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Temperature adaptation in two bivalve species from different thermal habitats: energetics and remodelling of membrane lipids

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Summary

We compared lipid dynamics and the physiological responses of blue mussels *Mytilus edulis*, a cold-adapted species, and oysters *Crassostrea virginica*, a warmer-water species, during simulated overwintering and passage to spring conditions. To simulate overwintering, animals were held at 0°C, 4°C and 9°C for 3 months and then gradually brought to and maintained at 20°C for 5 weeks to simulate spring–summer conditions. Changes in lipid class and fatty acid composition were related to clearance rate and oxygen consumption.

We found major differences between species in triglyceride (TAG) metabolism during overwintering. Mussels used digestive gland TAG stores for energy metabolism or reproductive processes during the winter, whereas oysters did not accumulate large TAG stores prior to overwintering. Mussel TAG contained high levels of 20:5n-3 compared to levels in oysters and in the diet. This may help to counteract the effect of low temperature by reducing the melting point of TAG and thus increasing the availability of storage fats at low temperature. Mussels seemed better able to mobilise 20:5n-3 and 18:4n-3 than other fatty acids.

We also found that both bivalves underwent a major remodelling of membrane phospholipids. The unsaturation index decreased in the gills and digestive glands of both species during the early stages of warming, principally due to decreases in 22:6n-3 and 20:5n-3. In digestive glands, the unsaturation index did not increase with decreasing temperature beyond a threshold attained at 9°C whereas a perfect negative relationship was observed in gills, as predicted by homeoviscous adaptation. The presence of digestive enzymes and acids in the digestive gland microenvironment may lead to specific requirements for membrane stability. That oysters had lower metabolic rates than mussels coincides with a lower unsaturation index of their lipids, as predicted by Hulbert's theory of membranes as metabolic pacemakers. Both species showed increased 20:4n-6 levels in their tissues as temperature rose, suggesting an increasing availability of this fatty acid for eicosanoid biosynthesis during stress responses.

The contrast between the species in TAG dynamics and the similarity of their phospholipid remodelling emphasises the essential functional role of membrane phospholipid structure and the contrasting use of TAG by oysters and mussels during overwintering.

Key words: lipid, fatty acid, triglyceride, phospholipid, homeoviscous adaptation, mollusc, temperature adaptation, acclimation.

Introduction

Temperature, due to its impact upon all levels of biological organisation, is a crucial determinant of the biogeography and physiological characteristics of poikilotherms. Indeed, temperature alters the velocity of chemical and enzymatic reactions, rates of diffusion, membrane fluidity and protein structure (Hochachka and Somero, 2002). The thermal sensitivity of membrane processes is due to the strong effect of temperature on the physical properties of membrane lipids, which in turn have a major influence on associated proteins. A decrease in temperature usually reduces membrane fluidity, which can lead to membrane dysfunction. Poikilotherms usually counteract this temperature effect by remodelling membrane lipids, a process known as homeoviscous adaptation (HVA), *via* changes in phospholipid headgroups, fatty acid composition and cholesterol content that compensate for the effect of temperature on membrane structure (Sinensky, 1974; Hazel, 1995). Many intertidal organisms, which commonly withstand variations in temperature of 20–30°C on a daily basis and encounter even wider thermal ranges on a seasonal basis, are able to regulate membrane fluidity in response to thermal change. For example, the mussel *Mytilus californianus* exhibits strong seasonal variations in membrane fluidity that are consistent with HVA (Williams and Somero, 1996). Similarly, membrane fluidity in gill phospholipids of the sea scallop *Placopecten magellanicus* is positively correlated with 20:5n-3 and negatively correlated

with acclimation temperature, presumably helping to maintain membrane function at low temperatures (Hall et al., 2002). Finally, a major remodelling of lipids consistent with HVA occurs in hard clams *Mercenaria mercenaria* exposed to a gradual cooling from ~24°C to 0°C and acclimatisation at <0°C (Pernet et al., 2006b).

Blue mussels *Mytilus edulis* and eastern oysters *Crassostrea virginica* are two eurythermal suspension-feeding bivalves widely distributed along the east coast of North America. *M. edulis* ranges from Baffin Island to North Carolina (Gosling, 1992; Fisk et al., 2003), whereas *C. virginica* is mainly found in the southern part of North America, from the Gulf of St Lawrence to the Gulf of Mexico (Galtsoff, 1964). In the Gulf of St Lawrence, *C. virginica* is restricted to warm shallow bays and estuaries whereas *M. edulis* is found almost everywhere. This reflects the thermal preferences of the two species: they both tolerate a minimum temperature of –2°C but maximal and optimal temperatures for *M. edulis* are much lower (27°C and 10–20°C respectively) than those of *C. virginica* (36°C and 20–30°C, respectively) (Thompson and Newell, 1985; Shumway, 1996). Therefore, low overwintering temperatures have been suggested as a potential explanation for sporadic overwintering mortalities of *C. virginica* in Atlantic Canada (Lavoie, 1995), whereas temperatures >20°C in this area coincided with summer mortality in certain populations of *M. edulis* (Myrand and Gaudreault, 1995; Tremblay et al., 1998).

