Overwinter mortality and spring growth in selected and non-selected juvenile *Mercenaria mercenaria*

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ABSTRACT: Juvenile cultured northern quahogs or hard clams *Mercenaria mercenaria* can experience significant mortalities during their first winter, and cultured *M. mercenaria* var. *notata* clams, selected for fast growth, may be more susceptible. In the present study, selected and non-selected juvenile clams were overwintered off the north shore of Long Island, NY from November 2007 to June 2008 in order to understand the physiological, genetic, and environmental mechanisms contributing to overwinter mortality. Selected clams had a cumulative mortality of approximately 47 % while the non-selected clams experienced 25 % mortality. A decrease in carbohydrate reserves coincided with the mortality in both clam strains, but time-specific loss of carbohydrates was greater in the selected clams. Metabolic activity, as estimated by the activity of the electron transport system (ETS), was high during the winter-to-spring transition and was later reduced, presumably when clams became acclimated to the warmer temperature in May. Growth occurred after the observed reduction in ETS activity and was greater in the selected clams. This reduction in metabolic activity may allow for the balance of available energy to switch from maintenance to growth. These results support previous work suggesting a genetic component to overwinter mortality and demonstrate the significant effects of seasonal transitions on hard clam growth and energetics.

KEY WORDS: Hard clam · *Mercenaria mercenaria* var. *notata* · Overwinter mortality · Aquaculture · Biochemical composition · Metabolism · Growth · Energy

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INTRODUCTION

The hard clam, *Mercenaria mercenaria*, has historically been an important marine resource in the USA (Belding 1912) and has now become an important aquacultured product. The success of hard clam aquaculture and stock enhancement programs is impeded from the mid-Atlantic region north through to Atlantic Canada due to excessive mortality over the winter (Bricelj et al. 2007, Zarnoch & Schreibman 2008). Although predation can have a significant impact on juvenile hard clam populations (Ford 2001, Kraeuter 2001), overwinter mortality has also been observed in populations protected from predators. This suggests physiological and/or environmental mechanisms contribute to the mortality. This mortality was shown to be variable from year to year and from site to site (Zarnoch & Schreibman 2008). Furthermore, recent

evidence suggests that juvenile hard clams selected for fast growth may be more susceptible to overwinter mortality. Thus selection for fast growth may be associated with physiological trade-offs with other fitness-related traits (Angilletta et al. 2003, Bayne 2004).

Zarnoch & Schreibman (2008) have shown that significant mortality (up to 1 % d⁻¹) occurs in the spring following a severe winter, at a time when water temperatures are rising but food levels are low. During this period, a rapid decline in carbohydrate content is observed. The clams, which are inactive at <5°C (Loosanoff 1939), are stimulated by an increase in water temperature. The increase in metabolic activity should be supported by the ambient food supply, but if food levels are low or nutritional quality of the food is poor, the clams must rely on stored carbohydrates to maintain the increased metabolic activity. Mortality occurred when carbohydrate levels fell below 10 % of the

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tissue dry weight (DW) and has been termed 'winter-spring mortality' (Zarnoch & Schreiber 2008).

A loss of carbohydrates has also been associated with overwinter mortality in studies conducted in Atlantic Canada. In laboratory experiments, juvenile clams (6 to 8 mm shell length [SL]; obtained from an aquaculture facility in Massachusetts, USA) were held at 1°C for ~12 wk. There was a linear decrease in carbohydrate content together with significant mortality (~70%). In addition, clams held at 7°C also experienced significant mortality (~50%), suggesting that this temperature also caused physiological stress. Clams held at 12°C experienced no significant mortality (Bricelj et al. 2007). Although the relationship between mortality and carbohydrates is similar to the findings of Zarnoch & Schreiber (2008), the timing of the mortality was not associated with spring warming since the clams were held in the laboratory at different constant temperatures for several months. This suggests that an alternative environmental or physiological mechanism might be responsible for the mortality. Another intriguing finding of Bricelj et al. (2007) is that in separate trials, clams selected for fast growth and held at 1°C experienced 70% mortality versus 30% mortality for non-selected clams held under identical conditions. This was the first work to suggest a genetic component to the overwinter mortality of juvenile hard clams.

