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# Lipid remodeling in wild and selectively bred hard clams at low temperatures in relation to genetic and physiological parameters

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## Summary

**A temperature decrease usually induces an ordering effect in membrane phospholipids, which can lead to membrane dysfunction. Poikilotherms inhabiting eurythermal environments typically counteract this temperature effect by remodeling membrane lipids as stipulated in the homeoviscous adaptation theory (HVA). Hard clams, *Mercenaria mercenaria*, can suffer high overwintering mortalities in the Gulf of St Lawrence, Canada. The selectively bred *M. mercenaria* var. *notata* can have higher overwintering mortalities than the wild species, thus suggesting that the two varieties have different degrees of adaptation to low temperatures. The objective of this study was to investigate the changes in lipid composition of soft tissues in wild and selected hard clams in relation to their metabolic and genetic characteristics. Clams were placed at the northern limit of their distribution from August 2003 to May 2004; they were exposed to a gradual temperature decrease and then maintained at <0°C for 3.5 months. This study is the first to report a major remodeling of lipids in this species as predicted by HVA; this remodeling involved a sequential response of the phospholipid to sterol ratio as well as in levels of 22:6n-3 and non-methylene interrupted dienoic fatty acids. Hard clams showed an increase in 20:5n-3 as temperature decreased, but this was not maintained during overwintering, which suggests that 20:5n-3 may have been used for eicosanoid biosynthesis as a stress response to environmental conditions. Selectively bred hard clams were characterized by a higher metabolic demand and a deviation from Hardy–Weinberg equilibrium at several genetic loci due to a deficit in heterozygote frequency compared with wild clams, which is believed to impose additional stress and render these animals more vulnerable to overwintering mortality. Finally, an intriguing finding is that the lower metabolic requirements of wild animals coincide with a lower unsaturation index of their lipids, as predicted by Hulbert's theory of membranes as pacemakers of metabolism.**

Key words: lipid composition, sterol, fatty acid, homeoviscous adaptation, bivalve, winter acclimatization.

## Introduction

Membrane-based processes are generally highly sensitive to changes in temperature. This sensitivity is due to the strong effect of temperature on the physical properties of membrane lipids, which in turn have a strong influence on the proteins they surround (Hochachka and Somero, 2002). A decrease in temperature usually induces an increase in the overall packing order of membrane phospholipids (i.e. a decrease in membrane fluidity) and elevated temperatures decrease membrane order. Large changes in membrane order can lead to membrane dysfunction. Poikilotherms inhabiting eurythermal environments usually counteract this temperature effect by remodeling membrane lipids, a process known as homeoviscous adaptation (Sinensky, 1974; Hazel, 1995). Remodeling membrane lipids during thermal acclimation or acclimatization commonly involves changes in phospholipid headgroups, acyl-chain composition and cholesterol content (Hazel and Williams, 1990). These biochemical responses render the membrane more or less fluid, which is believed to be a compensation for the direct effect of temperature change on membrane structure. Many intertidal organisms commonly withstand variations in temperature of 20–30°C on a daily basis and encounter even wider thermal ranges on a seasonal basis. Several studies have shown that marine bivalves are able to regulate their membrane fluidity in response to temperature changes. For example, the mussel *Mytilus californianus* exhibits strong seasonal variations in membrane fluidity that are consistent with homeoviscous adaptation (Williams and

Somero, 1996). A recent study showed that membrane fluidity in the gills of the sea scallop *Placopecten magellanicus* is positively correlated with the level of 20:5n-3 and negatively correlated with acclimation temperature, which may represent a mechanism to maintain membrane function at low temperatures in this species (Hall et al., 2002).

Unexplained overwintering losses of juvenile first-year hard clams *Mercenaria mercenaria* in different years and at different grow-out sites have been reported in Atlantic Canada (T. Landry, personal communications). It has also been reported that the selectively bred hard clam *M. mercenaria* var. *notata*, a variant of the native hard clam distinguished by brown bands on the shell surface, which usually shows improved growth performance (Chanley, 1961), suffer higher overwintering mortalities than the wild species (Bricelj et al., 2006). This evidence indicates that the two varieties of hard clams might show different degrees of adaptation to low temperatures. However, little is known about the physiological and biochemical responses of this species to low temperatures, especially in juveniles, as most prior studies have focused on the physiology of adults.

The hard clam is an infaunal suspension-feeding bivalve occurring in shallow and turbid estuarine environments and is widely distributed along the east coast of North America from the baie des Chaleurs (Gulf of St Lawrence, Canada) to the Florida Keys. In Canada, *M. mercenaria* is mainly found from the baie des Chaleurs in New Brunswick to Cape Breton Island in Nova Scotia at both the intertidal and subtidal depths. This eurythermal species can tolerate temperature between 0 and 30°C with optimum growth at 20°C (Grizzle et al., 2001). Because of the difficulties in producing fast-growing hard clams using native Canadian stocks, the PEI Department of Fisheries, Aquaculture and Environment set up trials to determine if *M. mercenaria* var. *notata* could be utilized successfully in eastern Canada. Commercial hatcheries in the US used the variety *notata* in a selective breeding program, apparently with no strict application of quantitative genetics (Hadley et al., 1991), to distinguish it from wild stock and to develop a more rapidly growing animal (Chanley, 1961). In 1997, a sample of 60 adult hard clams from the Aquaculture Research Corporation in Dennis, Massachusetts, USA was imported to the Ellerslie Shellfish Hatchery in PEI. The $F_1$ generation was placed at six grow-out sites to assess the performance of the variety *notata* in the field for 5 years and was then recovered to produce the $F_2$ in 2003.

In our study, wild ($F_1$) and selectively bred hard clam juveniles from the Ellerslie hatchery were placed in the field at their northern distribution limit (Neguac, NB, Canada) from August 2003 to May 2004. Over the course of this study, animals were exposed to a gradual lowering in temperature from ~24 to 0°C over 4 months and maintained at overwintering temperatures <0°C for 3.5 months; they were regularly sampled for lipid analysis. Our present investigation focuses on the changes in lipid class and fatty acid composition in wild and selectively bred hard clams in relation to their metabolic requirements and genetic characteristics as evaluated by the degree of heterozygosity. Several studies have shown negative correlations between heterozygote deficiency and survival in marine bivalves exposed to stressful environmental conditions, and this relationship was attributed to higher metabolic requirements for homozygous individuals. For example, mussel stocks susceptible to summer mortality had a lower degree of heterozygosity and showed higher oxygen consumption rates than mussels from resistant stocks, thus suggesting that the energy expenditure for maintenance was higher for mussels from susceptible stocks (Tremblay et al., 1998; Myrand et al., 2002). These results are in agreement with several other studies on molluscs that indicated that more heterozygous individuals show more efficient protein synthesis and a higher scope for growth compared to more homozygous individuals (Hawkins et al., 1989), which results in higher growth and survival rates (Zouros and Foltz, 1987; Koehn, 1991; Mitton, 1993). There are similarities between our study and previous work done on mussels: there is high seasonal mortality, populations are characterized by a heterozygote deficit, and relationships exist between the genetic make-up and physiology. The novelty of our study is that we examined a different species in which the mortality occurs in winter, and the physiological character that we relate to the genetic difference is lipid biochemistry.

## Materials and methods

### *Animals*

Hatchery-reared clams *Mercenaria mercenaria* L. ($F_1$) and *M. mercenaria* var. *notata* ($F_2$) were obtained from Ellerslie, Prince Edward Island (Canada). Clams were air-shipped to the Centre Aquicole Marin de Grande-Rivière (CAMGR, Quebec, Canada) for genetic and metabolic analyses in June 2003, and to the Coastal Zone Research Institute (CZRI, Shippagan, New Brunswick, Canada) for the field experiment in July, where they were regularly sampled for lipid analysis. Upon arrival at CAMGR, 200 clams of each variety (mean shell length 9.4±1.2 mm) were numbered and acclimated to laboratory conditions for 21 days prior to measuring individual oxygen consumption rates. Clams were kept in four 40-l aquaria with light aeration. The salinity was 30, the natural photoperiod was followed and the temperature was maintained at 15°C. Clams were fed a mixed suspension of *Chaetoceros muelleri* and *Isochrysis galbana* three times per day ($10\times10^3$ cell $ml^{-1}$, 50:50 by cell number). Microalgal stocks were obtained from the Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME, USA). Algae were batch-cultured in 20 l carboys as described elsewhere (Pernet et al., 2003).