In the Gulf of St Lawrence, where mortality of *C. virginica* occasionally occurs, 0°C is a typical overwintering temperature. No mortality has been reported along the central Atlantic coasts, where 4°C is a typical overwintering temperature. The southern Atlantic coast from Chesapeake Bay to South Carolina, which has overwintering temperatures of about 9°C, supports the highest annual growth rate of *C. virginica* (Shumway, 1996). Oysters are quiescent at 0°C, begin to feed at 4°C, and start to grow at 9°C (Loosanoff, 1958).

In this study, mussels and oysters from the Gulf of St Lawrence were overwintered at 0°C, 4°C and 9°C in the laboratory for 3 months. The temperature was gradually raised to and held at 20°C for 5 weeks to simulate spring–summer conditions in the Gulf of St Lawrence. Animals were regularly sampled for lipid analysis and physiological measurements. We focussed our study on changes in lipid class and fatty acid composition of digestive glands and gills in relation to clearance rates and oxygen consumption. Digestive glands are the main site of extra- and intracellular digestion; they typically store large amounts of neutral lipids. In contrast, gills are involved in particle processing and gas exchange, and gill lipids consist mainly of sterols and phospholipids. We predicted that (1) bivalves would counteract thermal effects on membrane fluidity by remodelling membrane lipids as stipulated by the HVA, (2) membrane lipids of *M. edulis*, a species adapted to harsh Canadian winters, would be more unsaturated than those of *C. virginica*, a species that is less tolerant to cold, and (3) inter-specific differences in metabolic rates would be related to membrane unsaturation, as predicted by Hulbert's theory of membranes as metabolic pacemakers (Hulbert and Else, 1999; Hulbert and Else, 2005).

Unlike studies that only focus on the thermal effects on membrane lipids, we also examined the dynamics of storage lipids in relation to overwintering temperature. In marine bivalves, lipids are primarily stored as triglyceride (TAG) droplets in digestive glands (Giese, 1966), and the synthesis, storage and use of TAG usually show pronounced seasonal cycles: TAG are sequestered during periods of high food availability in late summer and fall, and are subsequently used for maintenance metabolism during periods of reduced feeding in the winter and for the initiation of gametogenesis (De Zwaan and Mathieu, 1992; Thompson et al., 1996). Because TAG are not linked to membrane function, they seem less likely than membrane lipids to change with temperature. However, TAG can only be mobilised if they are in a fluid state (Florant, 1998), a fact that poikilotherms living at low temperatures must counteract if lipid mobilisation is to continue.

Materials and methods

Animals

Adult mussels *Mytilus edulis* (Linnaeus 1758) and oysters *Crassostrea virginica* (Gmelin 1791) were obtained from two adjacent aquaculture sites: Havre de Shippagan (47°46′N; 64°41′W, lease NB3D, mussel) and Baie de Miscou (47°52′N; 64°33′W, lease NB3K, oyster), Gulf of St Lawrence, NB, Canada. Water temperature at the time of collection was 8°C and salinity was 29. Animals were transported on 15 November 2004 to the Coastal Zone Research Institute (CZRI, Shippagan, NB, Canada). Upon arrival, 102 animals of each species (mean shell length=61.9±1.9 mm for mussels and 79.8±3.5 mm for oysters) were numbered with bee tags and acclimated to laboratory conditions for 63 days prior to starting the experiment. Animals were equally distributed in two 300-l tanks with light aeration; the salinity was 29‰, the natural photoperiod was followed, and the temperature was maintained at 9°C. The two species were maintained together in these tanks over the entire experiment. Seawater was circulated through 1/2 or 1/5 HP external chillers (J&L Aquatics, Burnaby, BC, Canada) to maintain the required temperature; the water temperature in each tank was controlled separately. Animals were fed a mixed suspension of *Chaetoceros muelleri* (CHGRA) and *Isochrysis galbana* (TISO). These two algal species showed adequate characteristics as food for several bivalve species and complementary profiles in essential fatty acids (Pernet et al., 2003). Animals were fed every 2 days at 20×10^3 cells ml^{-1} (50:50 of each algal species by cell number). When seawater temperature was raised on the 12 April (see next section), the algal concentration offered to mussels and oysters was increased from 20 to 50×10^3 cells ml^{-1}. The daily ash-free dry mass (AFDM) of the algal ration varied from 0.3–0.5% of the mean AFDM of one animal during the overwintering period and increased to 2.3–3.2% during the spring simulation period. Variation in food ration within each experimental period was due to the successive removal of animals used for lipid analyses. Microalgal stocks were obtained from the Centre for Culture of Marine Phytoplankton (West Boothbay Harbor, ME, USA). Algae were batch-cultured in 20-l carboys (Pernet et al., 2003) and sampled three times during the experiment for fatty acid determination (Table 1). Temperature was monitored every hour in each tank throughout the experiment with submersible Vemco 8-bit Minilog-TR data loggers (Shad Bay, NS, Canada).