Laboratory studies by Pernet et al. (2006) demonstrated that oxygen consumption was ~33% lower in non-selected versus selected hard clams, suggesting that there may be an energetic advantage to cold-adapted clams reducing their energetic needs during overwintering. If metabolic rates were higher in selected clams in the field during spring warming when food levels were low (as in the model proposed by Zarnoch & Schreiber 2008), greater mortality would be expected in these clams due to their higher, unmet metabolic demands. This hypothesis requires testing, but measures of oxygen consumption have limitations when used in field studies. The individuals can become stressed due to handling, simulating food and sediments is problematic, and results may not be indicative of ambient field conditions (Cammen et al. 1990). An alternative methodology to estimate oxygen utilization in a way more suitable for *in situ* studies is the measurement of the activity of the electron transport system (ETS). This assay estimates total aerobic potential of an organism by measuring the activity of the rate-limiting step in O₂ utilization for ATP production. It has been used to measure potential O₂ utilization in bivalves and correlates well with direct measures of oxygen utilization (Madon et al. 1998, Fanslow et al. 2001, García-Esquivel et al. 2001).

The overwinter mortality of selected and non-selected clams was compared in a combined field and

laboratory study (Gionet et al. 2009) in eastern Canada. Mortality was negligible in both the field and laboratory from August through April, but increased markedly in May. Mortality up to ~50% was observed in selected clams, while the non-selected clams had mortality of ~10%. The authors found a lower concentration of glucose and lipids in the selected clams and suggested that the higher glucose concentration in the non-selected clams may help reduce the freezing point of the clams' fluids, thus improving survival.

In the present study, selected and non-selected juvenile clams were overwintered under *in situ* conditions off the north shore of Long Island, NY in order to: (1) determine if selected clams experience greater overwinter/winter-spring mortality than non-selected clams, (2) determine if greater metabolic activity during spring warming results in higher mortality in selected clams, and (3) use a comparative approach to better understand the physiological, genetic, and environmental mechanisms responsible for overwinter mortality in selected and non-selected clams.

MATERIALS AND METHODS

Stock populations. Experimental *Mercenaria mercenaria* populations of both strains (selected and not selected for fast growth) were produced in spring 2007 by the Cornell Cooperative Extension Shellfish Hatchery, Centerport, NY, using standard hatchery procedures (Castagna 2001). However, this hatchery does not cull small or slow-growing clams during the larval and nursery phases. Non-selected clams were produced from wild broodstock collected from the Greater Huntington-Northport Harbor Complex (Huntington, NY). The selected clams were produced from broodstock obtained from the Frank M. Flowers & Sons Shellfish Hatchery (Oyster Bay, NY). These selected broodstock are *M. mercenaria* var. *notata*, which is a naturally occurring variant with brown zigzag bands on the shell (Chanley 1961). They grow faster than wild stock. Since *notata* clams occur in very low frequencies in natural populations (Chanley 1961, Humphrey & Walker 1982), aquaculturists breed this trait into clams to distinguish cultured clams from wild clams. Summer grow-out of the juvenile clams was performed in Huntington Harbor (Huntington, NY) using a floating upwelling system. Through the entire production and grow-out process, both strains were treated identically. In November 2007, clams of a common SL (~11 mm) were chosen for the study.

Experimental design and sampling. The overwintering study was conducted from November 2007 to June 2008 at Centerport Harbor on the north shore of Long Island, NY. The clams were deployed in wooden boxes

(30 × 30 × 15 cm) with a closed bottom and an open top covered with 6.4 mm aperture plastic mesh after stocking to exclude predators and retain the clams. Each was filled with sediment from the study site that was sieved to <12 mm. Thirty clams of either the selected (10.74 ± 0.23 mm SL) or non-selected (10.68 ± 0.99 mm SL) clams were stocked in each box. A total of 100 boxes were numbered and deployed subtidally (~1 m depth at mean low water) at the site. The boxes were placed directly on the bottom and spaced ~0.5 m apart. Half of the boxes (n = 50) contained selected clams and the other half contained non-selected clams. At monthly intervals from December through March, 5 boxes containing the selected clams and 5 boxes containing the non-selected clams were sampled. From April through June the remaining boxes were sampled biweekly and either 5 or 10 boxes (June) were sampled for each clam strain.