Clams of each variety (mean shell length 11.0±1.2 mm) were transferred on 15 July 2003 to Neguac, NB, in the Gulf of St Lawrence (47°N; 65°W), the northern distribution limit of this species. Seed were set at two tidal locations: individuals close to the littoral zone (i.e. subtidal site) were submerged during low tide, but individuals near the upper end of the distribution (i.e. intertidal site) were exposed for 6–8 h between high tides. Clams were held under predator-proof mesh covers mounted

on 1.8×1.2×0.3 m steel tables at an initial stocking density of ~6000 clams $m^{-2}$ (Kraeuter et al., 1998). Tables were filled with sediment that had been sieved to remove predators and gravel. One table was used per combination of tidal location and variety. To achieve replication, 72 subsections within each table were delimited and randomly chosen for sampling. On 30 October, before winter ice set in, a group of clams was overwintered subtidally in the field while another group was transferred to the laboratory at CZRI until 16 May 2004 to allow sampling during winter. In the laboratory, animals were held in a 630 l tank continuously supplied with 12 μm sand-filtered seawater. Clams were fed 10 cells $\mu l^{-1}$ Instant Microalgae Shellfish Diet 1800® (Reed mariculture, Campbell, CA, USA). Animals were not fed between 17 December and 28 April, when seawater temperature was <0°C.

### *Variety characterization*

#### *Genetic*

Juvenile hard clams were stored at –80°C at CAMGR for later electrophoretic study of enzymes as described (Tremblay et al., 1998). A small piece of muscle was homogenized in an approximately equal volume of homogenization buffer (0.2 mol $l^{-1}$ Tris-HCl, pH 8.0, with 30% sucrose, 1% polyvinyl–polypyrrolidone, 0.1% NAD, 5 mmol $l^{-1}$ dithiothreitol and 1 mmol $l^{-1}$ phenylmethylsulfonyl fluoride), centrifuged at 15 000 ***g*** for 30 min at 4°C, and the supernatant applied to a horizontal cellulose acetate gel (Hebert and Beaton, 1989). Eight polymorphic allozymes were analyzed: glucose phosphate isomerase (*GPI**, EC 5.3.1.9), phosphoglucomutase (*PGM**, EC 5.4.2.2), mannose phosphate isomerase (*MPI**, EC 5.3.1.8), pyruvate kinase (*PK**, EC 2.7.1.40), alkaline phosphatase (*ALP**, EC 3.1.3.1), fumarate hydratase (*FUM**, EC 4.2.1.2), isocitrate dehydrogenase (*IDH**, EC 1.1.1.42) and leucine aminopeptidase (*LAP**, EC 3.4.11.1). Alleles were named A, B, etc., in order of electrophoretic mobility, such that the slowest allele was called A. For each variety, 144 clams were individually analyzed.

#### *Metabolism*

After 21 days of acclimation to laboratory conditions, maximal oxygen consumption ($\dot{V}_{O2max}$ or maximum metabolic rate) was approximated by feeding the clams at a maximal rate for 4 days by increasing the food ration to $15\times10^3$ cells $ml^{-1}$ while minimum oxygen consumption ($\dot{V}_{O2min}$ or standard metabolic rate) was measured after starving the same clams for 14 days. Pseudofaeces were observed during measurement of $\dot{V}_{O2max}$. Oxygen uptake was measured for 50 animals of each variety in individual 50 ml chambers. Clams were kept individually in their metabolic chambers for 60 min before starting measurement. Empty shells were used as a procedure blank. Six chambers were used simultaneously, which allowed us to measure five clams and one blank (empty shell) at a time. Animals that remained closed in the chamber were excluded from physiological analysis. Oxygen consumption for an individual clam was determined by sealing the chamber and measuring the reduction in $\%O_2$ with a YSI (5331) polarographic analyzer and electrode (Yellow Springs, OH, USA). Seawater was well-mixed with a magnetic stirrer. The output signal was monitored continuously on a chart recorder until a decrease of at least 20% $O_2$ was reached. After oxygen uptake, each set of clams was frozen at –80°C for later determination of dry mass, which was measured after drying at 70°C for 72 h. Respiration rate was expressed as the rate expected for a standard clam with a dry mass of 1 g by applying the allometric correction ($ml^{-1}$ $g^{-1}$ dry mass $h^{-1}$) described by Widdows and Jonhson (Widdows and Jonhson, 1988).

### *Temperature measurements*

Temperature was recorded every hour with 8-bit Minilog-TR data loggers from Vemco (Shad Bay, Nova Scotia, Canada) at two tidal locations from 11 August until 30 October 2003. The data loggers were anchored to the sand tables and placed on the sand. Subtidal temperature monitoring continued in the field until 16 May 2004. We also monitored water temperatures in the laboratory holding tank.

### *Lipid analysis*

#### *Sampling*

Sampling took place in the field from 15 August until 6 October and then in the laboratory until 16 May. Clams were sampled simultaneously in the field and in the laboratory on 16 May to ensure that there was agreement between results from the field and the laboratory. Every 1–2 months, clams were removed from five randomly chosen subsections per table for determination of shell length, whole tissue dry mass, and lipid composition of soft tissues. Clams were carefully dissected and ~300 mg wet mass of tissue, corresponding to a pool of 5–10 individuals per subsection, was frozen at –80°C for later determination of dry mass and lipid composition.

#### *Lipid classes*

Lipids were extracted following the method of Folch et al. (Folch et al., 1957), spotted onto S-III Chromarods (Iatron Laboratories Inc., Tokyo, Japan), and separated into aliphatic hydrocarbons, sterol and wax esters, ketones, triacylglycerols, free fatty acids, free fatty alcohol, free sterols, diacylglycerols, acetone mobile polar lipids and phospholipids (Parrish, 1999). Chromarods were scanned by a flame ionization detection system (FID; Iatroscan Mark-VI, Iatron Laboratories Inc., Tokyo, Japan) and chromatograms were analyzed using integration software (Peak Simple version 3.2, SRI Inc.).

#### *Fatty acids*

Fatty acid methyl esters (FAME) were prepared using 12% $BF_3$ in $CH_3OH$ following the American Oil Chemists' Society method (AOCS, 1989). FAME were run on an SRI 8610C gas chromatograph equipped with a DB-Wax fused-silica capillary column (30 m×0.25 mm ID ×0.25 μm film thickness; Supelco, Bellfonte, PA, USA). Hydrogen was used as the carrier gas (flow velocity: 80 cm $s^{-1}$ at 145°C). FAME were introduced into a glass liner (uniliner, drilled, 4 mm, 6.3×78.5 mm; Restek, Bellfonte, PA, USA) maintained at

300°C. The temperature ramp was 58°C for 4 min at 40 psi (275.79 kPa), followed by an increase of 20°C $min^{-1}$ to 170°C at 20 psi (137.895 kPa), followed by an increase of 1°C $min^{-1}$ to 180°C, and finally by an increase of 2°C $min^{-1}$ to 220°C, where it was held for 5 min. The detector was maintained at 260°C. FAME were identified by comparison of retention times with known standards (37 component FAME Mix, PUFA-3, BAME and menhaden oil; Supelco Bellefonte, PA, USA) and quantified with tricosanoic acid (23:0) as an internal standard. The non-methylene interrupted dienoic fatty acids (22:2 NMI) were clearly identified by the comparison of our chromatograms with those from Kraffe De Laubarede (Kraffe De Laubarede, 2003) for the same species. Chromatograms were analyzed using integration software (Peak Simple version 3.2, SRI Inc).

### *Statistical analyses*

Allelic and genotypic frequencies for the polymorphic loci were obtained using GENETIX 4.05 (Belkhir et al., 1998). The fixation index (*F*is), which represents the Mendelian equilibrium and deviation from Hardy–Weinberg equilibrium, was calculated using GENEPOP 3.3 (Raymond and Rousset, 1995). Index of genetic differentiation, *F*st, was calculated using methods of Weir and Cockerham (Weir and Cockerham, 1984) and significant differences between allelic frequencies were tested using the Fisher exact test; both of these tests are implemented in GENEPOP 3.3. Significance levels for statistical tests were adjusted according to the sequential Bonferroni procedure (Rice, 1989).

A repeated-measures analysis of variance (ANOVA) was conducted to determine differences in oxygen consumption between the two varieties of hard clam, *M. mercenaria* and the selectively bred *M. mercenaria* var. *notata*, under two feeding regimes (high and starvation) in the laboratory. Since maximal oxygen consumption was approximated by feeding the clams at a maximal rate for 4 days and minimum oxygen consumption was measured after starving the same individuals for 14 days, the two measurements of oxygen consumption constituted a repeated measures factor.