Table 1. *Fatty acid composition of microalgal species used in diet fed to mussels and oysters*

Variable	*Chaetoceros gracilis* (CHGRA)	*Isochrysis galbana* (TISO)	Mixed diet (CHGRA:TISO, 50:50 by cell number)
Fatty acid composition (mol %)			
14:0	7.3±1.1	14.2±0.4	11.0±0.4
16:0	9.3±0.6	11.9±0.6	10.7±0.1
18:0	1.5±0.3	1.5±0.2	1.5±0.2
∑ SFA	19.4±1.3	29.0±0.1	24.6±0.6
16:1n-7	30.1±3.4	2.6±0.8	15.3±1.8
18:1n-9	0.9±0.3	16.5±3.9	9.3±2.1
18:1n-7	1.1±0.1	1.9±0.2	1.5±0.1
∑ MUFA	33.9±3.5	24.7±1.1	29.0±1.1
16:2n-6	0.9±0.4	0.6±0.8	0.7±0.4
16:2n-4	4.1±0.5	0.6±0.4	2.2±0.3
18:2n-6	0.9±0.5	10.5±0.8	6.1±0.6
16:3n-4	10.4±2.0	0.0±0.0	4.8±0.8
18:3n-6	0.7±0.2	1.3±1.1	1.0±0.7
18:3n-3	0.3±0.3	5.9±3.4	3.3±1.7
18:4n-3	0.9±0.1	10.7±2.0	6.2±1.0
20:4n-6	2.6±0.7	0.7±0.4	1.6±0.4
20:5n-3	22.1±1.3	1.0±0.4	10.8±0.6
22:5n-6	0.2±0.4	2.2±0.5	1.3±0.4
22:6n-3	1.9±0.2	10.4±0.7	6.5±0.3
∑ PUFA	46.7±2.9	45.8±1.0	46.1±0.9
Unsaturation index	221.8±8.0	200.4±6.2	210.2±4.6
Total fatty acids (nmol 10^{-6} cells)	5.6±1.0	6.5±1.0	6.1±1.0

Fatty acid composition is given as mol % of total fatty acid.
The biochemical composition of the mixed diet was calculated from that of its individual constituents.
All values represent the mean ± s.d., N=3 replicates.
Only fatty acids contributing >1% in at least one species are reported.

Experimental design

Mussels and oysters were randomly divided between the three experimental treatments in duplicate tanks on 17 January 2005: one group was maintained at 9°C and two groups experienced a gradual temperature decrease (~0.5°C/day), one to 4°C and one to 0°C (Fig. 1A). Thus, all animals reached the desired overwintering temperature by 31 January, after which these temperatures were maintained for 12 weeks. Mussels and oysters that had overwintered at 9°C, 4°C and 0°C were then warmed by ~1°C/day starting on 19, 15 and 12 April, respectively, to simulate spring–summer conditions. A 1°C/day increase is representative of field conditions in the spring (Bricelj et al., in press). When animals attained 20°C on 4 May, they were held at this temperature for 5 weeks, until 8 June. Digestive glands and gills were sampled on 17 January, before applying the overwintering temperatures; on 31 January, after attaining the overwintering temperature; on 14 February, after short-term winter acclimation; on 12 April, which reflects long-term winter acclimation; on 4 May, after attaining the summer temperature; and on 8 June, which reflects long-term summer acclimation (Fig. 1).

