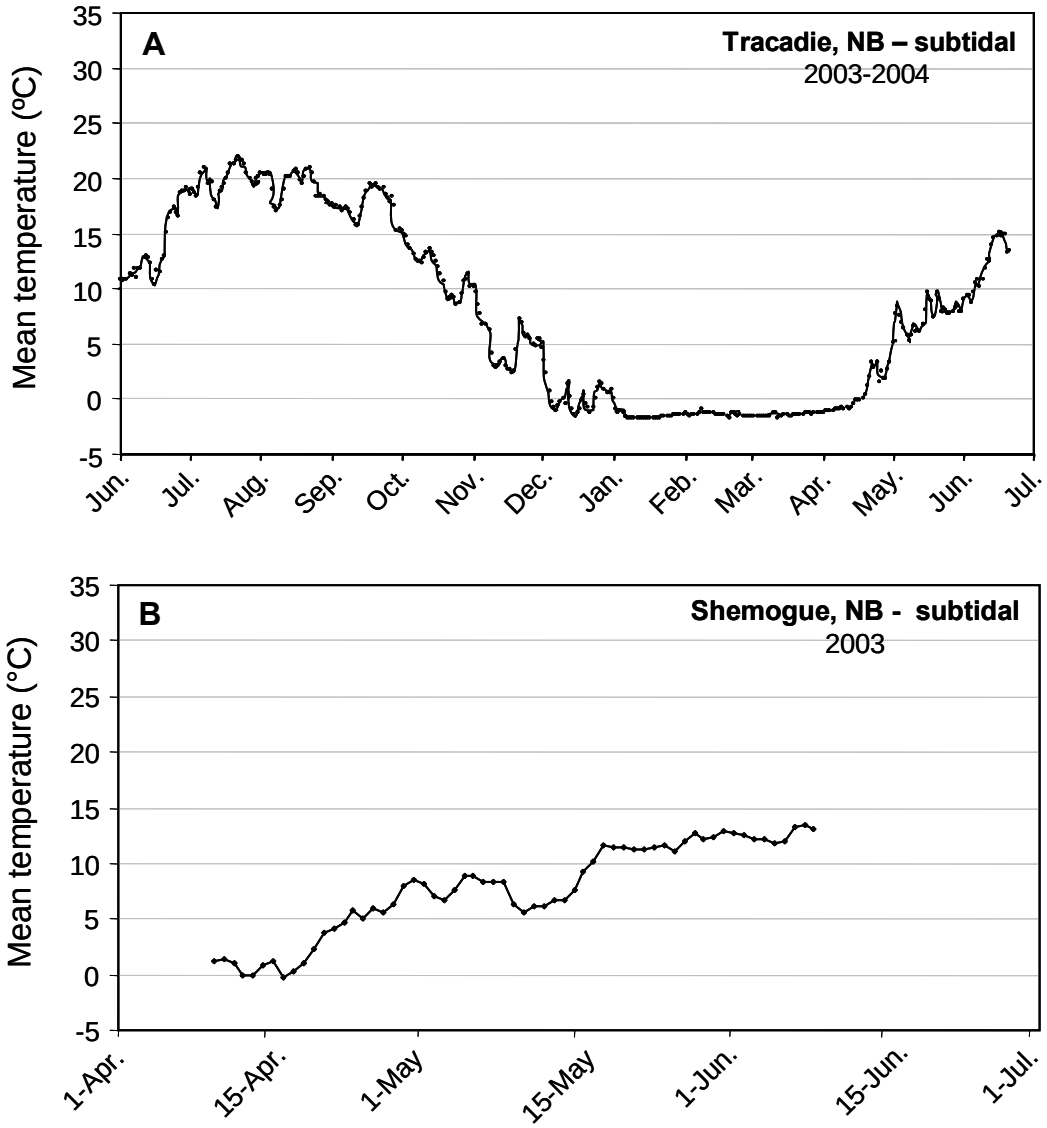

Figure 4. Seasonal temperature profiles at two subtidal growout sites: A) Tracadie, NB and B) Shemogue, NB (Source, DFO Moncton).

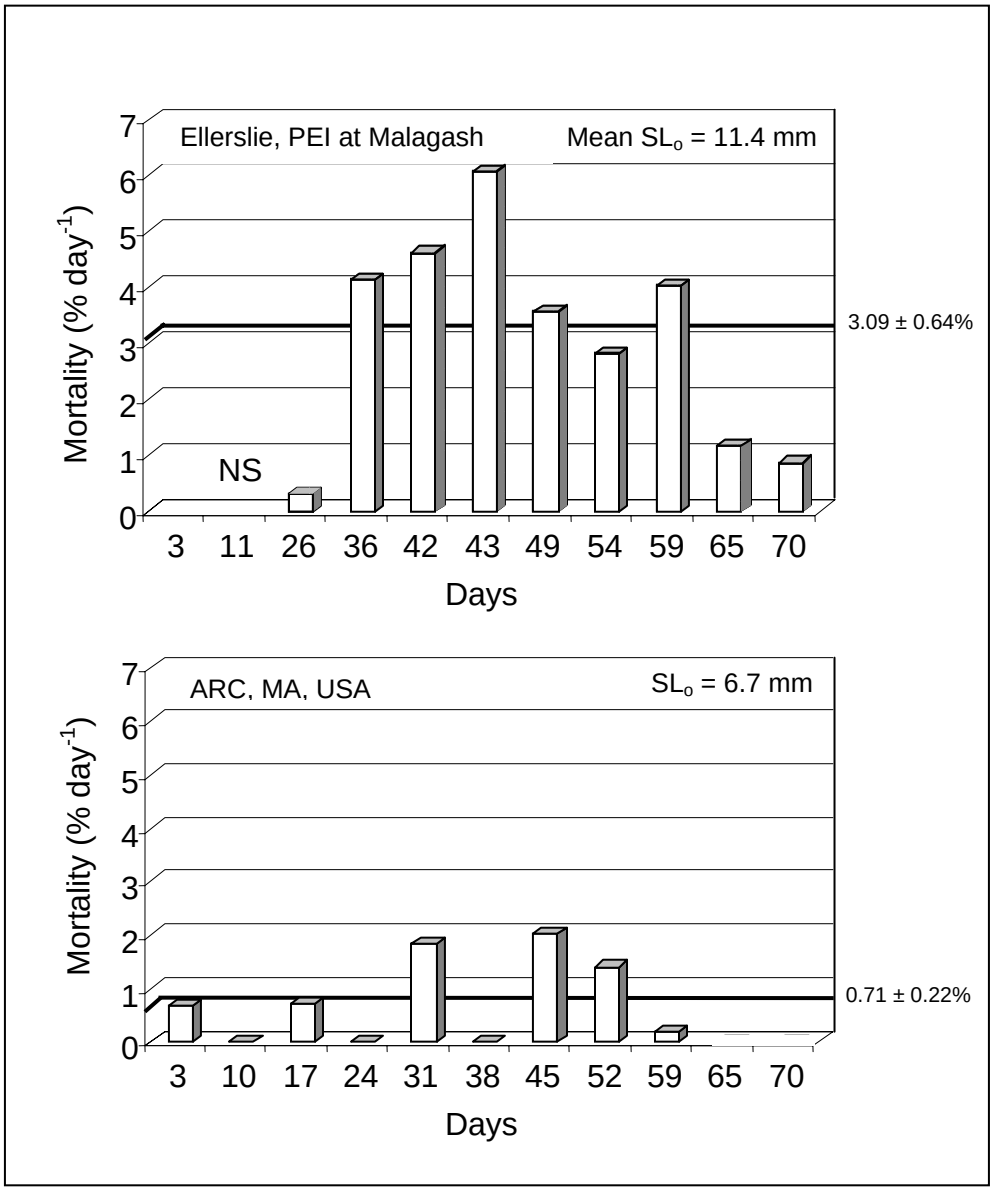

Figure 5. Results of a preliminary trial in which juvenile hard clams, *Mercenaria mercenaria* (notata variety), pre-conditioned in a land-based nursery with ambient seawater at Bay Enterprises Ltd. in Malagash, NS, were held in IMB's MRS land-based nurseries at 7ºC (see methods). Daily mortalities (% loss day^{-1}) of this batch are compared to those of ARC clams used in subsequent experiments (Fig. 9). Horizontal line indicates mean ± SE of daily mortalities over the experimental period; initial shell length (mean SL_o ± SE) at each site is also shown. NS = not sampled.