Three-way ANOVAs were conducted to determine differences in the phospholipid to sterol ratio and the unsaturation index (average number of double bonds per acyl chain) as a function of time, tidal location and variety. A three-way multiple analysis of variance (MANOVA) was carried out on major polyunsaturated fatty acids (PUFA), namely 22:6n-3, 20:5n-3 and 22:2 NMI, as a function of time, tidal location and variety.

Owing to logistical limitations, only one table was used per combination of tidal location × variety, which means that the experiment was not replicated and the interaction between the two experimental factors cannot be estimated. Therefore, the unit of replication used in these analyses was the subsection of the table instead of the table itself. In theory, our approach violated a fundamental assumption of the ANOVA, which is that each measurement on each table is independent of every other measurement. However, the independence of data among samples is a biological issue: biological (behavioral and ecological) processes cause non-independence among replicates [(Underwood, 1997), p. 179]. Here we supposed that the biochemical response of clams to each other when on the table would have no serious outcome for statistical tests on data derived from animals in groups. Indeed, a previous study clearly showed that there was no effect of clam density on growth or survival of clams maintained at the initial stocking density used in our study [6000 clams $m^{-2}$ (Kraeuter et al., 1998)]. Finally, another limitation of our approach is that it did not allow us to unambiguously separate out the interaction of location × variety from among-table differences.

Where differences were detected, Tukey's HSD multiple comparison tests were used to determine which means were significantly different. Residuals were screened for normality using the expected normal probability plot and further tested using Shapiro–Wilk. Residuals never showed significant deviation from normality for any variable. Homogeneity of variance–covariance matrices were graphically assessed and further tested using the Levene test for each variable. Analyses were carried out using SPSS 10.0 (SPSS Inc., Chicago, IL, USA).

## Results

### *Variety characterization*

Of the eight loci scored, wild *Mercenaria mercenaria* showed only three polymorphic loci (with a dominant allele frequency <0.8) compared with five polymorphic loci in the selectively bred *M. mercenaria* var. *notata* (Table 1). The wild variety of *Mercenaria* showed significant deviation from Hardy–Weinberg equilibrium as measured with *F*is for only two loci (*ALP** and *FUM**), whereas the selectively bred hard clam showed a significant heterozygote deficiency for *MPI**, *ALP**, *FUM**, *IDH** and *LAP**. The overall *F*is values for both strains indicate significant deviations from Hardy–Weinberg equilibrium. However, the Fisher's $\chi^2$ test was markedly higher for *M. mercenaria* var. *notata* (376.4) compared with the wild variety, where the Fisher's $\chi^2$ test was 44.7. Furthermore, it must be kept in mind that the *F*is value of *ALP** in the wild variety included a dominant allele frequency of 0.96, which biases the statistical test because of the predominant influence of rare alleles (Hummel et al., 2001). The two tested varieties of hard clams showed significant differences in their allelic frequencies for *GPI**, *PGM**, *MPI** and *ALP** (Fisher exact test for each of these four loci: $\chi^2=\infty$; d.f.=16; $P<0.001$). The overall *F*st value between the two varieties of clam was 0.032 ($P<0.001$), which indicates a high level of genetic differentiation between them.

The oxygen uptake of the selectively bred *M. mercenaria* var. *notata* showed increases of 22 and 33% in their mass specific rates compared with those of the wild type after feeding to satiety and after food deprivation, respectively (Fig. 1, variety effect: $P=0.024$, $F=5.33$, d.f.=1; feeding regime effect: $F=171.41$, d.f.=1, $P<0.001$). There was no interaction between clam variety and feeding regime ($F=1.16$, d.f.=1, $P=0.286$).

Table 1. *Allele frequencies at eight enzyme loci of wild M. mercenaria and selectively bred M. mercenaria var. notata*

| | *GPI** | *PGM** | *MPI** | *PK** | *ALP** | *FUM** | *IDH** | *LAP** | All loci |
|---|---|---|---|---|---|---|---|---|---|
| *Mercenaria mercenaria* (wild) | | | | | | | | | |
| A | 0.00 | 0.02 | 0.15 | 0.13 | 0.04 | 0.78 | 0.20 | 0.23 | |
| B | 1.00 | 0.98 | 0.83 | 0.87 | 0.96 | 0.21 | 0.40 | 0.46 | |
| C | 0.00 | 0.00 | 0.02 | 0.00 | 0.00 | 0.01 | 0.40 | 0.32 | |
| $H_o$ | – | 0.03 | 0.24 | 0.21 | 0.27 | 0.31 | 0.62 | 0.58 | 0.25 |
| $H_e$ | – | 0.03 | 0.28 | 0.23 | 0.67 | 0.35 | 0.64 | 0.64 | 0.28 |
| *F*is | – | 0.01 | 0.17 | 0.08 | **0.59** | **0.10** | 0.03 | 0.10 | **0.10** |
| *N* | 144 | 144 | 144 | 144 | 144 | 144 | 144 | 144 | 144 |
| *M. mercenaria* var. *notata* (selected) | | | | | | | | | |
| A | 0.07 | 0.14 | 0.19 | 0.14 | 0.21 | 0.69 | 0.24 | 0.29 | |
| B | 0.93 | 0.86 | 0.64 | 0.86 | 0.79 | 0.30 | 0.37 | 0.44 | |
| C | 0.00 | 0.00 | 0.17 | 0.00 | 0.00 | 0.01 | 0.39 | 0.27 | |
| $H_o$ | 0.19 | 0.26 | 0.25 | 0.22 | 0.06 | 0.06 | 0.42 | 0.44 | 0.22 |
| $H_e$ | 0.22 | 0.24 | 0.53 | 0.23 | 0.32 | 0.46 | 0.65 | 0.65 | 0.39 |
| *F*is | 0.04 | –0.09 | **0.53** | 0.09 | **0.83** | **0.86** | **0.36** | **0.33** | **0.44** |
| *N* | 143 | 144 | 144 | 144 | 144 | 144 | 144 | 144 | 144 |

$H_o$, the observed proportion of heterozygotes per locus; $H_e$, Hardy–Weinberg proportions of heterozygotes; *F*is, deviation from Hardy–Weinberg equilibrium; and *N*, sample size. Loci that show significant deviation from Hardy–Weinberg equilibrium once corrected with a sequential Bonferroni procedure are in bold.

Therefore, selectively bred hard clams were characterized by a higher heterozygote deficiency and a higher $\dot{V}_{O_2}$ than the wild *M. mercenaria*.

### *Temperature measurements*

Seawater temperature varied between –1°C and 24°C over the study period, thus covering the entire range of the hard clam's thermal tolerance. Temperature profiles recorded in the field and in the laboratory showed three distinct periods (Fig. 2A). The first period, from 11 August to 15 December, was characterized by a marked decrease in temperature from a maximum of 23.7°C to 0°C, corresponding to a reduction of ~0.2°C day$^{-1}$. The overwintering period, from 15 December to 5 April, was characterized by stable temperatures of –0.1±0.3°C in the laboratory and –1.6±0.2°C in the field. Finally, the third period, from 5 April until the end of the study, was characterized by a marked temperature increase, up to 8–9°C, which is typical of spring. Averages and standard deviations in daily temperatures were similar between intertidal and subtidal locations except at the end of September, when intertidal temperatures seemed somewhat more variable than those recorded at the subtidal location (Fig. 2B).

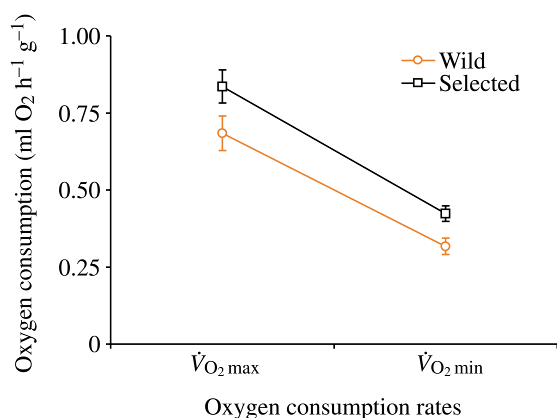


Fig. 1. Minimum and maximum oxygen consumption rates (mean ± s.e.m.) for two varieties of juvenile hard clams: wild *Mercenaria mercenaria* (*N*=31) and selectively bred *M. mercenaria* var. *notata* (*N*=42).