At each sampling, all sediment from a box was sieved over a 2 mm mesh at the site. Live and dead clams (identified as 'gaping' or a 'clapper') were enumerated and percent cumulative mortality (%MO) was calculated directly for each box as: %MO = (dead clams/total clams) × 100. The boxes were treated as replicate plots and the mean %MO for each sampling date was determined from these replicates. Water temperature at the site was measured twice per day by an *in situ* temperature logger (Onset) and is shown as the mean temperature (±SE) for the periods between consecutive sampling dates. On each sampling date, water samples were collected and analyzed for size-fractionated chlorophyll *a* (chl *a*) to estimate food availability. This was determined in triplicate for each sample/size-class (<180 µm [total size fraction], 5 to 28 µm, <5 µm) and then analyzed spectrophotometrically following Parsons et al. (1984).

Growth. All live clams collected from the replicate boxes were pooled, transferred to the laboratory on ice, and immediately processed for condition index and for measuring the activity of the ETS. The SL of a random sample of 30 live clams from each strain was measured. These clams were then carefully shucked and the tissue was removed. The wet tissue weight and shell weight were determined separately. The tissue and shell samples were dried at 60°C for >48 h, then reweighed to obtain the DW. Instantaneous growth rate (*k*) was calculated for SL and tissue DW for samples collected in May and June using the equation:

$$k = (\ln x_2 - \ln x_1) / (t_2 - t_1) \quad (1)$$

where x_2 and x_1 represent SL or tissue DW at times (*t*) t_2 and t_1 (Grizzle et al. 2001). The *k* coefficient was then multiplied by 100 to express growth as % d⁻¹. A condition index was calculated as a ratio of tissue DW to SL × 1000 (Lucas & Beninger 1985). The dried tissue sam-

ples of each clam strain was pooled into 2 groups (selected and non-selected), placed in polypropylene vials, and stored at -20°C for later determination of total protein, lipid, and carbohydrate content. Additionally, 6 to 9 live clams of each strain were measured and carefully shucked. The tissue was removed, weighed, and immediately used to measure ETS activity.

ETS activity. The ETS activity for each clam strain was determined using the method of Owens & King (1975) as modified by Madon et al. (1998), where INT-tetrazolium is reduced to INT-formazan when substituted for oxygen as the terminal electron acceptor. The modifications of Madon et al. (1998) included larger volumes of samples and reagents, increased concentration of NADH, and eliminating NADPH as a substrate. All tissues and reagents were kept in an iced water bath (0 to 4°C) during the procedures. Tissue samples were placed in 3 ml of ETS-B solution (9.0 mg MgSO₄, 1.5 g polyvinylpyrrolidone [PVP], and 2.0 ml of Triton X-100 in 0.1 M phosphate buffer, pH 8.5) and homogenized for three 20 s intervals. The homogenizer was rinsed with 1.0 ml of ETS-B solution and the volume of the homogenate was recorded. From the homogenate, 2.0 ml subsamples were removed and placed in centrifuge tubes. Samples were centrifuged at 4°C for 20 min at 10 000 × *g* and then 100 µl of the supernatant was removed from the centrifuge tube and placed into a culture tube to which 2.25 ml of substrate solution (10 mg NADH and 50 µl of Triton X-100 in 0.1 M phosphate buffer), 2.25 ml of phosphate buffer, and 1.0 ml of INT solution (100 mg INT-tetrazolium chloride in double distilled water, pH 8.5) were added. These culture tubes were incubated for 20 min at the ambient temperature (±1°C) of Centerport Harbor on the sample date by placing them in a water bath or cold room. The reaction was stopped by adding 1.0 ml of a quench solution (50 % formalin, 50 % 1 M H₃PO₄). The absorbance of each sample was measured spectrophotometrically at 490 nm. The absorbance value was corrected by a turbidity blank, which consisted of 4.0 ml of ETS-B solution and 1.0 ml of quench solution. ETS activity was calculated using the following equation (García-Esquivel et al. 2001):

$$\text{ETS } \mu\text{mol O}_2 \text{ h}^{-1} \text{ g DW}^{-1} = [(E_{\text{corr}} \times V_{\text{hom}} \times 60/t \times 1 \times V_{\text{rxn}}) / (V_{\text{inc}} \times \text{DW} \times 31.8)] \quad (2)$$

where E_{corr} is the corrected sample absorbance (cm⁻¹), V_{hom} is the total homogenate volume (ml), *t* is the incubation time (min), 60 is used to express the activity per hour, V_{rxn} is the final reaction volume (ml), V_{inc} is the volume of homogenate used in the reaction assay (ml), and DW is the DW of the tissue sample in the total homogenate (g).