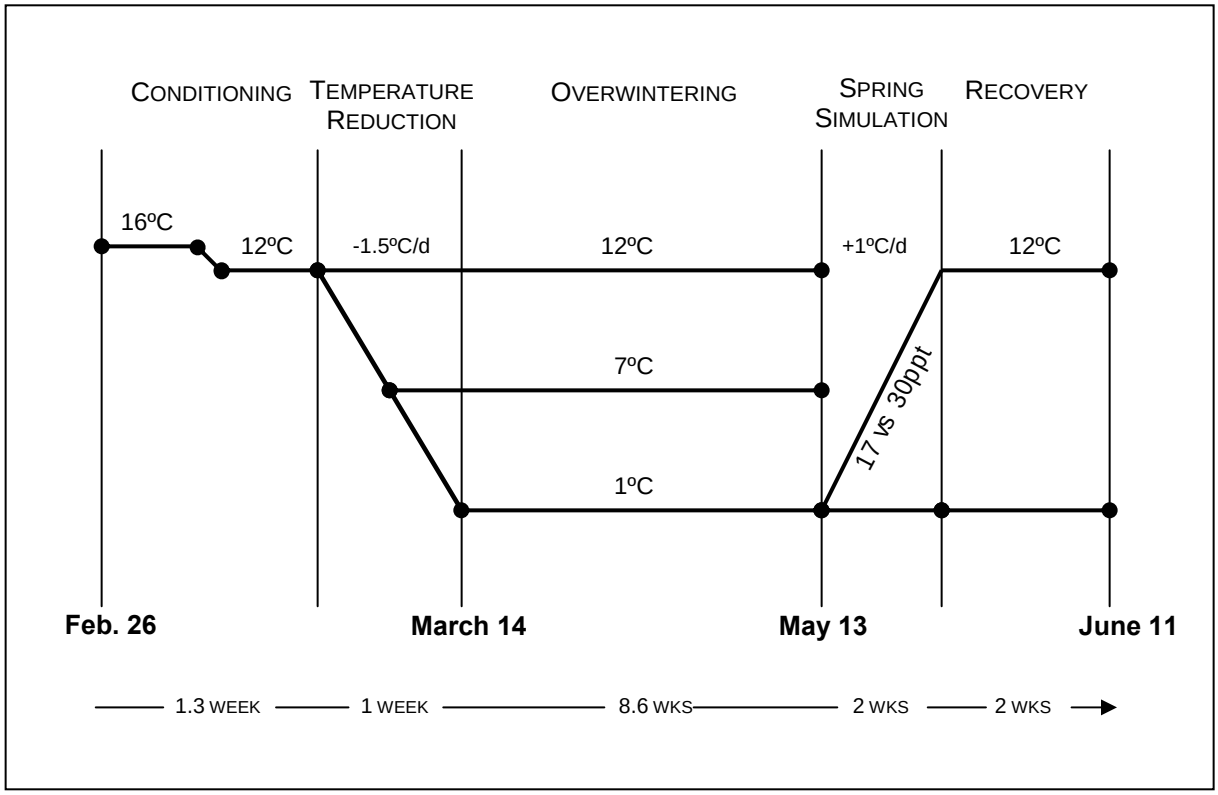

Figure 6. Schematic of experimental protocol for low temperature and spring simulation experiments with *Mercenaria mercenaria* (ARC clams) (see text). Dates and duration of each experimental phase are indicated.

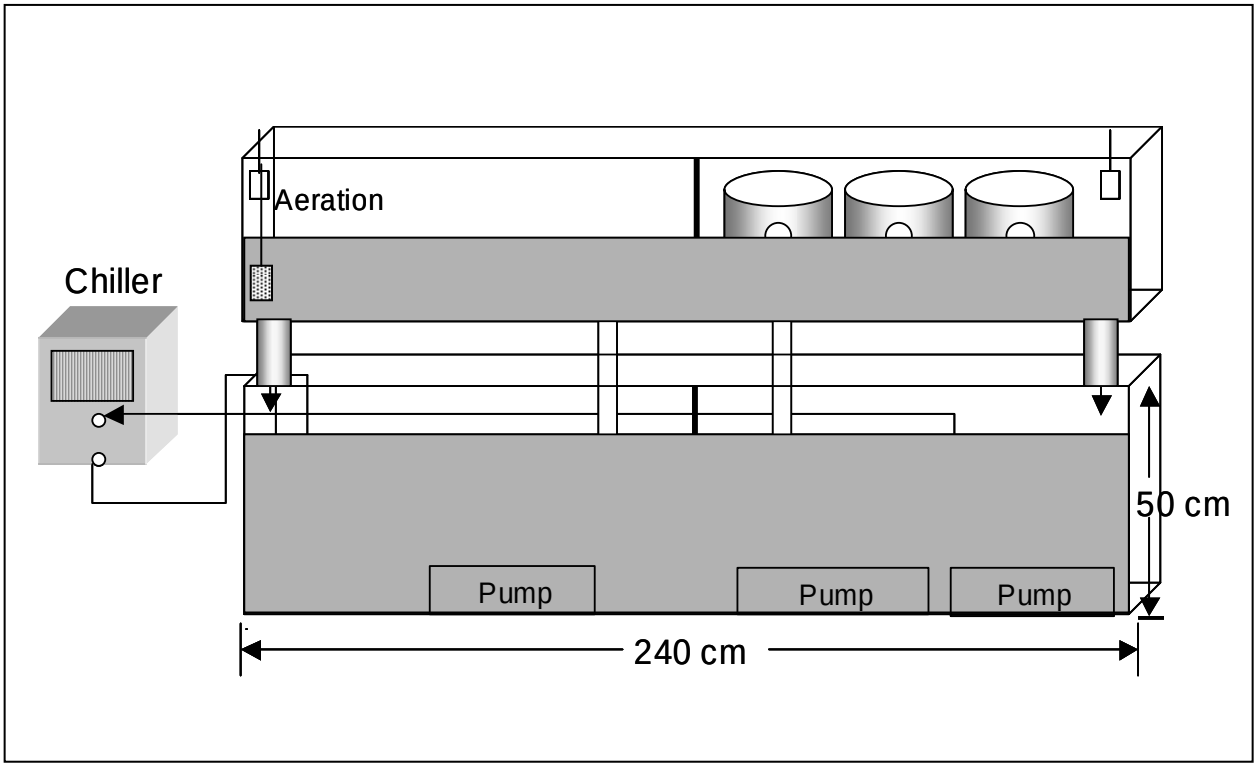

Figure 7. Schematic of the nursery system used at IMB's MRS for the overwintering and spring simulation experiments with ARC quahogs.