### *Lipid analysis*

#### *Lipid classes*

Lipid classes detected in juvenile hard clams in this study were sterols, acetone-mobile polar lipids and phospholipids. Minor amounts of triglycerides (⩽0.5%) were occasionally detected and are not discussed further. Acetone-mobile polar lipids, a lipid class that 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). The phospholipid to sterol ratio, generally considered as an indicator of membrane fluidity, varied as a function of time, tidal location and variety (Fig. 3). Indeed, wild *M. mercenaria* held at the intertidal location were characterized by a 1.4-fold increase in their phospholipid to sterol ratio between Augusts and October, immediately followed by a rapid decrease to initial values in December before overwintering. *M. mercenaria* held at the subtidal location showed a similar pattern of variation but exhibited a rate 2.6 times higher than their intertidal counterparts (respectively 0.049 and 0.131 d$^{-1}$). Interestingly, the selectively bred *M. mercenaria* var. *notata* was characterized by a phospholipid to sterol ratio that was stable with time, irrespective of tidal location.

*Fatty acids*

Major fatty acids separated from the total lipid extracts of clams were, in order of abundance, 22:6n-3 (18.8%), 16:4n-3 (10.7%), 16:0 (10.6%), 22:2 NMI (8.5%), 20:5n-3 (8.0%), 18:0 (4.8%), 20:4n-6 (4.5%) and 16:1n-9 (3.8%), which together accounted for ~70% of the total fatty acids (Table 2).

The unsaturation index, which is the number of double bonds per 100 molecules of fatty acids, increased significantly, by 7.2% between October and December before overwintering, and remained elevated until the end of the study period. This increase in the unsaturation index coincided with a reduction in the seawater temperature in the field from 13.7°C to 0°C. The unsaturation index also varied as a function of tidal location (intertidal>subtidal) and variety of hard clam (*M. mercenaria* var. *notata*>*M. mercenaria*; Fig. 4). A stepwise multiple regression model using groups of fatty acids as explanatory variables and the unsaturation index as the response variable showed that the unsaturation index was positively correlated with PUFA ($y$=5.9×PUFA–83.5; $r^2$=0.928, $N$=171, $F$=2169.9, d.f.=1, $P$<0.001; Fig. 4). A second regression model using individual PUFA as explanatory variables showed that variations in the unsaturation index were attributable to 22:6n-3 ($y$=4.18×22:6n-3+228.7; $r^2$=0.892, $N$=171, $F$=17485.9, d.f.=1, $P$<0.001; Fig. 4). Indeed, 22:6n-3 increased significantly by 29.9 and 34.6% in *M. mercenaria* var. *notata* and *M. mercenaria,* respectively, between October and December before overwintering, and remained elevated until the end of the study period, as observed for the unsaturation index. The selectively bred *M. mercenaria* var. *notata* showed a 2.7% increase in 22:6n-3 compared to the wild variety.

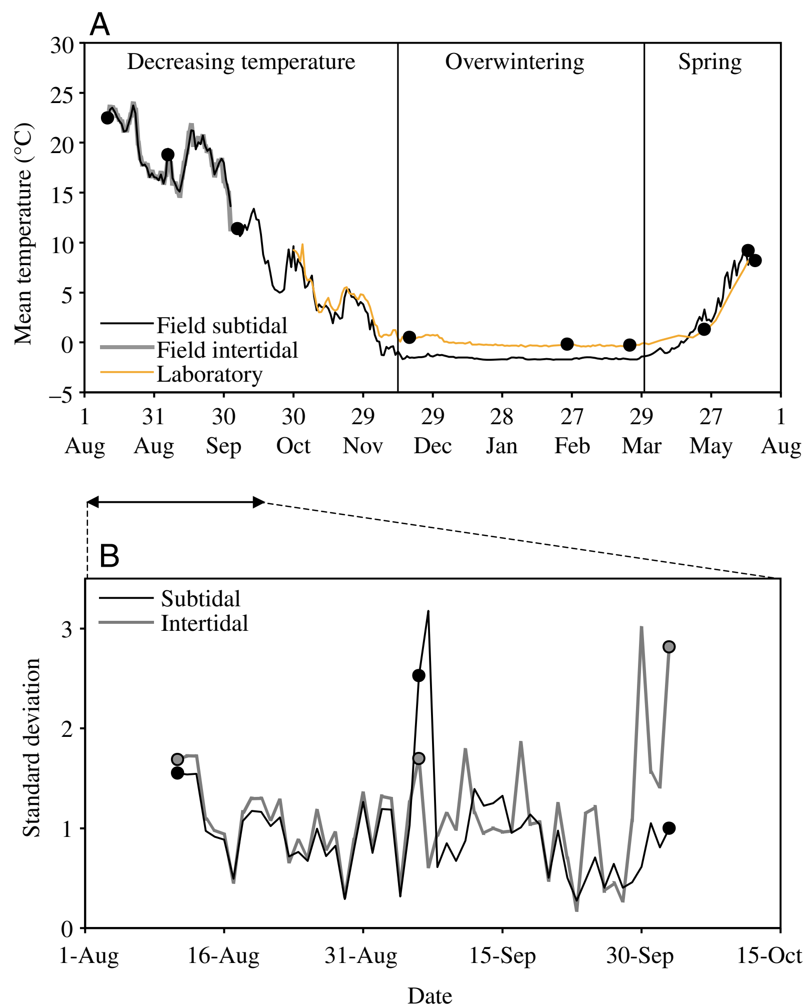


Fig. 2. Temperature recordings in the field at Neguac, Miramichi Bay (New Brunswick, Canada), at two tidal locations from 11 August until 6 October 2003. Subtidal temperature monitoring continued in the field until 16 May 2004, and temperature was monitored in the laboratory holding tank from 6 October 2003 until 16 May 2004. Filled circles indicate clam samplings. (A) Mean daily temperature at intertidal and subtidal locations and in the laboratory. (B) Standard deviation of temperatures at intertidal and subtidal locations.

The fatty acid 20:5n-3, an eicosanoid precursor of many biologically active lipids, increased by 19.2% in intertidal clams, followed by a rapid decrease to initial values in December before overwintering (Fig. 5). Clams held at the subtidal location showed a delayed increase in 20:5n-3 compared with their intertidal counterparts. The selectively bred *M. mercenaria* var. *notata* attained a lower level of 20:5n-3 during the fall than the wild *M. mercenaria* (Fig. 5).

Finally, the non-methylene interrupted dienoic fatty acids (22:2 NMI), a group of long-chain PUFA naturally biosynthesized by mollusks, increased by 23.6% between August and December, whereas seawater temperature decreased from ~23°C to 0°C, and remained constantly high (8.8%) during the overwintering period (Fig. 6). The selectively bred *M. mercenaria* var. *notata* showed a 4.8% reduction in 22:2 NMI compared to the wild variety.

Lipid data originating from clams overwintered in the laboratory were in good agreement with those from animals overwintered in the field (see Table 2 and Figs 3–6).

## Discussion

The phospholipid to sterol ratio in the wild hard clam *M. mercenaria* increased transiently with the lowering of temperature between August and October whereas it remained constant in the selectively bred *M. mercenaria* var. *notata*, thus suggesting that the capacity for adjusting the phospholipid to sterol ratio in response to environmental temperature may have been lost during the selection for fast-growing animals. Cholesterol is an essential component in animal membranes, with multiple effects on their physical properties including membrane fluidity, phase behavior, thickness, and permeability (Crockett, 1998). Although the