Biochemical analyses. Subsamples of the pooled dried tissue were used to determine the proximate biochemi-

cal composition of the selected and non-selected clams. Total carbohydrate content was determined in triplicate using the phenol-sulfuric acid method of Dubois et al. (1956). A calibration curve was constructed using glucose as a standard. Proteins were quantified by organic nitrogen analysis with a CHN elemental analyzer using acetanilide as a standard, and a nitrogen-to-protein conversion factor of 5.8 (Gnaiger & Bitterlich 1984). Total lipid content was estimated gravimetrically using the chloroform-methanol extraction of Folch et al. (1957). Results are expressed as biochemical content per unit DW and per individual clam. Since biochemical composition was determined from a pooled group of clams, the reported variance represents methodological variability, not variability among replicate boxes. Changes in carbohydrate composition were also described as changes in a carbohydrate budget (CB_x ; Smolders et al. 2004) which was calculated as:

$$CB_x = [(T_x - T_{x-1}) \times (Y_x - Y_{x-1})/2] + (T_x - T_{x-1}) \times (Y_{x-1} - Y_0) \quad (3)$$

with T_x being the number of days deployed in the boxes, Y_x being the carbohydrate composition at time x , and $x-1$ being the composition at the previous measurement. Carbohydrate proximate composition was converted to its energy equivalent using an enthalpy of combustion of 17 kJ g^{-1} and results were expressed as mJ ind.^{-1} .

Statistical analyses. A 2-way ANOVA was used to determine significant differences among sample dates and clam strains (selected and non-selected) for all measured variables. When a significant effect was found, a Tukey post hoc test was employed to deter-

mine the differences between strains for each sample date. Student's t -tests were used to compare carbohydrate energy budgets between strains on each sample date. Adequate data transformations were performed where necessary; however, all data are presented as untransformed. Data are shown as the mean $\pm$ SE. Statistical tests were conducted using SPSS version 15.

RESULTS

Overwinter mortality

Significant effects of sample date, strain type, and their interaction were observed for %MO sample $^{-1}$ (Fig. 1). Mortality increased from December through March for the selected clam strain and then did not change through April and May, and into June. In contrast, no significant differences were observed in non-selected clams through the sampling period. There were no significant differences in %MO observed between clam strains from December through February; however, there were differences between strains in all measurements from March through June. During this period the average %MO sample $^{-1}$ was approximately 47 and 25 % for selected and non-selected clams, respectively.

Environmental parameters

In fall 2007, water temperature in Centerport Harbor decreased rapidly and remained $<5^\circ\text{C}$ until the end of March. Temperature increased steadily through the

Fig. 1. *Mercenaria mercenaria*. Percent mortality sample $^{-1}$ of selected and non-selected clams held in sediment-filled boxes and sampled monthly or biweekly between November 2007 and June 2008. Each value is the mean $\pm$ SE of 5 boxes except in June when 10 boxes strain $^{-1}$ were sampled. Each box originally contained 30 clams. Results of 2-way ANOVA (sample date $\times$ strain, $D \times S$) are shown. Intra-strain means not sharing the same letters were significantly different between sampling dates as indicated by a Tukey post hoc test. The difference between the strains on each sampling date is marked as significant (*) or not significant (ns).

Fig. 2. Mean temperature ($\pm$ SE) for each sampling interval and total chl *a* concentration (mean $\pm$ SE) measured at the overwintering site in Centerport Harbor, NY

spring and was 16°C on the final sampling date (Fig. 2). Size-fractionated chl *a* showed negligible amounts of chl *a* in the $<5 \mu\text{m}$ fraction and no statistical differences (Student's *t*-test; $p > 0.05$) between the total ($<180 \mu\text{m}$) and $5\text{--}28 \mu\text{m}$ fraction. Therefore, only the data for total chl *a* are presented (Fig. 2). Mean chl *a* was $4.5 \mu\text{g l}^{-1}$ in November and December as the water temperature was falling. In January, the winter-spring bloom occurred and resulted in chl *a* levels $>100 \mu\text{g l}^{-1}$. The levels decreased through April and remained relatively stable through the remainder of the sampling period with a mean of $15.5 \mu\text{g l}^{-1}$.