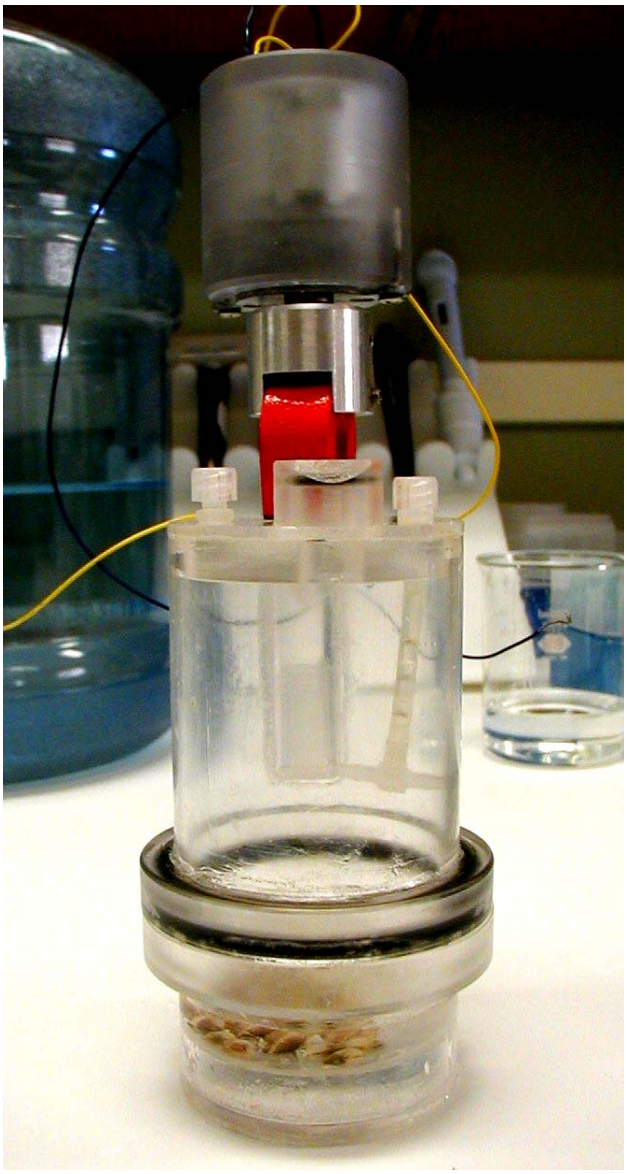

Figure 8. Chamber used for the determination of clearance rates and rates of oxygen consumption of juvenile quahogs, *Mercenaria mercenaria*.

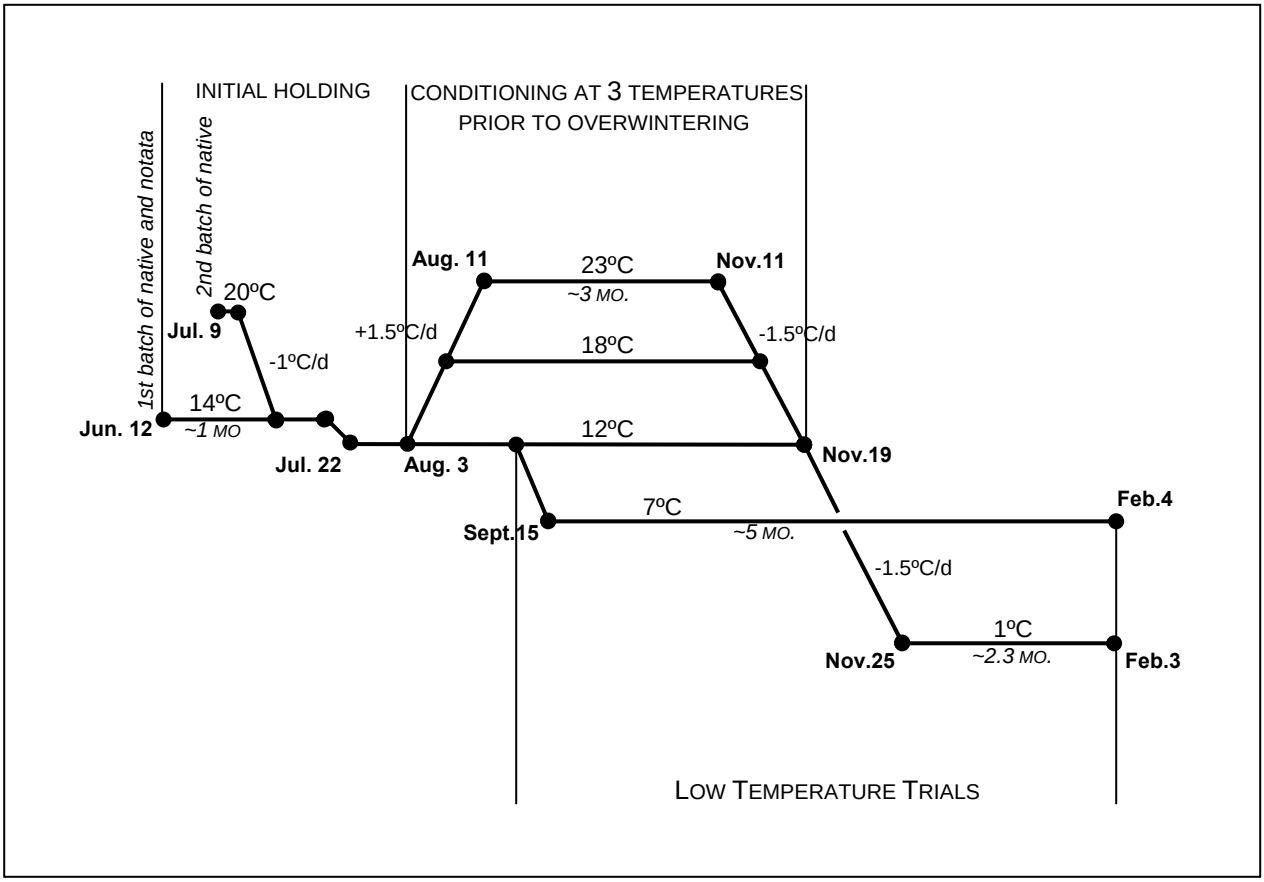

Figure 9. Schematic of the prior holding history and experimental treatments of juvenile clams, *Mercenaria mercenaria* (from Ellerslie, PEI). Comparison of the performance of notata and native varieties conditioned at 12, 18 and 23°C and then overwintered at 1°C from Nov. 25 to Feb.3 (results summarized in Fig. 14); and comparison of the two clam varieties during long-term holding at 7°C, from Sept. 15 to Feb. 4 (results shown in Fig. 13) (see methods).