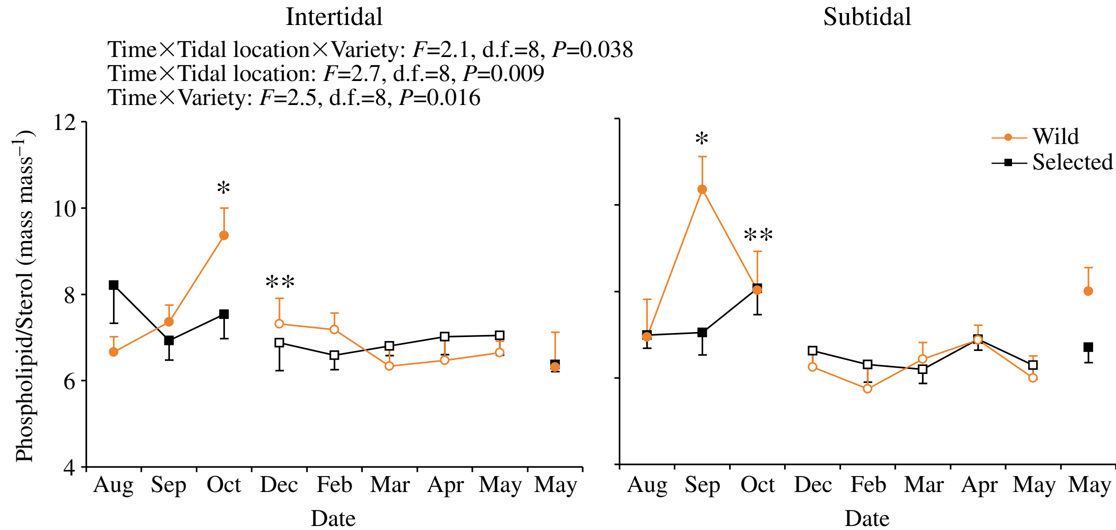


Fig. 3. Phospholipid to sterol ratios (mean ± s.e.m.) for two varieties of juvenile hard clams, wild *Mercenaria mercenaria* (circles) and selectively bred *M. mercenaria* var. *notata* (squares), as a function of time. Clams were placed in the field in August 2003 at two tidal locations and overwintered subtidally and in the laboratory between October 2003 and May 2004. Sampling took place in the field until October (filled symbols) and then in the laboratory until May (open symbols). Clams were sampled simultaneously in the field and in the laboratory in May to validate agreement between field and laboratory results. The first time at which a significant increase occurred is indicated by a single asterisk; double asterisks indicate the time at which the value returned to the initial level.

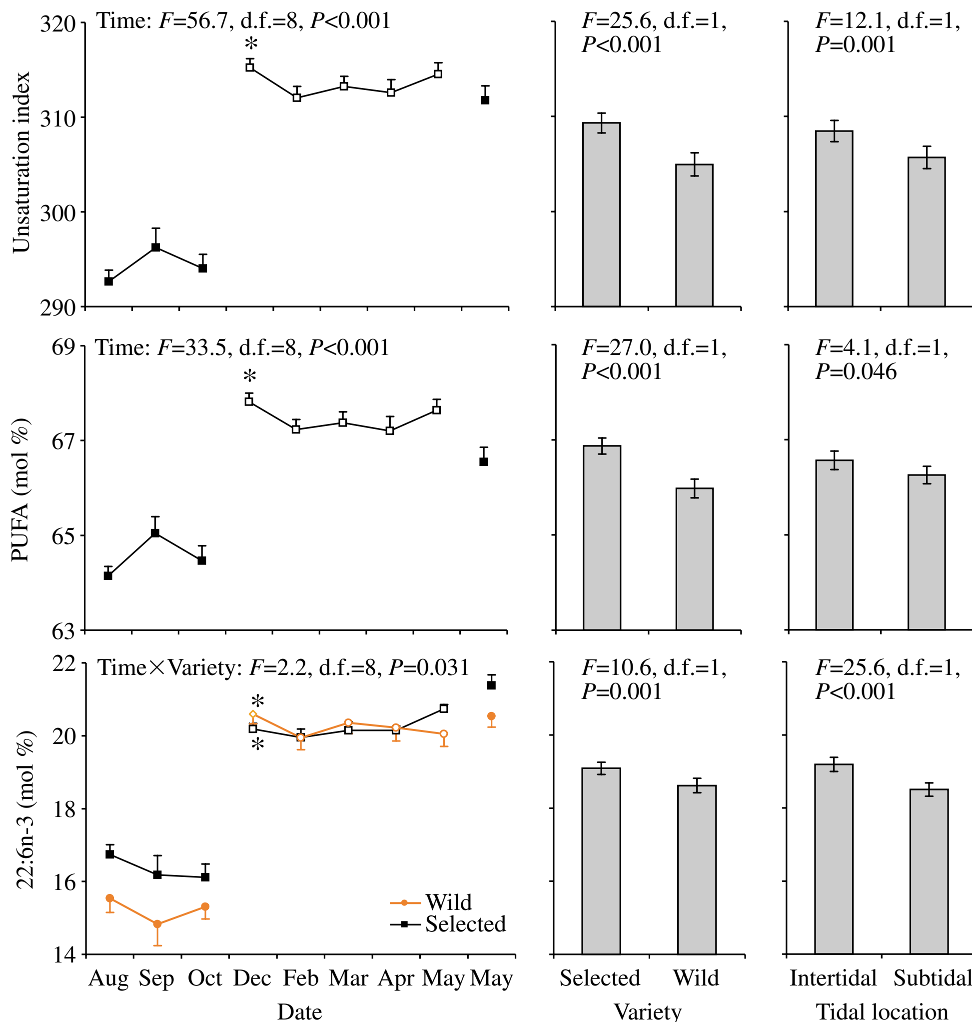


Fig. 4. Unsaturation index and mol % of polyunsaturated fatty acids (PUFA) and docosahexaenoic acid (22:6n-3) in juvenile hard clams as a function of time, variety and tidal location (mean ± s.e.m.). Unsaturation index and mol % of PUFA as a function of time are means of the two clam varieties. The unsaturation index is calculated as the sum of the mol % of each unsaturated fatty acid multiplied by the number of double bonds within that fatty acid. Clams were placed in the field in August 2003 at two tidal locations and overwintered subtidally and in the laboratory between October 2003 and May 2004. Sampling took place in the field until October (filled symbols) and then in the laboratory until May (open symbols). Clams were sampled simultaneously in the field and in the laboratory in May to validate agreement between field and laboratory results. The first time at which a significant increase occurred is indicated by a single asterisk.

Table 2. *Fatty acid composition of two varieties of juvenile hard clams, wild Mercenaria mercenaria and selectively bred M. mercenaria var. notata, as a function of time*