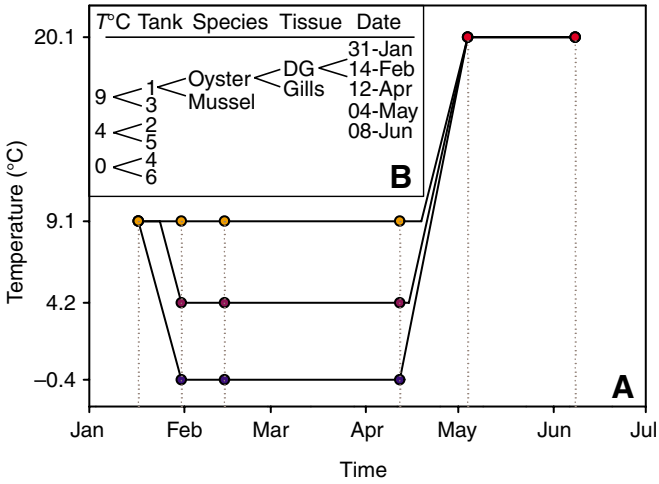

Fig. 1. (A) Experimental protocol for low temperature and spring–summer simulation experiment with mussel *Mytilus edulis* and oyster *Crassostrea virginica*. Circles indicate dates of clearance rate measurements and lipid sampling. Routine $\dot{V}_{O_2}$ was measured on 12 April, 4 May and 31 May and $\dot{V}_{O_2min}$ was measured on 8 June after starving the animals used for routine $\dot{V}_{O_2}$. (B) Schematic of the split-split plot experimental design. T°C=9, 4 or 0. See Materials and methods for further details.

Physiological measurements

Six mussels and six oysters per tank were used for physiological measurements. Physiological rates were

individually measured in 600 ml chambers; animals were held in their metabolic chambers for 1 h before measurements began. Six chambers were used simultaneously, which allowed us to measure five animals and one control (empty shell) at a time. Animals that remained closed in the chamber were excluded from physiological analysis. On 12 April, mussels and oysters for physiological measurements were sacrificed for dry mass determination, which was measured after drying at 70°C for 72 h; dry masses were used to calculate mass-standardised physiological rates for the overwintering period. Physiological rates were measured on other animals during the spring–summer simulation, which occurred between 12 April and 8 June. Again on 8 June, mussels and oysters used for physiological measurements were sacrificed for dry mass determination to normalise physiological rates for the spring–summer simulation period. Physiological rates were converted to mass-specific rates for animals of 1 g dry mass using appropriate weight exponent (b=0.7) in the allometric equations. The allometric equation is

$$Y = aX^b , \quad (1)$$

where Y=physiological rate, X=body mass and a and b are fitted parameters.

Clearance rate

Clearance rate (CR) is defined as the volume of water cleared of suspended particles per unit time and biomass (Widdows and Johnson, 1988). The CR was determined on 17 and 31 January, 14 February, 12 April, 4 May and 8 June for each temperature treatment. Six animals per tank of each species were used for CR measurement (12 animals per overwintering temperature and species). Animals were removed from their holding tank and maintained individually in their experimental chambers in which the suspension was mixed *via* gentle aeration from the bottom of the chamber. Before beginning particle concentration measurements, animals were left undisturbed for at least 1 h to allow their valves to open and feeding to begin. The CR was determined using a static system in which the decrease in particle concentration was monitored inside the chambers. At the beginning of the incubation period, individual animals were provided TISO at an initial concentration of 10×10^3 cells ml^{-1}. Food particles were counted every 10 min for 60 min with an electronic particle counter (Beckman Coulter-counter Z1^{TM}) fitted with a 100-μm aperture tube. The greatest difference between two consecutive measurements was used to calculate CR (Gilek et al., 1992):

$$CR = [\Delta C_{max}–S]V\, t^{-1}\, m^{-1} , \quad (2)$$

where ΔC_{max}=greatest difference in particle concentration between two consecutive measurements, S=sedimentation constant (0.049 and 0.059 for mussels and oysters, respectively) calculated as the mean exponential decline in particle concentration in the chambers without animal, V=volume of suspension, t=time between measurements and m=dry tissue mass.

Oxygen consumption

Routine oxygen consumption ($\dot{V}_{O_2}$, or routine metabolic rate) was determined at the end of the overwintering period on 12 April in each temperature treatment and during the spring–summer simulation on 4 May and 31 May. Minimum oxygen consumption ($\dot{V}_{O_2min}$, or standard metabolic rate) was measured at the end of the experiment on 8 June, after starving the animals for 8 days. Oxygen consumption for an individual animal 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 in the chamber was 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. Respiration was then expressed as ml O_2 h^{-1} g^{-1} tissue dry mass.