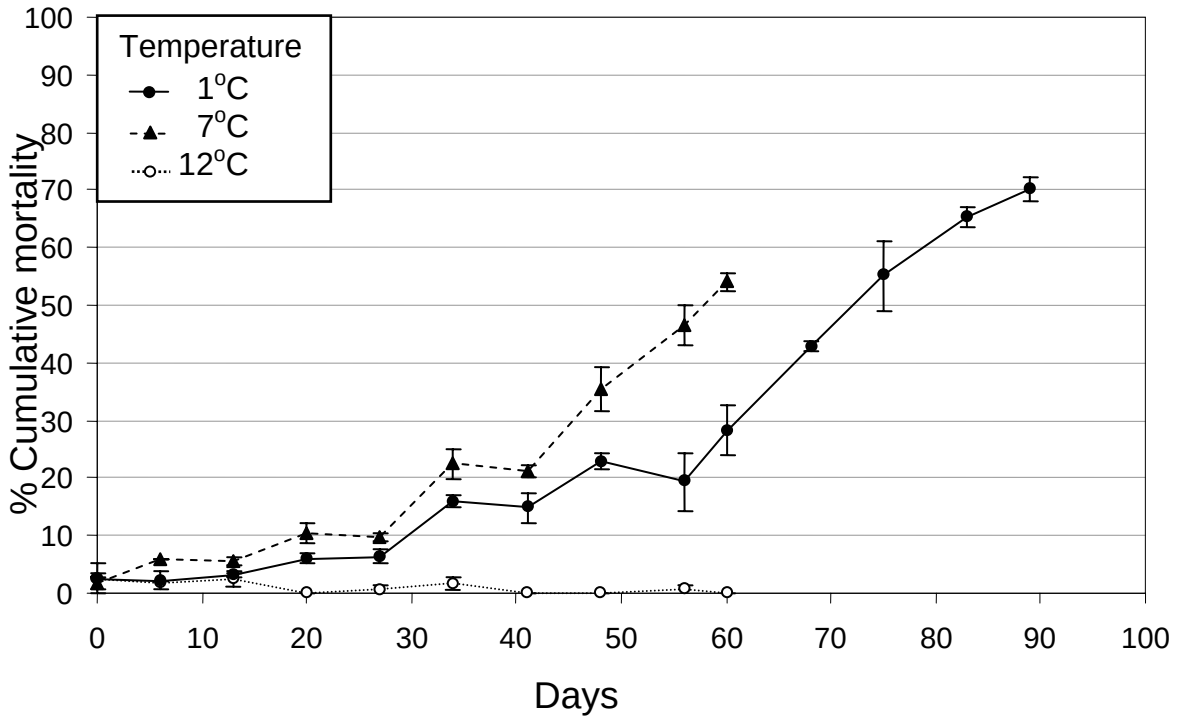

Figure 10. Percent cumulative mortalities (mean ± SE) of *Mercenaria mercenaria* (ARC source). Quahogs were held at three temperatures: 1ºC (simulated overwintering), 7ºC (at feeding threshold), and 12ºC (control, temperature at which positive growth is known to occur). Quahogs held in IBM's MRS land-based nurseries; mortalities recorded between March 14 and May 13, 2003 for clams held at 7ºC and 12ºC, and from March 14 to June 11 for clams held at 1ºC. Mortality prior to the temperature drop (March 6) was negligible, averaging 0.01%.

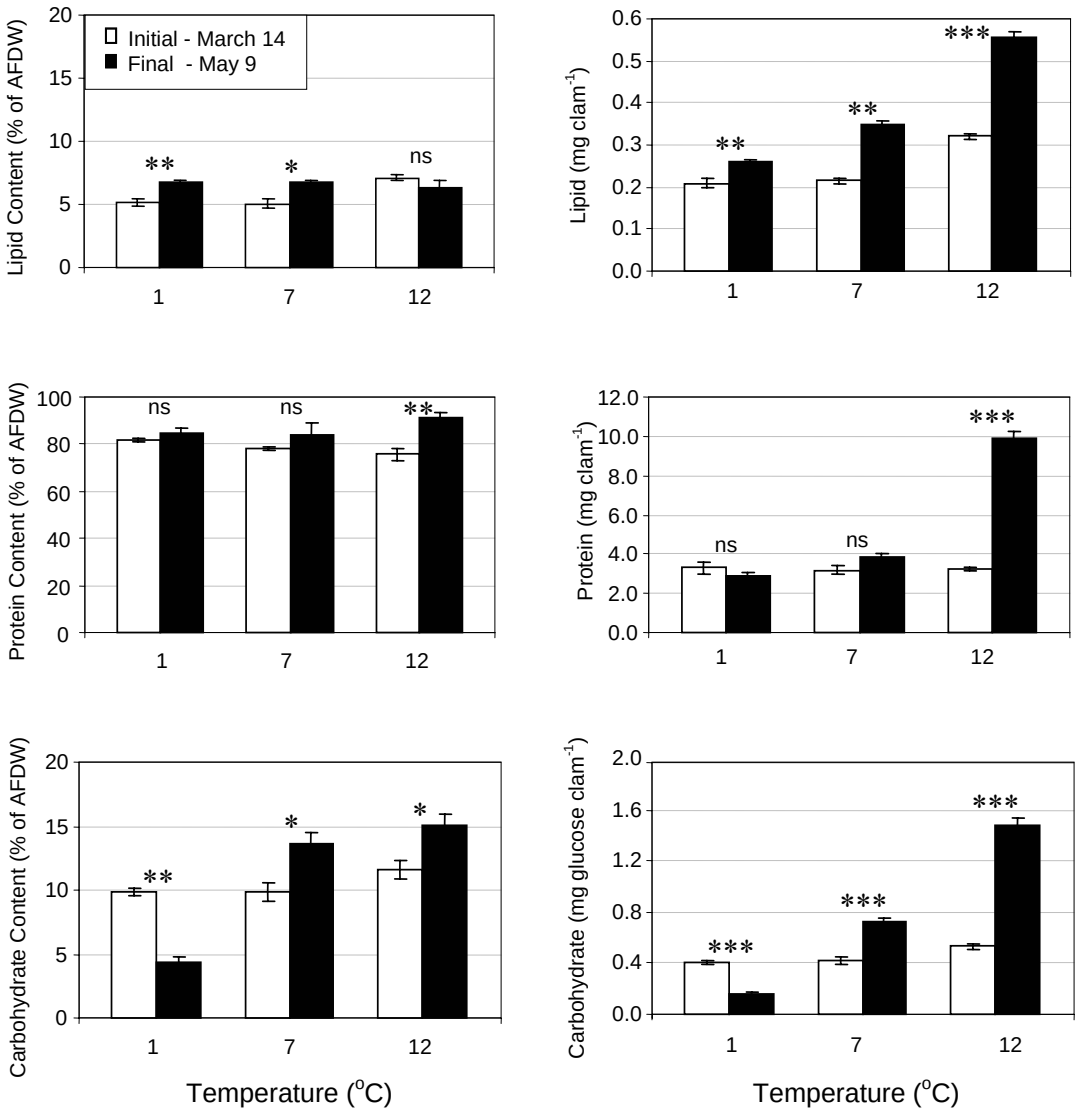

Figure 11. *Mercenaria mercenaria* juveniles (ARC source). Initial and final mean (±SE) of total lipid, protein and carbohydrate content (as % of ash-free dry weight) of juvenile quahogs held at three experimental temperatures. Results of ANOVAs comparing initial vs. final parameters (n = 3 upwellers) are shown; ns = non-significant, *** = $p \leq 0.001$, ** = $0.001 < p \leq 0.01$, * = $p < 0.05$.