Proximate biochemical composition

Protein, carbohydrate, and lipid concentrations ($\mu\text{g mg DW}^{-1}$) were significantly different among dates (ANOVA; $p < 0.001$) as were the interactions between date and strain (ANOVA; $p \leq 0.024$). No significant effect of strain was found (ANOVA; $p > 0.05$) (Figs. 3 to 5). Protein, carbohydrate, and lipid contents clam^{-1} were significantly affected by date, strain, and the interaction of date and strain (Fig. A1 in Appendix 1). Protein concentration remained constant from November through April. In May and June, protein concentration decreased (Fig. 3) and increased when expressed as clam^{-1} (Fig. A1a). There was clearly more protein clam^{-1} in selected clams compared to non-selected clams (Fig. A1a). The carbohydrate concentrations were initially 143.08 ± 8.29 and $151.63 \pm 11.81 \mu\text{g mg DW}^{-1}$ for selected and non-selected clams, respectively. Carbohydrate concentrations decreased through April, reaching values as low as 104.71 ± 13.14 and $88.25 \pm 2.34 \mu\text{g mg DW}^{-1}$ for selected and non-selected clams, respectively (Fig. 4). Carbohydrates clam^{-1} remained constant in both clam strains through the win-

Fig. 3. *Mercenaria mercenaria*. Protein content (mean $\pm$ SE) of selected and non-selected clams sampled monthly or bi-weekly between November 2007 and June 2008. Results of 2-way ANOVA (sample date $\times$ strain, $D \times S$) are shown. ns: not significant

ter months. In May and June, both carbohydrate concentration and carbohydrate clam^{-1} increased significantly in both strains, although greater values were observed in selected clams (Fig. 4, Fig. A1b). Lipid concentration varied between 61 and $86 \mu\text{g mg DW}^{-1}$ for both strains throughout the study (Fig. 5). Lipid content clam^{-1} was higher in the selected clams throughout the study and increased significantly in both strains in May and June (Fig. A1c).

The carbohydrate energy budget (Fig. 6) decreased for all samples collected from December through

Fig. 4. *Mercenaria mercenaria*. Carbohydrate content (mean $\pm$ SE) of selected and non-selected clams sampled monthly or bi-weekly between November 2007 and June 2008. Results of 2-way ANOVA (sample date $\times$ strain, $D \times S$) are shown. ns: not significant

Fig. 5. *Mercenaria mercenaria*. Lipid content (mean $\pm$ SE) of selected and non-selected clams sampled monthly or bi-weekly between November 2007 and June 2008. Results of 2-way ANOVA (sample date $\times$ strain, $D \times S$) are shown. ns: not significant

the beginning of May. A significantly (Student's *t*-test; $p < 0.05$) greater loss in the carbohydrate energy budget was observed in the selected clams from January through early April. At the end of May and into June, the carbohydrate energy budget increased for both strains, but the increase was greater in selected clams (Fig. 6).

ETS activity

A significant effect of date (ANOVA; $p < 0.0001$) was observed on the ETS activity of the selected and non-selected clams over the study period, which is expected since the assays were conducted at ambient temperatures measured in the field. There was no observed effect of strain and the interaction of date and strain (Fig. 7). ETS activity was approximately $102 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g DW}^{-1}$ in both strains in November 2007 and then decreased significantly in December as water temperature decreased (Fig. 1). ETS activity remained very low through the winter months when water temperature was $< 5^\circ\text{C}$ and then increased in April as the water temperature began to rise. ETS activity reached the highest observed values of $127.27 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g DW}^{-1}$ for selected and $151.53 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g DW}^{-1}$ for non-selected clams in early May. Following this peak, ETS activity decreased in late May and June to a mean value of $68.35 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g DW}^{-1}$ for both strains.