Lipid analysis

Tissue sampling

Mussel and oyster gills and digestive glands were sampled on 17 and 31 January, 14 February, 12 April, 4 May and 8 June in each temperature treatment for determination of lipid class and fatty acid compositions. 2–3 mussels and oysters were randomly sampled in each tank for determination of shell length, tissue AFDM, and lipid composition. Animals were dissected and ca. 300 mg wet mass of tissue were stored in lipid-free amber glass vials with Teflon^{TM}-lined caps under nitrogen in 1 ml dichloromethane at –80°C for later determination of lipid composition. Algae were filtered on GF/C filters precombusted at 450°C and stored in amber glass vials as previously described for tissues.

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, TAG, 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 (Iatroscan Mark-VI, Iatron Laboratories Inc., Tokyo, Japan) and chromatograms were analyzed using integration software (Peak Simple version 3.2, SRI, Torrance, CA, USA).

Neutral and polar lipid separation

Lipids were separated into neutral lipids (including triglycerides, free fatty acids and sterols) and polar lipids (including mainly phospholipids and minor amounts of glycolipids) using column chromatography on silica gel hydrated with 6% water as previously described (Pernet et al., 2006a). Briefly, the 100 mg columns were preconditioned with 1 ml of methanol and 1 ml of chloroform. Samples (200 μl) of lipid corresponding to ~1 mg of lipid were loaded onto the solid-phase extraction column. Samples were gently drawn into the solid phase with a slight vacuum. Columns were washed with 1 ml chloroform–methanol (98:2 v/v) to elute neutral lipids followed by 5 ml of methanol to elute polar lipids. The fractions eluted were collected in 7 ml tubes positioned in a vacuum manifold apparatus. The vacuum was adjusted to generate a flow rate of ~1 ml min^{-1}.

Fatty acids

Fatty acid methyl esters (FAME) from neutral and polar lipids

were prepared using 12% BF_3 in CH_3OH following the American Oil Chemists' Society method (AOCS, 1989). FAME were run on a Varian CP3900 gas chromatograph equipped with a ZB-wax fused-silica capillary column (20 m×0.18 mm i.d.×0.18 μm film thickness; Supelco, Bellfonte, PA, USA). Helium was used as the carrier gas (flow velocity: 1 ml min^{-1}). FAME were injected at 250°C at a 1:10 split ratio. The temperature ramp was 140°C for 0.2 min, followed by an increase of 40°C min^{-1} to 170°C, followed by an increase of 4°C min^{-1} to 185°C, and finally by an increase of 2°C min^{-1} to 230°C. The detector was maintained at 260°C. FAME were identified by comparison of retention times with known standards (37 component FAME Mix, PUFA-3 and menhaden oil; Supelco Bellefonte, PA, USA) and quantified with nonadecanoic acid (19:0) as an internal standard. Chromatograms were analyzed using the Galaxie chromatography data system (version 1.9.3.2, Varian, Mississauga, ON, Canada).

Statistical analyses

Analyses of variance (ANOVAs) were conducted to determine differences in initial characteristics of the neutral and polar lipids between the two bivalve species, *M. edulis* and *C. virginica*, before exposure to overwintering temperatures. One-way ANOVAs were conducted to determine differences between the two species in the relative TAG concentration, the fatty acid composition and the unsaturation index [average number of double bonds per acyl chain as calculated in Logue et al. (Logue et al., 2000)] of the neutral lipids in digestive glands. Minor amounts of TAG (<0.5%) were occasionally detected in gills and were not analyzed further. Two-way ANOVAs were conducted to determine differences in the initial characteristics of the membrane lipids as a function of bivalve species and tissue (gills and digestive glands). Dependent variables were the phospholipid to sterol ratio, the fatty acid composition and the unsaturation index of the polar lipids. The unit of replication used in these analyses was the rearing tank in which the animals were maintained (N=2).

Three-way split-split plot ANOVAs were conducted to determine differences in the physiological rates (CR and $\dot{V}_{O_2}$) and the neutral lipids of digestive glands, i.e. the relative TAG concentration, the unsaturation index and the major polyunsaturated fatty acids (PUFA), namely 22:6n-3, 20:5n-3 and 18:4n-3, as a function of overwintering temperature, species and date. The unit of replication was the tank in which the overwintering temperature was applied (N=2 for each temperature). The main plots were overwintering temperature levels (0, 4 and 9°C), subplots were species levels (mussel and oyster), and sub-subplots were sampling dates. Four-way split-split plot ANOVAs were used to determine differences in the phospholipid to sterol ratio, the unsaturation index and major PUFA of the polar lipid fraction, namely 22:6n-3, 20:5n-3, 20:4n-6, 22:2 and 20:2 NMI, as a function of overwintering temperature, species, tissue and date (Fig. 1B). Features of the four-way split-split plot experimental design were similar to those of the three-way plot except that sub-subplots also included tissue levels (gills and digestive glands). Here we used a mixed linear model, which models not only the means of our data but their variances and covariances. The need for covariance parameters arose because the experimental units on which the variables were measured were grouped into clusters and repeated measurements were taken on the same experimental unit. The repeated option was applied to the interaction terms 'Date' and 'Tissue×Date' for the three-way and four-way split-split plot experimental designs, respectively. These terms were combined with the covariance structure of the matrix to take into account spatial and temporal dependence (SAS Institute 2002).