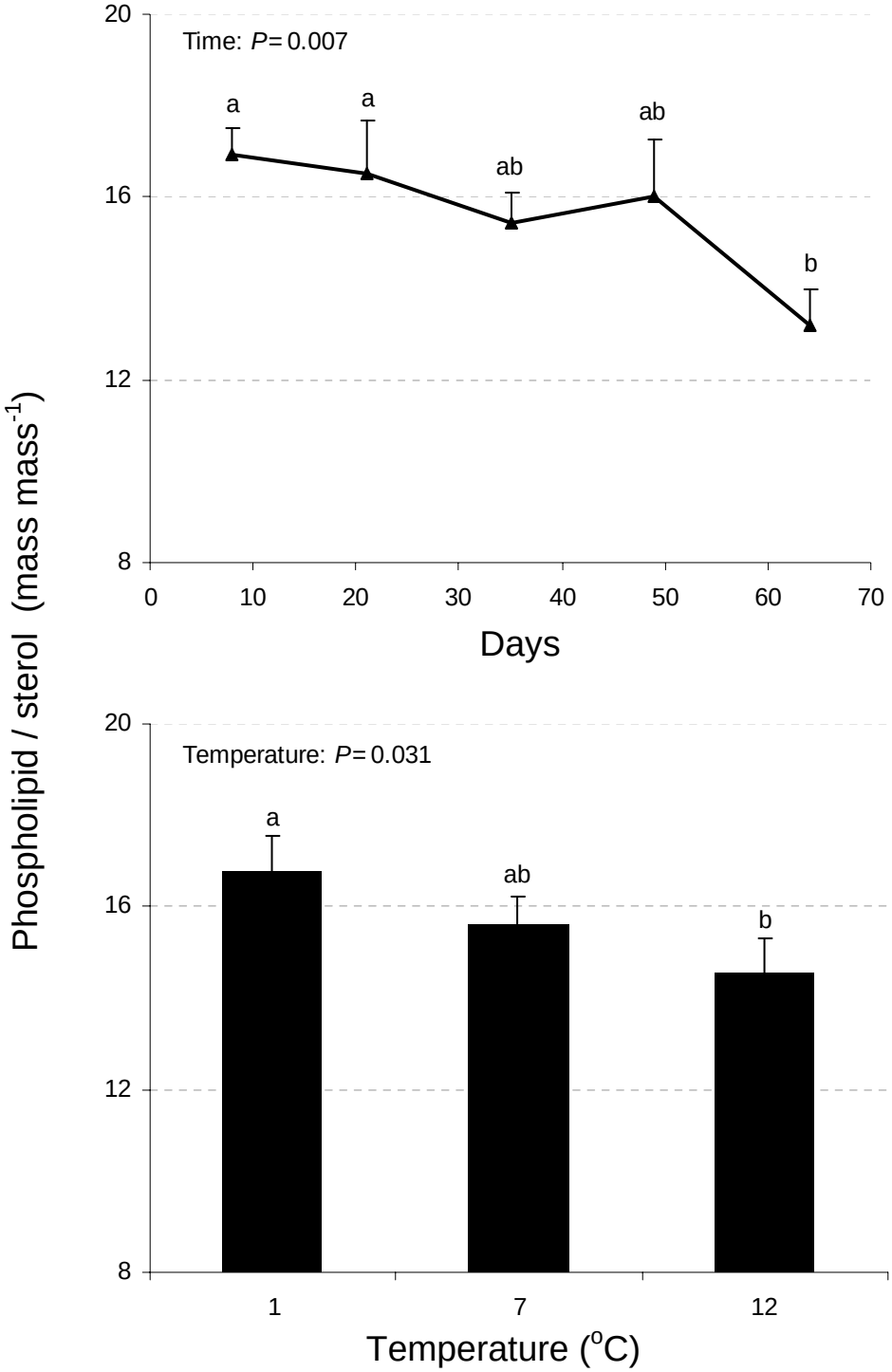

Fig. 12. Upper graph: Phospholipid (PL) to sterol (ST) ratio for juvenile hard clams as a function of time (averaged for all three temperature treatments). Lower graph: PL to ST ratio for juvenile hard clams as a function of temperature (all time-points averaged). Only the main effect is shown as the interaction between temperature and time was not significant (P=0.061)

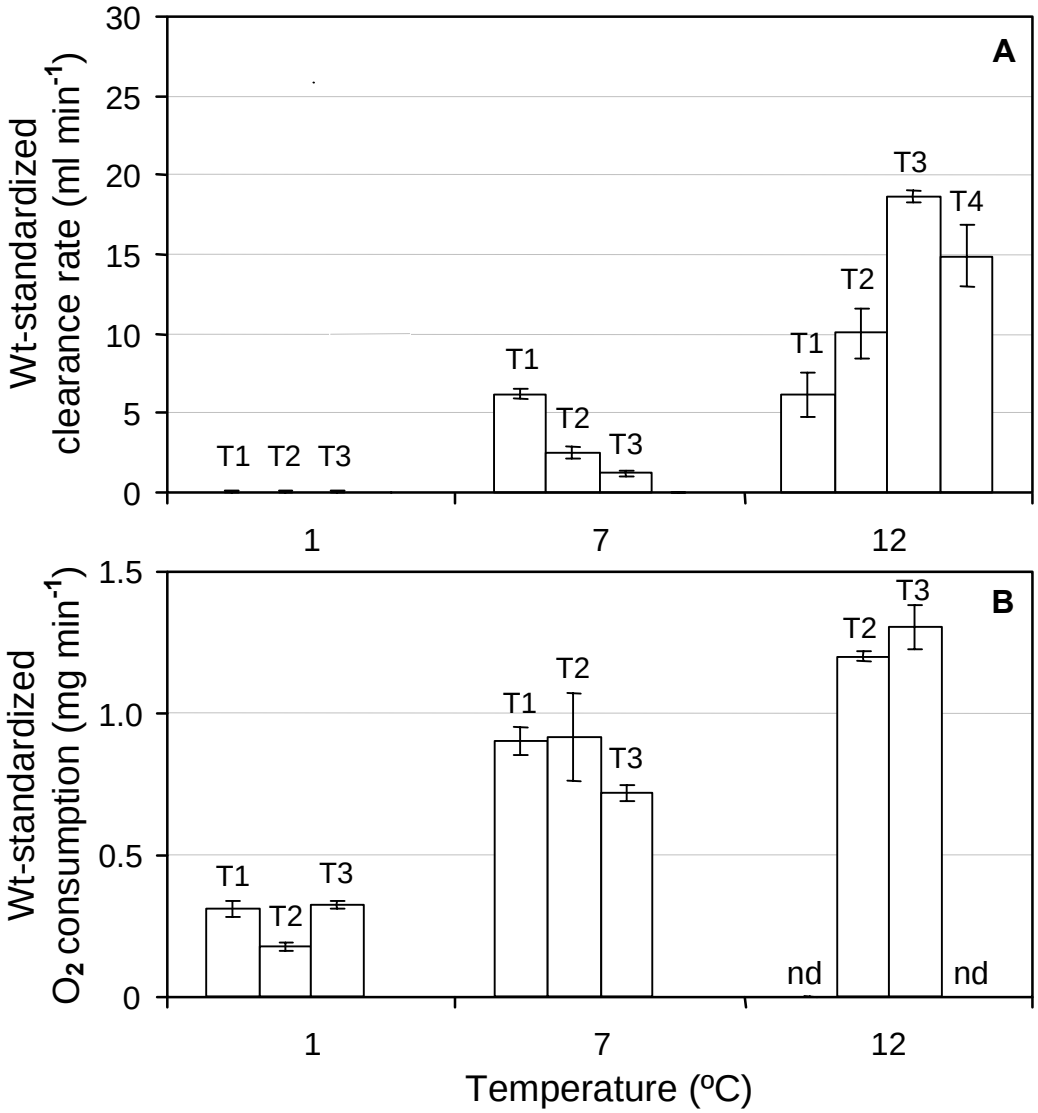

Figure 13. Clearance rate (A) and oxygen consumption rate (B) of *Mercenaria mercenaria* juveniles (ARC source) at 1, 7 and 12°C, for a standard clam 1 g in whole body ash-free dry weight. Values indicate mean ± SE; nd = not determined. Trial dates: T1 = between March 26 and April 3; T2 = April 9 to 11, T3 = April 24 to April 30, and T4 = June 2 (they represent ~ 2, 4, 6 and 10 wks of acclimation at a constant experimental temperature respectively).