Growth and condition index

Analysis of measurements of SL, tissue DW, and condition index indicated a significant effect (ANOVA; $p < 0.0001$) of date, strain, and the interaction of date and

Fig. 6. *Mercenaria mercenaria*. Carbohydrate energy budget (mean $\pm$ SE) of selected and non-selected clams sampled monthly or bi-weekly between November 2007 and June 2008. *Significant difference (Student's *t*-test; $p < 0.05$) between strain types

strain (Fig. 8). In general, selected clams had higher observed mean tissue DW and condition index than non-selected clams. Mean SL was also higher in selected clams; however, there was greater variability in SL in non-selected clams as indicated by the large error bars (Fig. 8a). No significant decreases in tissue DW or condition index were observed during the winter months despite the decreases in carbohydrates (Figs. 4 & 6). SL, tissue DW, and condition index did increase significantly for both strains in late May and June along with ETS activity. Selected clams had a greater increase in condition index during May and June and also had greater SL and tissue DW growth

Fig. 7. *Mercenaria mercenaria*. Electron transport system (ETS) activity (mean $\pm$ SE) of selected and non-selected clams sampled monthly or bi-weekly between November 2007 and June 2008. Results of 2-way ANOVA (sample date $\times$ strain, $D \times S$) are shown. ns: not significant

Fig. 8. *Mercenaria mercenaria*. (a) Shell length, (b) tissue dry weight, and (c) condition index of selected and non-selected clams sampled monthly or biweekly between November 2007 and June 2008. Results of 2-way ANOVA (sample date $\times$ strain, $D \times S$) are shown

rates. Calculated instantaneous growth rates were 0.79 and 0.68 % d^{-1} on the basis of SL, and 3.25 and 2.89 % d^{-1} on the basis of tissue DW, for selected and non-selected clams, respectively.

DISCUSSION

The present field study demonstrated a significant genetic component in overwinter mortality in juvenile hard clams. During the overwintering period of 2007–2008, hard clams selected for fast growth experienced mortality of approximately 47% and non-selected clams had mortality of 25%. There was no significant difference in %MO among the sample dates in non-selected clams. In contrast, a significant increase in %MO was observed in selected clams between February and late March. This higher mortality coincided with an increase in water temperature. Zarnoch & Schreibman (2008) described a winter-spring mortality (up to 0.99 % d^{-1}) of juvenile hard clams (SL ~10 mm) when water temperature increased above 5°C and food levels, as measured by chl *a* concentration, were low. In the present study, mortality was observed through the winter in both strains, but mortality increased significantly in the selected clams from February to March. Water temperature reached 4°C during the first week of March and then fluctuated between 4 and 6°C through March and early April. Hard clams are inactive when water temperatures are <5°C (Loosanoff 1939, Ansell 1964). It is likely that the small changes in temperature during March moved the juvenile hard clams from an inactive to active physiological state resulting in physiological stress and thus additional mortality. Our results are consistent with other similar studies (Bricelj et al. 2007, Gionet et al. 2009). Gionet et al. (2009) found an increase in mortality (up to 47.2%) in selected clams (SL ~9 mm) during May when water temperature increased above 5°C in a field trial conducted in Atlantic Canada; non-selected clams (SL ~6 mm) had mortality up to 10.8%, with most of it also occurring in May. Gionet et al. (2009) and Zarnoch & Schreibman (2008) found negligible mortality occurring during the winter months and significant mortality occurring as water temperatures were increasing in spring. Gionet et al. (2009) did not report food availability at their field site so it is unclear if this had any influence on clam mortality.

The greatest mortality in the present study was not related to food availability as chl *a* concentration was >12.5 $\mu g\ l^{-1}$ from February through June. In addition the winter-spring phytoplankton bloom peaked in February when water temperatures were <5°C, clams were inactive, and significant mortality was observed. Between December and February, mean percent mortality was 21.3 and 13.7% for the selected and non-selected clams, respectively. Bricelj et al. (2007) conducted several laboratory trials aimed at understanding the causes of overwinter mortality in general and to specifically inform aquaculture efforts in Atlantic Canada. These authors held selected and non-selected

clams (SL ~6 to 8 mm) at 1, 7, and 12°C for periods up to 150 d. The greatest mortality (>70 % cumulative mortality) occurred in the 1°C treatment with selected clams. Therefore, the work of Bricelj et al. (2007) also demonstrates that mortality occurred during the winter period (1°C) and that it had a distinct genetic component.