Where differences were detected, least-square means 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. When necessary, data were log+1 or 1/square root(x) transformed to achieve normality of residuals and homogeneity of variances. Homogeneity of variance–covariance matrices was graphically assessed. Analyses were carried out using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA).

Results

Physiological measurements

Clearance rate (CR) varied as a function of temperature×species×date (Fig. 2A; P=0.029, F=2.57, d.f.=8).

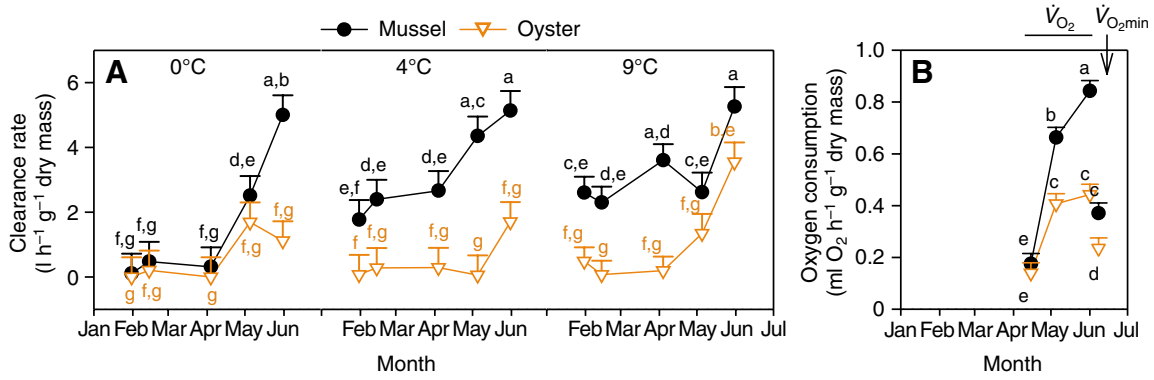

Fig. 2. (A) Clearance rate (CR) and (B) oxygen consumption rate of mussels and oysters for a standard animal 1 g in whole body dry mass (mean ± s.e.m, N=2 tanks). CR was affected by the combination of temperature, species and date whereas oxygen consumption was affected only by the combination of species and date. Routine $\dot{V}_{O_2}$ was measured on 12 April, 4 May and 31 May, and $\dot{V}_{O_2min}$ was measured on 8 June after starving the animals used for routine $\dot{V}_{O_2}$.

During the overwintering period, mussels maintained at 4°C and 9°C showed higher CR than those maintained at 0°C, where the CR was <0.5 l h^{-1} g^{-1} dry mass; CR was similarly low in overwintering oysters. During the spring–summer simulation, the CR of mussels overwintered at 0°C increased more rapidly (by 16×) than the CR of mussels overwintered at higher temperatures (~2×), resulting in CR values of ~5.1 l h^{-1} g^{-1} dry mass for the three overwintering groups at the end of the experiment (Fig. 2A). As in mussels, the CR of oysters increased during the spring–summer simulation. However, the CR of mussels was always higher than that of oysters, where it varied between 1.1 and 3.6 l h^{-1} g^{-1} dry mass as a function of overwintering temperature. Oysters overwintered at 9°C showed a greater increase in CR than those overwintered at lower temperatures.

There was no effect of overwintering temperature on the oxygen uptake of mussels and oysters (Temperature effect: P=0.904, F=0.10, d.f.=2; Temperature×Species effect: P=0.809, F=0.23, d.f.=2; Temperature×Date effect: P=0.210, F=1.58, d.f.=6; Temperature×Species×Date effect: P=0.808, F=0.49, d.f.=6). At the end of the overwintering period, mussels and oysters showed similar rates of oxygen uptake (Fig. 2B, P=0.534), but the increase in $\dot{V}_{O_2}$ during the spring–summer simulation was greater in mussels (4.8×) than in oysters, where the $\dot{V}_{O_2}$ increased by only 3.2× (Species×Date effect: P<0.002, F=11.2, d.f.=3). After food deprivation, the oxygen uptake of mussels and oysters maintained at 20°C for 5 weeks decreased by 2.2 and 1.8× respectively. The $\dot{V}_{O_2min}$ of mussels was 1.6× higher than that of oysters at 20°C (Fig. 2B).