| | | Field | | | | Laboratory | | | | | | Field | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tidal | 2003-08-11 | | 2003-10-06 | | 2003-12-19 | | 2004-03-23 | | 2004-05-16 | | 2004-05-13 | |
| Fatty acid | location | Wild | Selected | Wild | Selected | Wild | Selected | Wild | Selected | Wild | Selected | Wild | Selected |
| 16:0 | I | 12.5 | 12.5 | 11.6 | 10.8 | 10.2 | 9.7 | 10.4 | 9.9 | 10.0 | 9.3 | 10.4 | 9.9 |
| | S | 12.6 | 11.6 | 11.7 | 11.0 | 10.5 | 10.1 | 10.2 | 10.1 | 9.9 | 9.5 | 11.0 | 10.6 |
| 17:0 | I | 1.1 | 1.2 | 1.0 | 1.2 | 1.1 | 1.1 | 1.1 | 1.1 | 1.2 | 1.2 | 1.1 | 1.1 |
| | S | 1.2 | 1.2 | 1.1 | 1.1 | 1.1 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.3 |
| 18:0 | I | 4.8 | 4.8 | 4.8 | 4.9 | 4.7 | 4.7 | 4.7 | 4.9 | 4.7 | 4.9 | 4.7 | 4.8 |
| | S | 5.0 | 4.7 | 4.7 | 4.8 | 4.7 | 4.7 | 4.8 | 4.9 | 5.0 | 4.9 | 5.0 | 4.9 |
| ΣSFA* | I | 18.8 | 19.0 | 18.2 | 17.0 | 16.1 | 15.5 | 16.5 | 16.2 | 15.9 | 15.5 | 16.5 | 16.1 |
| | S | 19.3 | 18.0 | 17.8 | 17.0 | 16.3 | 16.1 | 16.5 | 16.5 | 16.3 | 16.0 | 17.2 | 17.3 |
| 16:1n-9 | I | 2.9 | 2.9 | 3.6 | 3.7 | 4.0 | 3.7 | 4.2 | 3.7 | 4.1 | 3.9 | 4.5 | 3.7 |
| | S | 3.1 | 3.3 | 3.8 | 3.6 | 4.3 | 3.8 | 4.1 | 3.8 | 4.4 | 3.8 | 4.0 | 3.8 |
| 16:1n-7 | I | 1.5 | 1.7 | 2.1 | 1.6 | 1.5 | 1.6 | 1.5 | 1.6 | 1.7 | 1.9 | 1.4 | 1.6 |
| | S | 1.4 | 1.4 | 2.8 | 3.0 | 1.5 | 1.7 | 1.5 | 1.7 | 1.7 | 1.8 | 2.0 | 1.4 |
| 16:1n-5 | I | 1.3 | 1.2 | 1.5 | 1.6 | 1.6 | 1.6 | 1.6 | 1.5 | 1.6 | 1.6 | 1.6 | 1.5 |
| | S | 1.3 | 1.4 | 1.5 | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.3 |
| 18:1n-9 | I | 1.5 | 1.8 | 1.4 | 1.8 | 1.8 | 2.0 | 1.8 | 2.0 | 2.0 | 2.3 | 2.0 | 2.3 |
| | S | 1.6 | 1.8 | 1.5 | 1.9 | 1.7 | 2.0 | 1.8 | 2.0 | 1.9 | 2.1 | 2.1 | 2.1 |
| 18:1n-7 | I | 2.2 | 2.0 | 2.2 | 1.7 | 1.9 | 1.7 | 1.8 | 1.6 | 1.8 | 1.6 | 1.9 | 1.8 |
| | S | 2.2 | 1.8 | 2.6 | 2.2 | 1.8 | 1.8 | 1.9 | 1.8 | 1.8 | 1.7 | 2.2 | 2.0 |
| 20:1n-11 | I | 3.9 | 3.9 | 3.1 | 3.1 | 1.3 | 1.6 | 1.6 | 1.6 | 1.6 | 1.4 | 1.5 | 1.4 |
| | S | 3.9 | 3.9 | 3.0 | 3.0 | 1.6 | 1.6 | 1.6 | 1.5 | 1.5 | 1.3 | 1.5 | 1.4 |
| 20:1n-9 | I | 1.6 | 1.6 | 1.6 | 1.7 | 2.1 | 2.0 | 1.9 | 1.9 | 2.0 | 1.9 | 2.0 | 2.2 |
| | S | 1.5 | 1.6 | 1.4 | 1.5 | 1.8 | 1.9 | 1.8 | 1.9 | 1.7 | 1.8 | 2.1 | 2.2 |
| 20:1n-7 | I | 1.4 | 1.3 | 1.4 | 1.3 | 1.3 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 |
| | S | 1.4 | 1.4 | 1.5 | 1.4 | 1.3 | 1.3 | 1.3 | 1.2 | 1.2 | 1.2 | 1.3 | 1.2 |
| ΣMUFA† | I | 17.1 | 16.8 | 17.8 | 17.3 | 16.0 | 16.0 | 16.2 | 16.2 | 16.6 | 16.3 | 17.2 | 16.1 |
| | S | 17.1 | 17.3 | 18.7 | 18.6 | 16.2 | 16.3 | 16.4 | 16.1 | 17.1 | 15.6 | 17.3 | 15.8 |
| 16:2n-6 | I | 1.8 | 1.7 | 1.9 | 1.8 | 1.7 | 1.6 | 1.8 | 1.6 | 1.7 | 1.6 | 1.8 | 1.6 |
| | S | 1.9 | 1.8 | 1.9 | 1.7 | 1.7 | 1.5 | 1.7 | 1.6 | 1.8 | 1.6 | 1.8 | 1.7 |
| 20:2n-6 | I | 1.9 | 1.9 | 1.4 | 1.5 | 1.0 | 1.0 | 1.0 | 1.0 | 0.9 | 0.9 | 0.9 | 0.9 |
| | S | 1.9 | 1.9 | 1.3 | 1.3 | 1.0 | 1.0 | 0.9 | 0.9 | 0.9 | 0.9 | 0.9 | 0.8 |
| 22:2NMI | I | 7.1 | 6.4 | 8.7 | 8.6 | 9.2 | 8.5 | 9.0 | 8.7 | 9.3 | 8.7 | 9.1 | 8.3 |
| | S | 7.2 | 7.6 | 8.7 | 7.9 | 9.2 | 8.8 | 9.1 | 8.5 | 9.5 | 8.8 | 8.8 | 8.6 |
| 16:3n-4 | I | 1.6 | 2.1 | 1.9 | 1.8 | 2.0 | 2.3 | 2.0 | 2.2 | 2.1 | 2.4 | 2.2 | 2.3 |
| | S | 1.8 | 2.0 | 1.8 | 2.5 | 2.0 | 2.2 | 2.0 | 2.2 | 2.1 | 2.2 | 2.1 | 1.8 |
| 18:3n-3 | I | 1.1 | 1.4 | 0.6 | 0.7 | 0.5 | 0.7 | 0.3 | 0.7 | 0.4 | 0.5 | 0.5 | 0.5 |
| | S | 1.5 | 1.2 | 0.8 | 1.0 | 0.5 | 0.7 | 0.8 | 0.5 | 0.4 | 0.6 | 0.7 | 0.6 |
| 16:4n-3 | I | 12.1 | 13.2 | 10.5 | 12.3 | 9.7 | 10.5 | 10.2 | 10.9 | 9.5 | 10.8 | 9.1 | 9.9 |
| | S | 13.2 | 13.0 | 10.5 | 11.7 | 10.0 | 10.8 | 9.7 | 10.7 | 9.8 | 10.2 | 8.7 | 9.7 |
| 18:4n-3 | I | 0.7 | 0.9 | 0.6 | 0.5 | 0.7 | 0.8 | 0.6 | 0.8 | 0.5 | 0.6 | 0.6 | 0.7 |
| | S | 0.9 | 0.7 | 0.8 | 0.8 | 0.6 | 0.8 | 0.6 | 0.7 | 0.5 | 0.6 | 0.6 | 0.7 |
| 20:4n-6 | I | 4.2 | 3.9 | 4.3 | 4.6 | 4.5 | 4.6 | 4.3 | 4.5 | 4.6 | 4.6 | 4.5 | 4.4 |
| | S | 4.3 | 4.4 | 4.2 | 4.4 | 4.4 | 4.4 | 4.6 | 4.4 | 4.7 | 4.7 | 4.4 | 4.5 |
| 22:4n-6 | I | 2.1 | 1.5 | 2.3 | 1.9 | 2.4 | 2.6 | 2.2 | 2.1 | 2.5 | 2.4 | 2.0 | 2.6 |
| | S | 2.2 | 1.8 | 2.3 | 2.1 | 2.7 | 2.3 | 2.6 | 2.5 | 2.6 | 2.6 | 2.6 | 2.1 |
| 20:5n-3 | I | 7.8 | 7.8 | 8.9 | 8.0 | 7.7 | 8.1 | 7.5 | 7.9 | 7.6 | 7.6 | 7.3 | 7.4 |
| | S | 7.5 | 6.8 | 9.6 | 8.9 | 8.0 | 8.0 | 8.0 | 7.8 | 7.7 | 7.8 | 7.8 | 7.9 |
| 21:5n-3 | I | 1.7 | 1.7 | 1.9 | 1.7 | 2.1 | 1.9 | 2.1 | 1.8 | 2.0 | 2.0 | 2.1 | 2.0 |
| | S | 1.8 | 1.7 | 1.9 | 2.0 | 2.2 | 2.0 | 2.2 | 2.0 | 2.1 | 2.0 | 2.3 | 2.1 |
| 22:5n-6 | I | 1.6 | 1.5 | 1.4 | 1.5 | 1.4 | 1.6 | 1.4 | 1.5 | 1.5 | 1.6 | 1.5 | 1.4 |
| | S | 1.6 | 1.6 | 1.3 | 1.3 | 1.4 | 1.4 | 1.4 | 1.4 | 1.5 | 1.5 | 1.2 | 1.3 |
| 22:5n-3 | I | 2.6 | 2.5 | 2.7 | 2.8 | 3.0 | 3.0 | 3.0 | 3.0 | 3.1 | 3.0 | 3.0 | 3.0 |
| | S | 2.4 | 2.6 | 2.6 | 2.6 | 3.0 | 2.8 | 2.9 | 2.9 | 3.0 | 3.1 | 2.9 | 2.8 |