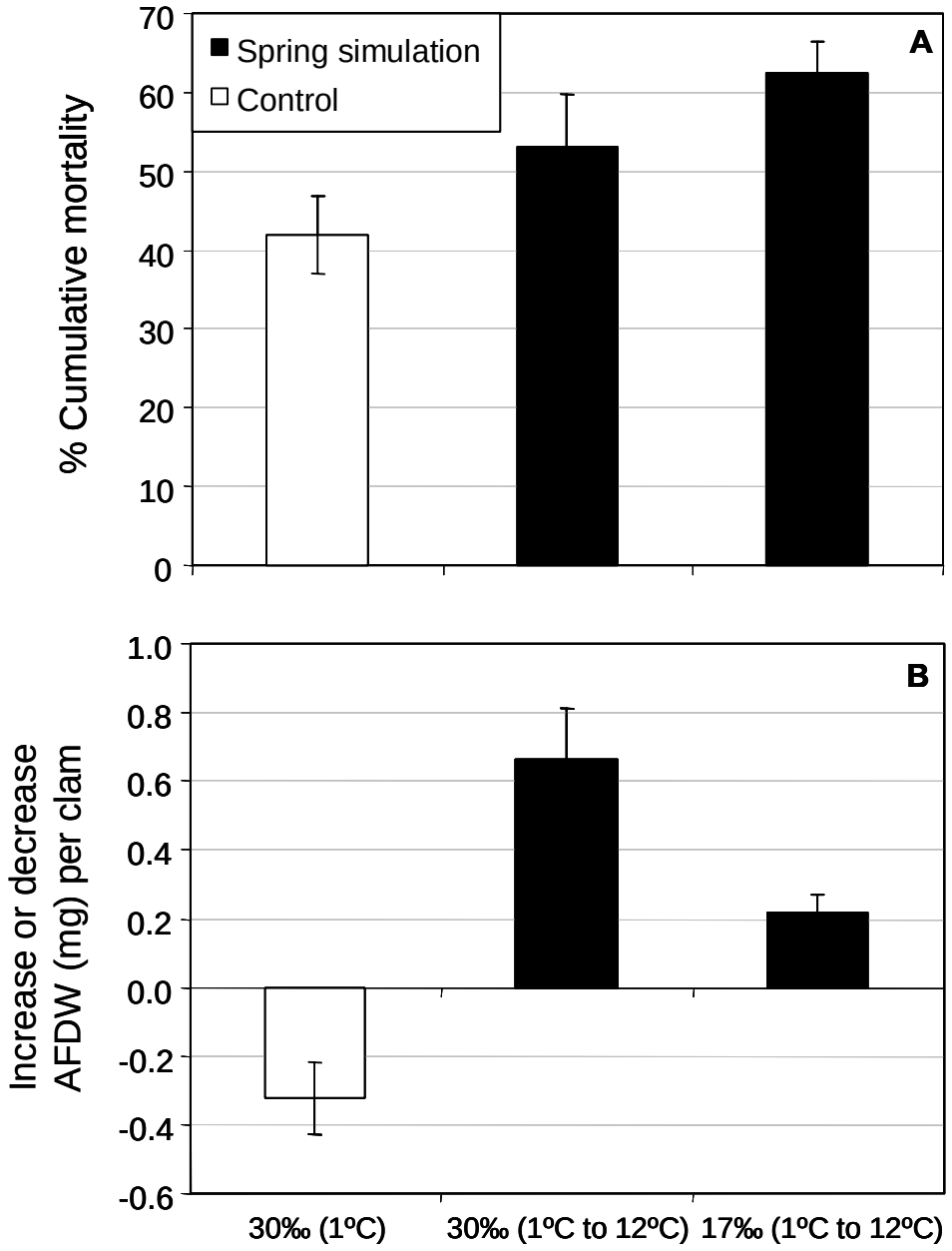

Figure 14. Percent mortality (A) and growth rate (B) (increase or decrease in ash-free dry weight) of *Mercenaria mercenaria* juveniles (ARC source; mean ± SE of 3 upwellers) subjected to a 2 wk experimental spring simulation (May 13 to May 28) following overwintering, involving: (i) temperature increase from 1 to 12ºC at $1^{o}C\ day^{-1}$ and ii) both temperature increase (1 to 12ºC at $1^{o}C\ day^{-1}$) and low salinity stress (2 wks at 17‰). These treatments were followed by a 2 wk recovery period (May 28 to June 11) at constant $12^{o}C$ and 30‰. Controls were maintained at $1^{o}C$ and 30‰ throughout. Mortalities and growth are calculated over the entire 4 wk period.

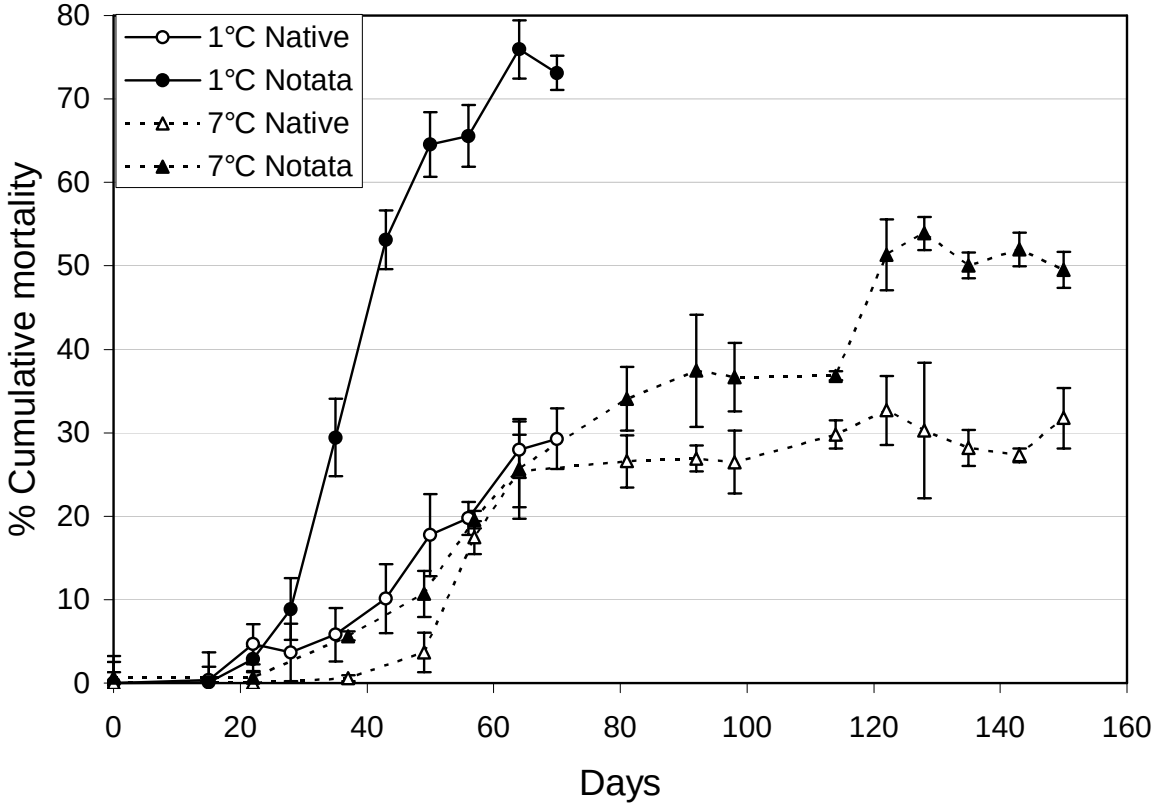

Figure 15. Cumulative mortalities of native vs. notata *Mercenaria. mercenaria* juveniles from the Ellerslie Shellfish Hatchery, PEI, at low temperatures. Note that experiments at 7 and 1°C were initiated at different times of the year (see below) and thus provide a direct comparison between clam varieties at a given temperature rather than between holding temperatures.