Storage lipids

The initial concentration of TAG in the digestive gland, expressed as mg g^{-1} AFDM, was 3.7× higher in mussels than in oysters (Table 2). TAG levels in mussel digestive glands decreased during the entire study, whereas TAG remained almost constant in oysters (Fig. 3, Table 3). During the entire study, mussels used 48.9 mg g^{-1} AFDM of TAG, which represented ~80% of the initial TAG level. The unsaturation index of digestive gland TAG was initially 21% higher in mussels than in oysters and in the diet (Table 2, Fig. 3). The species effect on the unsaturation index was mainly attributable to PUFA and more particularly to 20:5n-3, which was 1.6–1.8× higher in mussels than in oysters and the food, respectively. During the experiment, the unsaturation index of the digestive gland TAG varied as a function of species and date (Fig. 3, Table 3). In mussels, the unsaturation index remained elevated during overwintering and decreased only during the spring–summer simulation at 20°C. In oysters, the unsaturation index increased 12% during overwintering and decreased until attaining initial values when temperature was raised to 20°C. 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=5.2×PUFA–21.8; r^2=0.909, N=60, P<0.001; Fig. 3). A second regression model using individual PUFA as explanatory variables showed that variations in the unsaturation index were mostly attributable to 22:6n-3 for the two species (y=11.0×22:6n-3+111.7; r^2=0.734, N=60, P<0.001; Fig. 3) and 20:5n-3 (y=5.9×22:6n-3+151.7; r^2=0.735, N=60, P<0.001; Fig. 3). The fatty acid 22:6n-3 increased significantly by 16% in the digestive gland TAG in the two species during overwintering before decreasing until the end of the study. The fatty acids 20:5n-3 and 18:4n-3 in mussel digestive glands decreased during the entire study, whereas in oysters, 20:5n-3 decreased only when the temperature was increased to 20°C and 18:4n-3 remained constant (Fig. 3, Table 3).

The relative mobilisation of 17 fatty acids from mussel and oyster digestive glands was approximated as the ratio of their molar percentage (mol %) in initial TAG to that in TAG at the end of the study period (Fig. 4). A ratio greater than, equal to, or lower than unity indicates that the fatty acid is mobilised more, equally, or less readily than the total TAG-fatty acids,

Table 2. *Characteristics of the neutral lipids in digestive glands of mussels Mytilus edulis and oysters Crassostrea virginica sampled on 17 January before exposure to overwintering temperatures*

Variable	Mussel	Oyster	P
Triglyceride (mg g^{-1} AFDM)	57.1±20.4	15.3±5.7	NS
Fatty acid (mol %)			
14:0	5.7±0.2	3.0±0.3	**
15:0	0.6±0.1	1.0±0.2	NS
16:0	14.3±0.9	23.2±1.1	*
17:0	0.8±0.0	1.7±0.1	*
18:0	2.0±0.1	3.2±0.7	NS
∑ SFA	23.5±1.2	32.2±1.7	*
16:1n-7	11.5±0.0	4.1±0.6	**
16:1n-5	0.4±0.0	0.4±0.1	NS
18:1n-9	3.2±0.1	6.0±0.1	**
18:1n-7	3.0±0.2	2.8±0.2	NS
20:1n-11	0.8±0.0	1.4±0.1	*
20:1n-9	1.5±0.4	0.9±0.2	NS
20:1n-7	1.0±0.0	3.9±0.1	**
∑ MUFA	22.4±0.6	21.2±1.2	NS
16:2n-4	1.5±0.0	0.2±0.1	**
16:2n-6	1.0±0.1	0.9±0.1	NS
18:2n-6	2.5±0.0	2.5±0.2	NS
$20:2NMI_{i,j}$	1.3±0.1	2.6±0.4	NS
$22:2NMI_{i,j}$	1.1±0.0	3.3±0.2	**
18:3n-3	2.6±0.1	3.1±0.6	NS
18:4n-3	5.8±0.2	4.7±0.8	NS
20:4n-6	1.6±0.0	2.0±0.2	NS
20:5n-3	19.1±1.5	12.1±0.2	*
21:5n-3	1.6±0.0	0.7±0.2	*
22:5n-6	0.5±0.0	0.5±0.0	NS
22:5n-3	0.7±0.0	0.8±0.1	NS
22:6n-3	11.1±0.0	10.1±0.8	NS
∑ PUFA	53.5±1.8	45.9±0.7	*
Unsaturation index	259.9±7.6	215.6±0.7	*

All values represent the mean ± s.d.; N=3 replicate tanks, 2 animals per tank were sampled.