Table 2. *Continued*

| | | Field | | | | Laboratory | | | | | | Field | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tidal | 2003-08-11 | | 2003-10-06 | | 2003-12-19 | | 2004-03-23 | | 2004-05-16 | | 2004-05-13 | |
| Fatty acid | location | Wild | Selected | Wild | Selected | Wild | Selected | Wild | Selected | Wild | Selected | Wild | Selected |
| 22:6n-3 | I | 16.6 | 16.8 | 15.8 | 16.9 | 21.1 | 20.4 | 20.9 | 20.3 | 20.9 | 20.7 | 20.8 | 21.7 |
| | S | 14.5 | 16.7 | 14.6 | 15.2 | 20.1 | 20.0 | 19.8 | 20.0 | 19.2 | 20.8 | 20.3 | 21.1 |
| ΣPUFA‡ | I | 64.1 | 64.2 | 64.0 | 65.7 | 67.9 | 68.5 | 67.3 | 67.6 | 67.5 | 68.2 | 66.2 | 67.8 |
| | S | 63.6 | 64.7 | 63.5 | 64.4 | 67.5 | 67.6 | 67.2 | 67.5 | 66.6 | 68.4 | 65.5 | 66.9 |
| Σn-3 | I | 24.7 | 27.5 | 27.2 | 27.8 | 26.2 | 25.9 | 26.7 | 26.3 | 25.4 | 27.0 | 25.7 | 25.4 |
| | S | 26.3 | 26.4 | 25.8 | 28.4 | 26.2 | 26.3 | 26.1 | 25.6 | 26.6 | 26.3 | 28.6 | 24.9 |
| Σn-6 | I | 12.5 | 11.3 | 12.1 | 12.1 | 11.6 | 12.1 | 11.3 | 11.3 | 11.8 | 11.6 | 11.5 | 11.5 |
| | S | 12.7 | 12.1 | 12.1 | 11.7 | 11.7 | 11.4 | 11.9 | 11.6 | 12.2 | 11.9 | 11.4 | 11.0 |
| ΣUnsat. | I | 294.5 | 295.4 | 292.2 | 299.7 | 316.4 | 318.4 | 313.6 | 314.6 | 314.7 | 317.5 | 309.5 | 318.1 |
| | S | 286.8 | 294.9 | 289.1 | 294.2 | 313.2 | 313.5 | 311.5 | 313.3 | 307.9 | 318.0 | 307.4 | 313.4 |

Clams were placed in the field in August 2003 at intertidal (I) and subtidal (S) locations and overwintered subtidally and in the laboratory between October 2003 and May 2004. Sampling took place in the field until October and then in the laboratory until May. Clams were sampled simultaneously in the field and in the laboratory in May to validate agreement between field and laboratory results.

Only fatty acids contributing >1% for at least one treatment combination are reported.

*Includes a trace amount of 15:0.

†Includes trace amounts of 14:1n-7, 14:1n-5 and 15:1n-5.

‡Includes trace amounts of 17:1, 18:1n-5, 20:1n-5, 22:1n-11+13, 22:1n-9, 16:2n-4, 18:2n-6, 18:2n-4, 16:3n-3, 18:3n-6, 18:3n-4, 20:3n-6, 20:3n-3, 16:4n-1.

responses of cholesterol to temperature remain membrane-specific, some positive correlations between membrane cholesterol content, as determined by the cholesterol to phospholipid ratio, and acclimation temperature have been reported in several tissues of rainbow trout (Robertson and Hazel, 1995). A widely accepted notion is that cholesterol stabilizes membranes, i.e. it increases the order of the surrounding acyl chains in membranes in the fluid phase (Crockett, 1998). Therefore, the observed increase in the phospholipid to sterol ratio during the early stage of decreasing temperatures in *M. mercenaria* is consistent with a role for cholesterol in the membrane's homeoviscous response. It is worth mentioning that the sterol profile of marine bivalves consists of a complex mixture of $C_{26}$ to $C_{30}$ sterols (cholesterol=$C_{27}$), with each molecular species being characterized by a planar ring system with a 3β-hydroxyl group and a side chain of varying length (Knauer et al., 1998). However, an increase or decrease of one or more carbons in

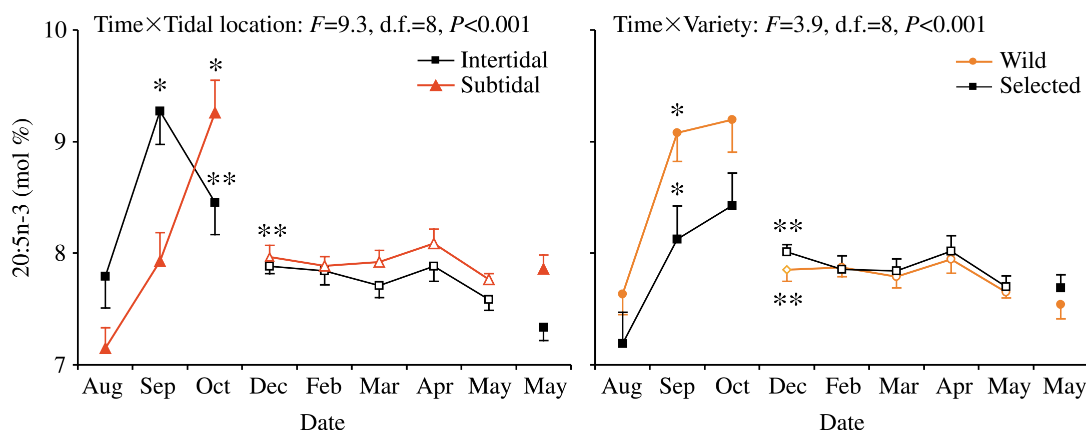


Fig. 5. mol % of eicosapentaenoic acid (20:5n-3) in juvenile hard clams as a function of time× tidal location and time× variety (mean ± s.e.m.). Clams were placed in the field in August 2003 at two tidal locations and overwintered subtidally and in the laboratory between October 2003 and May 2004. Sampling took place in the field until October (filled symbols) and then in the laboratory until May (open symbols). Clams were sampled simultaneously in the field and in the laboratory in May to validate agreement between field and laboratory results. The first time at which a significant increase occurred is indicated by a single asterisk; the time at which the value returned to the initial level is indicated by double asterisks.

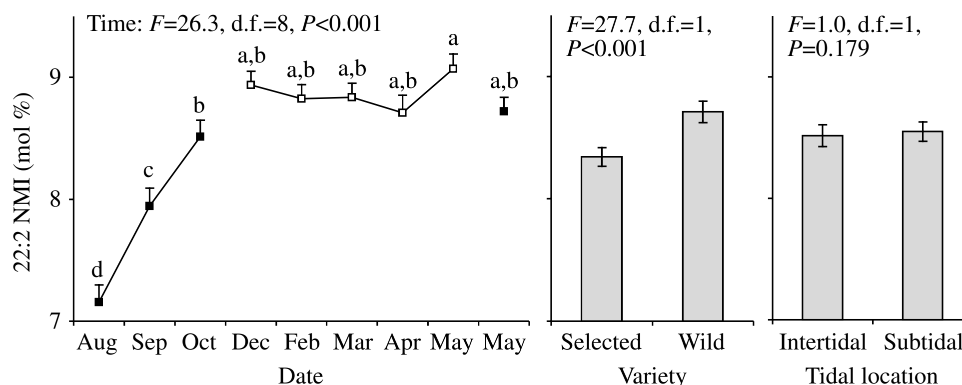


Fig. 6. Mol % of the total non-methylene interrupted dienoic fatty acids (22:2 NMI) in juvenile hard clams as a function of time, variety and tidal location (mean ± s.e.m.). Clams were placed in the field in August 2003 at two tidal locations and overwintered subtidally and in the laboratory between October 2003 and May 2004. Sampling took place in the field until October (filled symbols) and then in the laboratory until May (open symbols). Clams were sampled simultaneously in the field and in the laboratory in May to validate agreement between field and the laboratory results. Different letters indicate significant differences.

the side chain length reduces the ordering effect on membranes compared with cholesterol (Suckling et al., 1979), thus raising the need for caution in interpreting the modulation of sterols from marine bivalves in relation to cholesterol in other species.

The unsaturation index increased during the later stage of the temperature decrease between November and December, principally due to 22:6n-3, and is primarily responsible for the maintained differences in lipid composition between fall- and winter-acclimatized clams. Although membrane fluidity was not measured in our study, it is tempting to speculate that temporal variations in 22:6n-3 represent an adaptive response of the membranes to counteract the ordering effect of a decrease in the environmental temperature. In support of this hypothesis, several studies have shown that molecular species of phospholipids containing 22:6n-3 are important in controlling membrane fluidity during cold acclimation of fish (Dey et al., 1993; Buda et al., 1994; Tiku, 1996; Logue et al., 2000). It is noteworthy that absolute concentrations of 22:6n-3 increased from 266 to 305 $\mu$mol clam$^{-1}$ between October and December while total fatty acids decreased from 1679 to 1492 $\mu$mol clam$^{-1}$, thus suggesting that the machinery of membrane biogenesis favors the incorporation of 22:6n-3 at low temperatures. A reliance on 22:6n-3 for controlling membrane fluidity in hard clams could potentially limit their capacity for thermal acclimatization since bivalves cannot *de novo* synthesize long-chain PUFA (DeMoreno et al., 1976). Therefore, 22:6n-3 needs to be obtained preformed from the diet. Since hard clams cease feeding at temperatures of <6°C (Grizzle et al., 2001), the provision of exogenous 22:6n-3 prior to temperatures falling below 6°C is probably an important constraint for the long-term acclimatization of hard clams to low temperatures.