The observed mortality during early winter in the present study as well as the mortality observed by Bricelj et al. (2007) cannot be correlated with an increase in temperature (as in Gionet et al. 2009) or lack of food (as in Zarnoch & Schreibman 2008). There was, however, a relationship between mortality and loss of carbohydrates. In the present study, a decline in carbohydrate concentration occurred through the winter period and into early spring in both clam strains. Similarly, Bricelj et al. (2007) found a significant decrease in carbohydrate concentration in selected clams when comparing the initial measurement to the final (no carbohydrate data were given for the non-selected clams). The calculated carbohydrate energy budget in the present study provides a useful perspective on the greater mortality in the selected clams. The carbohydrate energy budget better reflects physiological changes between sampling periods than the absolute value of carbohydrate reserves (Smolders et al. 2004). Decreases in the carbohydrate energy budget were observed from December through early May in both clam strains, suggesting the clams were being challenged physiologically going into the winter period. Significantly greater decreases in the carbohydrate energy budget were observed in the selected clams compared to the non-selected clams from January through early April. These decreases coincided with the significant increase in mortality observed between February and March in the selected clams. Measurements of ETS activity during this period were near zero, thus the hard clams were likely using anaerobic pathways for catabolism of the carbohydrate stores. Anaerobic metabolism has not been well studied in hard clams and a breakdown of total metabolism into aerobic and anaerobic components, which has been done in other bivalves, is lacking for *Mercenaria mercenaria* (Grizzle et al. 2001). The present study and the data presented by Bricelj et al. (2007) suggest the need for future work on anaerobic metabolism in juvenile hard clams over the winter.

The effect of genetic selection was clearly evident during spring growth in juvenile hard clams as indicated by the energetic differences between the 2 clam strains. The carbohydrate energy budget increased in both clam strains during the last 2 sampling dates in late May and June, suggesting an improvement in physiological condition. Selected clams seemed to recover more rapidly from the overwinter stress as indicated by significantly greater increases in the carbohydrate energy budget. The last 2 sample dates were also when growth was observed. Again, selected

clams showed greater increases in tissue DW, SL, and thus condition index. The greater growth in selected clams was obviously due to previous selection for rapid growth, but it is also interesting that selection apparently also resulted in a more uniform SL distribution among the cohort. The error bars were much larger for the SL of non-selected clams compared to selected clams. In addition, it was also clear that variability in the tissue DW of non-selected clams was lower even though the units were different.

Another interesting observation regarding spring growth was that none was observed until May even though food availability and water temperature were sufficient to support growth before then. Chl *a* levels were >12.5 µg l⁻¹ from February through June, which is sufficient to support growth. Zarnoch & Schreibman (2008) suggested that chl *a* values >3 µg l⁻¹ can support growth during spring warming while lesser values may be insufficient. Growth occurs in hard clams at temperatures >7°C (Ansell 1968), while temperatures between 15 and 25°C have been shown to be optimal (Grizzle et al. 2001). Water temperature reached 7°C on 1 April and the maximum temperature recorded was 18°C in June. Therefore, the question remains, why did growth not occur in April despite appropriate food and water temperature? One possible explanation is the poor nutritional quality of the phytoplankton community. As previous work has shown, hard-clam growth and reproduction can be limited by phytoplankton communities dominated by microalgae of <5 µm diameter (Weiss et al. 2007, Newell et al. 2009). However, this is unlikely to be the explanation in the present study since size-fractionated chl *a* analyses did not reveal any microalgae in the <5 µm size class. The answer may be related to metabolic acclimation as indicated by the ETS activity data.

It was hypothesized that selected clams would have higher levels of ETS activity during spring warming, which would make them more susceptible to winter-spring mortality. As expected, ETS activity closely followed changes in ambient water temperature through the winter and early spring. However, there were no significant differences between the clam strains throughout the study, suggesting that the higher overwinter mortality in the selected clam strain was not associated with higher aerobic metabolic rates as estimated by ETS activity. ETS activity was also quite low while water temperatures were <5°C, which was the period when losses of carbohydrates were observed. Thus the juvenile hard clams were likely using anaerobic pathways for maintenance. Future work comparing anaerobic metabolism of selected and non-selected juvenile clams during the overwinter period may explain the greater losses of carbohydrates in the selected clams.