1°C trial: conducted between November 25, 2003 and February 3, 2004; the two clam varieties were held in separate upwellers within the same each tank (n = 2 replicate tanks; see methods); values represent means ± SE of two replicate tanks (3 subsamples per upweller from each tank). Initial mean SL: 8.8 and 8.5 mm for native and notata clams respectively.

7°C trial: holding at a 7°C was conducted between September 15, 2003 and February 4, 2004 (see Fig. 8); the two clam varieties were held in separate upwellers within one tank (see methods); values represent means ± SE of 3 subsamples from one upweller. Initial mean SL: 7.1 and 6.5 mm for native and notata respectively.

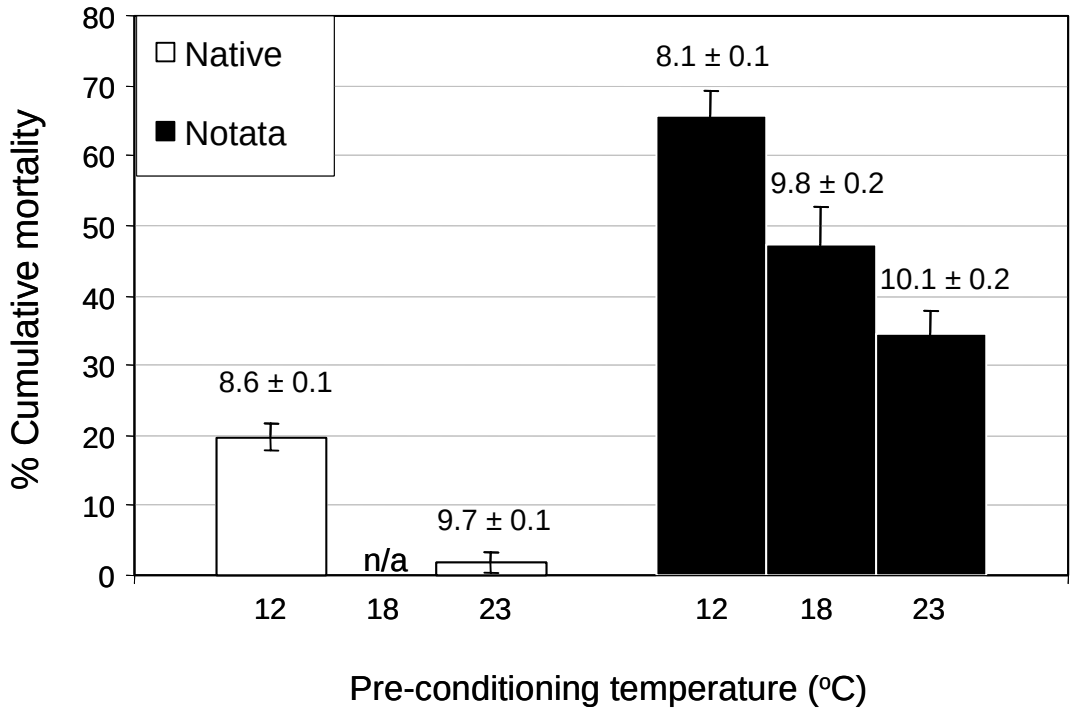

Figure 16. Summary graph showing the effect of pre-conditioning temperature (12°C, 18°C and 23°C) on cumulative mortalities of native and notata *Mercenaria mercenaria* juveniles overwintered at 1°C for 2.3 mo. in land-based nurseries at MRS. Mortality time series for clams pre-conditioned at 12°C is shown in Fig. 14. Mean SL of clams at the start of overwintering (± SE) shown above each bar. n/a: not available.

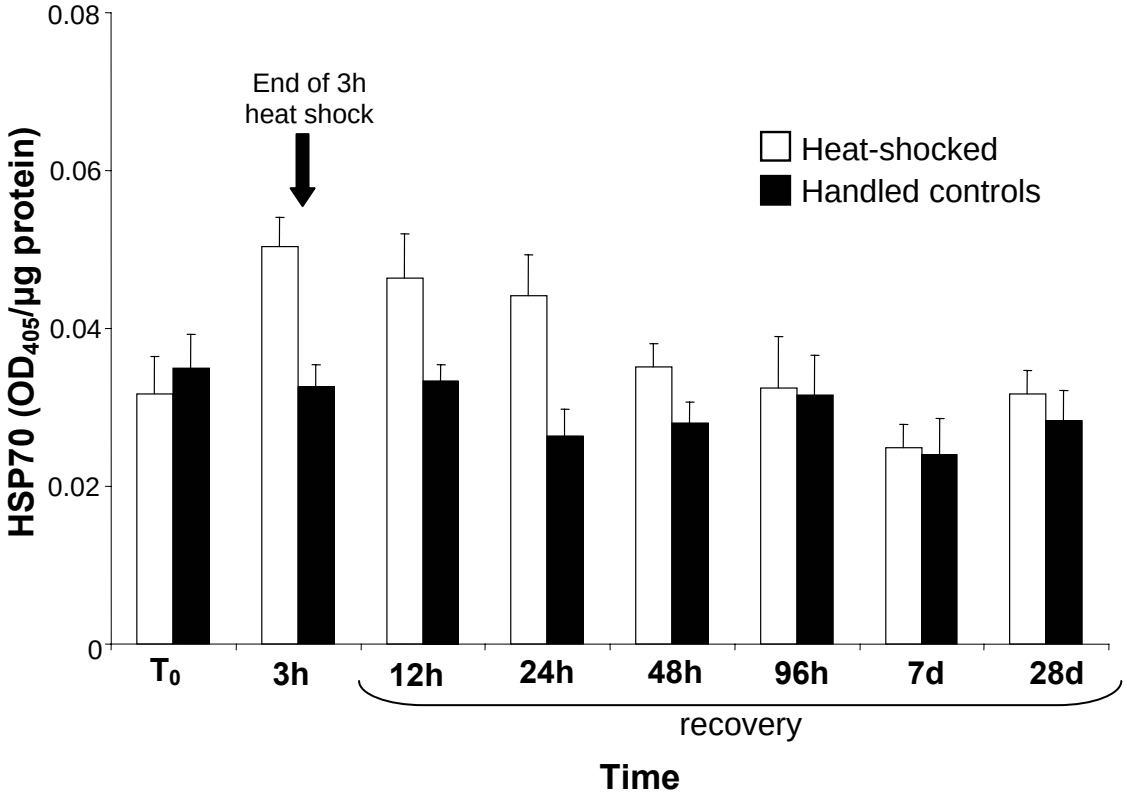

Figure 17. HSP 70 levels in juvenile quahog soft tissues from animals exposed to acute, 3 h heat shock (1^0C – 12^0C). Bars represent mean HSP70 level per µg of soluble protein ± standard error (n = 10 clams, mean shell length ± SE = 6.43 ± 0.06 mm).