Little is known about the time course of changes in lipid composition during thermal acclimatization of bivalves. Previous studies on rainbow trout showed that exposure to low temperatures led to rapid and transient changes in phospholipid head groups of kidney plasma membranes whereas PUFA increased gradually during cold acclimation (Hazel and Landrey, 1988a; Hazel and Landrey, 1988b). In hard clams, the early transient increase in the phospholipid to sterol ratio may be viewed as a short-term adjustment to a lowering of environmental temperatures whereas the later increase in the unsaturation index (attributable to PUFA) may be viewed as a long-term adjustment to winter temperature. The rapid response of the phospholipid to sterol ratio to a temperature decrease probably reflects the ease of use of sterol in biological membranes. It has been suggested that the use of cholesterol as a modulator may not require any expenditure of ATP or reduced cofactors (Crockett, 1998). Therefore, modulation of the phospholipid to sterol ratio probably provides a metabolically less expensive mechanism of membrane remodeling than *de novo* synthesis of PUFA-rich phospholipids. Alternatively, the influence of cholesterol on the physical properties of membranes is greater in more saturated membranes than in more unsaturated ones (Kusumi et al., 1986). Therefore, modulation of the phospholipid to sterol ratio may have a more pronounced effect in clams living in a warm environment, which have relatively saturated membranes, than in cold-acclimatized clams, which have relatively unsaturated membranes, thus explaining why the phospholipid to sterol ratio increases before the unsaturation index.

Juvenile hard clams exhibited an increase in 22:2 NMI during decreasing temperatures; these fatty acids remained elevated during overwintering. Although the functional role of 22:2 NMI fatty acids is not well understood, they are believed to be involved in the regulation of membrane fluidity in poikilothermic animals (Rabinovich and Ripatti, 1991). In contrast to 22:6n-3 and other essential PUFA, which need to be obtained preformed from the diet, NMI fatty acids are synthesized *de novo* by bivalves (Zhukova, 1991). Therefore, synthesis of 22:2 NMI fatty acids could represent an alternative to the selective incorporation of 22:6n-3 or other essential PUFA at low temperatures. This biochemical pathway could be particularly important when animals cannot acquire essential

PUFA from their environment because of low concentrations in phytoplankton or when feeding has ceased at low temperatures. In these cases, a higher biosynthesis of the NMI fatty acid in wild *Mercenaria* may provide an advantage at low temperatures by reducing the need for other essential PUFA.

*M. mercenaria* showed an increase in 20:5n-3 along with decreasing temperatures but did not maintain the high levels during overwintering. A recent study showed that 20:5n-3 increased by ~45% in gill membranes of the sea scallop *Placopecten magellanicus* after 21 days of acclimation at low temperature, which correlates with an increase in membrane fluidity (Hall et al., 2002). Therefore, it was suggested that 20:5n-3 may have contributed to the maintenance of the sea scallop's membrane fluidity at low temperatures. In our study, 20:5n-3 was not correlated with the lipid unsaturation index ($r^2$=0.185, $P$=0.249), thus indicating that a rise in 20:5n-3 did not seem to contribute to increasing membrane fluidity in hard clams. Therefore, 20:5n-3, which is generally considered as a precursor of biologically active metabolites (Stanley and Howard, 1998), may have been incorporated for eicosanoid biosynthesis as a stress response to the early lowering of environmental temperatures or other unmeasured factors in the field.

Wild *M. mercenaria* showed a 33% reduction in their standard $\dot{V}_{O_2}$ compared to the selectively bred *M. mercenaria* var. *notata*. A reduction in the maintenance metabolic rate generally provides an energetic advantage in cold-adapted organisms by reducing their energetic needs during overwintering (Peck, 2002; Petersen et al., 2003), which suggests that wild *M. mercenaria* were more adapted to overwintering conditions than their selectively bred counterparts. Furthermore, there was a smaller deficiency in heterozygote frequencies in wild clams ($F$is=0.10) compared with selectively bred hard clams, for which the $F$is was 0.44. Several studies have shown negative correlations between heterozygote deficiency and survival in marine bivalves exposed to stressful environmental conditions; this relationship was attributed to lower metabolic requirements for heterozygous individuals (Hawkins et al., 1989; Tremblay et al., 1998; Myrand et al., 2002). For example, mussel stocks susceptible to summer mortality had a lower degree of heterozygosity and showed higher oxygen consumption rates than mussels from resistant stocks, thus suggesting that the energy expenditure for maintenance was higher for mussels from susceptible stocks (Tremblay et al., 1998). Similarly, higher metabolic demands in selectively bred hard clams associated with a higher heterozygote deficiency probably impose a supplementary stress that renders these animals more vulnerable to overwintering mortality. Interestingly, the selective breeding of hard clams for improving growth performance resulted in an increase in heterozygote deficiency and an increase in oxygen consumption rates.

A previous study on wild populations of hard clam from the US showed a good fit to the Hardy–Weinberg equilibrium (Dillon and Manzi, 1992), thus suggesting that the marked reduction in heterozygote frequencies in the variety *notata*, which originate from Massachusetts, most likely emerged because of the long-term breeding program and associated loss of genetic variability in the hatchery in the US and/or in Canada. Another possibility is that differences in heterozygote frequencies between the two varieties of clams emerged within the generation sampled, between the time that the clams were bred in the hatchery and their shipment from the hatchery to our facility. In this case, the marked heterozygote deficiency characteristic of the variety *notata* would be attributable to the Wahlund effect, inbreeding and/or selection (Zouros, 1987; Beaumont, 1991; Bierne et al., 2000; Bierne et al., 2003). However, given the lack of information about the selective breeding program, it is not possible to further investigate the cause of the decrease in heterozygote frequencies observed in the selectively bred hard clam.

Wild and selected hard clams differed in their allelic frequencies at four of the eight loci examined, which means that biochemical and physiological differences cannot be associated only to heterozygosity. For instance, selection for particular allozyme alleles was first demonstrated in the blue mussel, *Mytilus edulis*, where salinity acted on the *LAP** allele (Koehn et al., 1980). More recently, the functional significance of variations in allele frequency at the *GPI** locus, a key enzyme regulating glucose metabolism, was investigated in populations of the beetle *Chrysomela aeneicollis* (Dahlhoff and Rank, 2000). This study showed that the functional properties of *GPI** allozymes are related to environmental conditions in which each genotype predominates, thus suggesting that the genotype is associated with local adaptation to temperature. In our study, biochemical and physiological differences between varieties of hard clams cannot be related to any particular genotype at any locus or functional property of allozyme.

Wild *M. mercenaria* also exhibited a lower unsaturation index than the selectively bred counterpart *M. mercenaria* var. *notata*. It was previously shown that the unsaturation index of membrane phospholipids is positively correlated with the basal metabolic rate of the animal (Hulbert and Else, 1999; Hulbert and Else, 2005). Indeed, membrane unsaturation increases the molecular activity of many membrane-bound proteins and consequently also increases some specific membrane leak–pump cycles and cellular metabolic activities, which suggest that membranes could act as pacemakers for overall metabolic activity. Therefore, the positive relationship between standard $\dot{V}_{O_2}$ and the unsaturation index between wild and selectively bred hard clams is in good agreement with Hulbert's theory of membranes as possible pacemakers of metabolism. It is tempting to speculate that the lower metabolic requirements of wild clams characterized by a lower heterozygote deficit could be related to a lower unsaturation index in their membrane phospholipids. However, there was only a 4% difference in the unsaturation index between wild and selectively bred hard clams; this could only have a marginal effect on membrane fluidity and cellular metabolic activity.

In conclusion, the data presented in this study provide evidence that a major remodeling of lipids occurred in hard clams exposed to a gradual lowering in temperature from ~24

to 0°C and then acclimatized at <0°C, as predicted by HVA. Furthermore, we showed that selectively bred hard clams were characterized by a higher metabolic demand and a marked deviation from Hardy–Weinberg equilibrium at several loci due to a heterozygote deficit compared to wild clams, which is believed to impose additional stress and render these animals more vulnerable to overwintering mortality. Finally, an intriguing finding is that the lower metabolic requirements of wild animals coincide with a lower unsaturation index of their lipids, as predicted by Hulbert's theory of membranes as pacemakers of metabolism.

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