Zarnoch & Schreibman (2008) suggested that winter-spring mortality occurs when metabolic activity is enhanced as clams become active during spring warming ($>5^{\circ}\text{C}$) and food levels are low ($\text{chl } a < 3 \mu\text{g l}^{-1}$). However, these authors did not measure metabolic activity. One aim in the present study was to test this hypothesis in the field. Measured ETS activity increased as water temperature increased from April through early May. It then decreased significantly in late May and remained at this level in June (Fig. 7). This decrease in ETS activity was interesting since water temperature was still increasing in early June and oxygen utilization would be expected to increase with temperature at least up to the maximum threshold where metabolic activity becomes curtailed ($\sim 27^{\circ}\text{C}$; Hamwi 1969). These last 2 samples also coincided with the period of significant spring growth. Therefore, we speculate that metabolic activity was exaggerated during the spring warming period (5 to 12°C) and then as acclimation occurred, metabolic activity may have been reduced. When metabolic activity is reduced, the balance of energetic demand then favors growth. This hypothesis corroborates and further elucidates the model of Zarnoch & Schreibman (2008) to explain winter-spring mortality. In the present study, the high food availability during spring warming, indicated by chl *a* levels $>12.5 \mu\text{g l}^{-1}$, was sufficient to support the exaggerated metabolic demands of the hard clams and thus not cause additional mortality. Accordingly, if food levels had been low ($\text{chl } a < 3 \mu\text{g l}^{-1}$), greater mortality would have been expected.

Another intriguing facet to the ETS activity data is the similarity between the measurements made during spring warming and the initial measurement made in November. Again, it is suspected that during spring warming, metabolic activity was exaggerated due to the changing temperature. ETS activity measurements in the fall and early spring were $>100 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g DW}^{-1}$ and conversely the measurements made where suspected acclimation occurred were much lower ($68 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g DW}^{-1}$). Therefore in the fall when water temperatures are decreasing, hard clams may be subject to exaggerated metabolic demands similar to what they experience during spring warming. This would then place higher energy demands on the clams, which would have to be met by ambient food supply. At the time the clams were deployed in November 2007, chl *a* concentration was approximately $3.5 \mu\text{g l}^{-1}$, which is similar to the low food levels ($<3 \mu\text{g l}^{-1}$) described as insufficient to support metabolic demand by Zarnoch & Schreibman (2008). Therefore, the fall-to-winter transition may also be physiologically stressful and leave hard clams challenged as they enter the winter. This may explain why hard clams were reducing their carbohydrate stores as early

as December. Ideally, future studies could take measurements of ETS activity in late summer or early fall to identify a change in ETS activity as temperatures decrease, which would suggest a lack of acclimation. These periods of temperature transition appear to be critical to understanding the physiological mechanisms associated with overwinter mortality of juvenile hard clams.

The present study provides further evidence for a genetic component to the overwinter mortality of juvenile hard clams. Mortality in selected clams was nearly twice that of non-selected clams. Interestingly, juvenile hard clams produced from an unselected Maine broodstock had $>99\%$ overwinter survival (Beal et al. 2009). It is unclear whether this high survival was due to the Maine clams being genetically adapted to survive overwinter conditions or if the culture methodology (Beal et al. 1995, 2009) used contributed to the high survival. Comparative studies of the performance of different hard clam stocks (e.g. geographic, selected, non-selected, etc.) would be useful.

Mortality in the present study was associated with a decrease in carbohydrate reserves as also observed by Zarnoch & Schreibman (2008) and Bricelj et al. (2007). In addition, the loss of reserves was greater in selected clams. Following the definition of physiological trade-offs by Angilletta et al. (2003), the present study may suggest a link between increased growth (via selection) and overwinter survival, which affects the fitness of genotypes and prevents these traits from evolving independently. We speculate that the greater loss of carbohydrate reserves in the selected clams during the overwinter period may be a physiological trade-off for increased growth rate. In order to fully explore this hypothesis, future work might examine the physiological processes responsible for differences in growth rate and couple it with an understanding of the genetic component of growth.

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Appendix 1

Fig. A1. *Mercenaria mercenaria*. (a) Protein, (b) carbohydrate, and (c) lipid content (mean $\pm$ SE) shown as mg clam⁻¹ of selected and non-selected clams sampled monthly or biweekly between November 2007 and June 2008. Results of 2-way ANOVA (sample date $\times$ strain, D $\times$ S) are shown