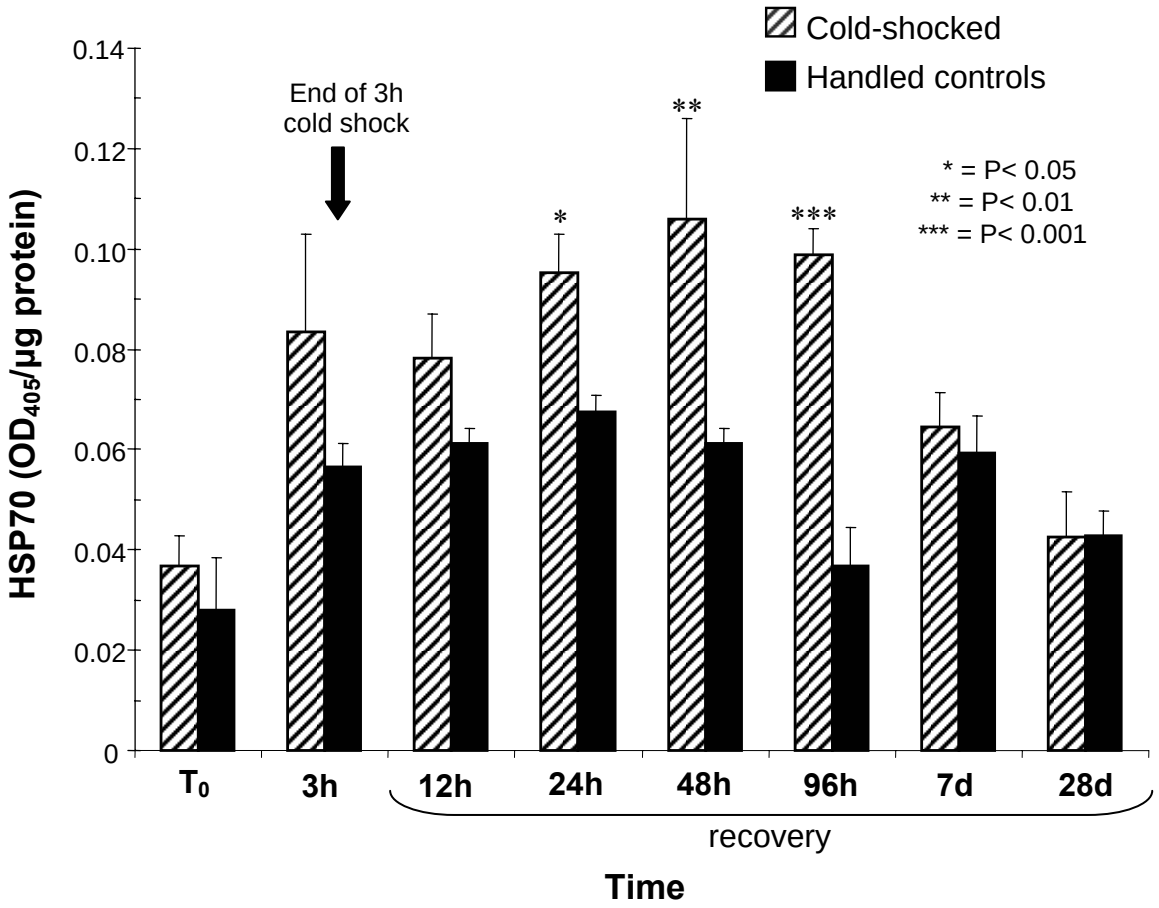

Figure 18. HSP 70 expression in juvenile quahog soft tissues from animals exposed to acute, 3 h cold shock (12^0C – 1^0C). Bars represent mean HSP70 level per µg of soluble protein ± SE (n = 10 clams, mean shell length ± SE = 6.45 ± 0.04 mm).

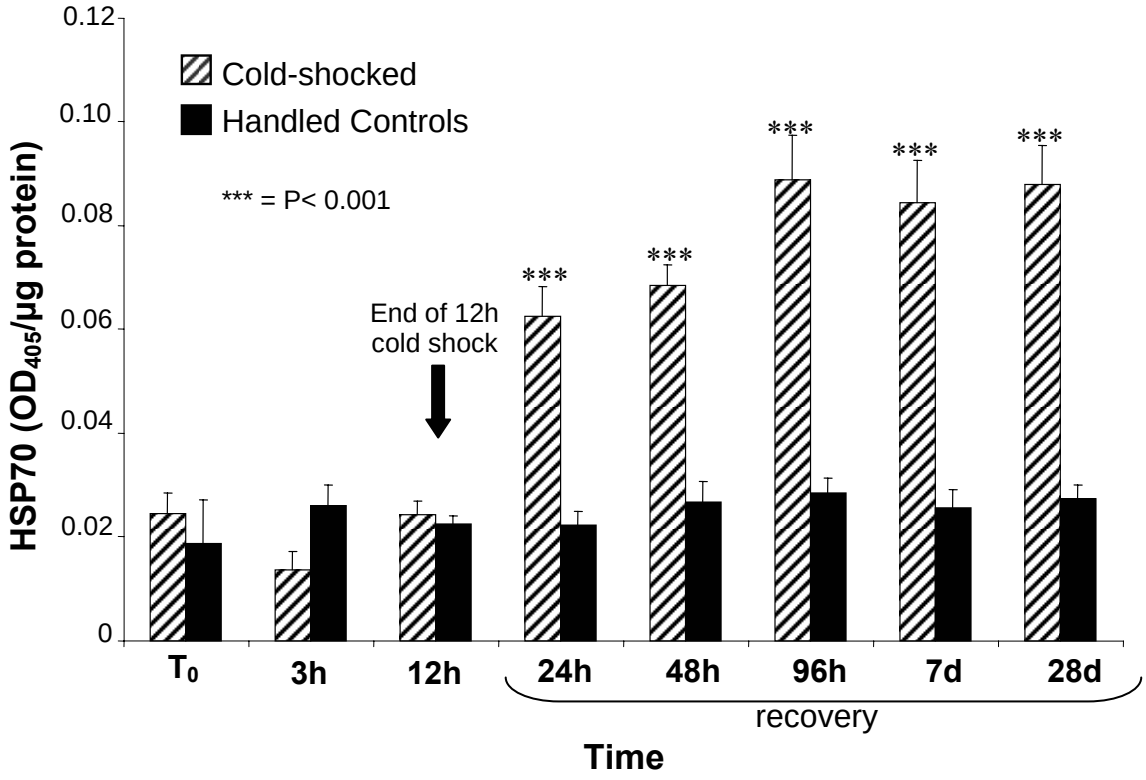

Figure 19. HSP 70 expression in juvenile quahog mantle tissue from animals exposed to acute, 12 h cold shock (12^0C – 1^0C). Bars represent mean HSP70 level per µg of soluble protein in mantle tissue ± SE (n = 5 clams, mean shell length ± SE = 10.40 ± 0.12 mm).

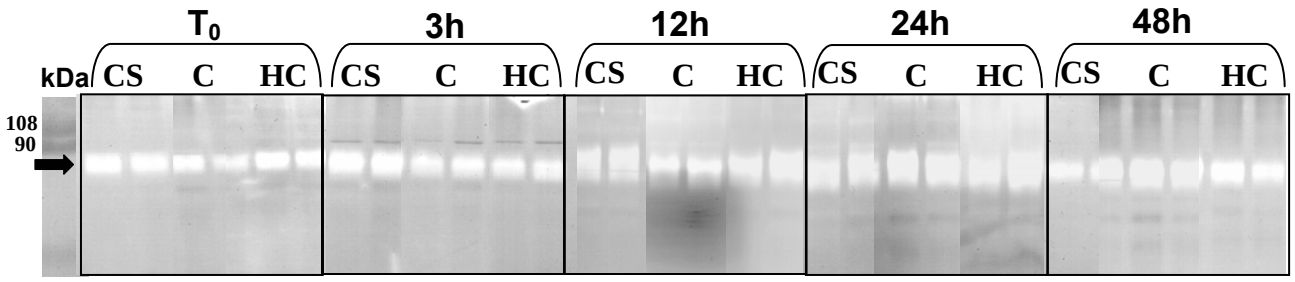

Figure 20. Protease activity in juvenile quahog mantle tissue from animals subjected to acute cold shock (20^{o}C-3^{o}C). Activity of the 70-80 kDa single band was further characterized as a metalloprotease (black arrow). The activity of this enzyme does not change over time (T_0-28 d, data from 72 h not shown) between cold-shocked (CS), control (C), and handled control (HC) groups. Zymography was conducted on all 10 animals of each treatment group; however, data for 2 animals only are presented.