*Significant differences (P<0.05); **highly significant differences (P<0.01); NS, no significant difference.

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

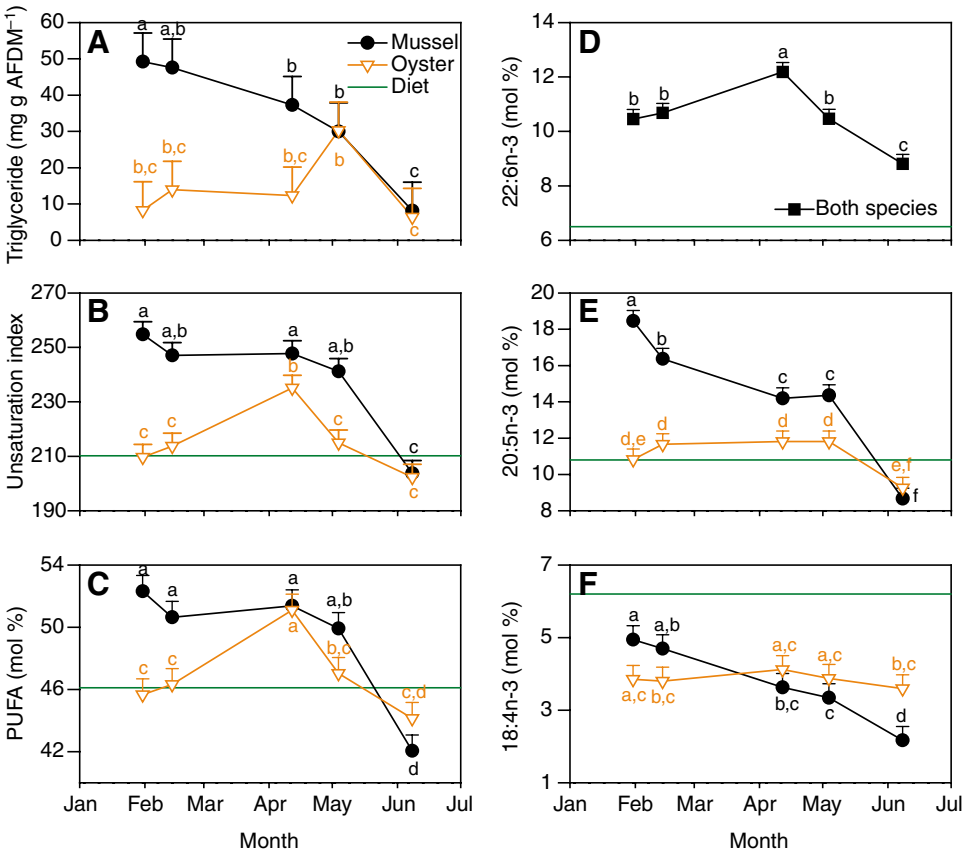

Fig. 3. Characteristics of the neutral lipids in mussel and oyster digestive glands as a function of time. Data presented here are (A) the concentration of triglycerides, (B) the unsaturation index and (C–F) the mol % of polyunsaturated fatty acids (PUFA; C), 22:6n-3 (D), 20:5n-3 (E) and 18:4n-3 (F). Values are means ± s.e.m, *N*=2–6 tanks. The green line indicates dietary values. The unsaturation index is calculated as the sum of the molar percentage of each unsaturated fatty acid multiplied by the number of double bonds within that fatty acid. Data from different overwintering temperatures were pooled as this effect was not significant. Different letters indicate significant differences.

Table 3. *Summary of the split-split plot three-way ANOVAs on the effect of overwintering temperature, bivalve species and sampling date on concentrations of triglyceride, unsaturation index, total PUFA and major individual PUFA of neutral lipids in digestive glands*

		Triglyceride		Unsaturation		PUFA		22:6n-3		20:5n-3		18:4n-3	
Source of variation	d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Main plot analysis													
T°C	2	0.3	0.749	0.2	0.813	0.1	0.917	0.7	0.564	0.5	0.671	0.4	0.699
Error A	3												
Subplot analysis													
Sp.	1	13.8	**0.034**	74.0	**0.003**	14.2	**0.033**	8.1	0.065	89.6	**0.002**	2.1	0.245
T°C × Sp.	2	2.5	0.227	2.4	0.241	1.8	0.309	3.3	0.176	0.2	0.850	2.7	0.214
Error B	3												
Sub-subplot analysis													
Date	4	4.1	**0.012**	18.1	**<0.001**	17.0	**<0.001**	11.7	**<0.001**	28.9	**<0.001**	5.3	**0.003**
T°C × Date	8	2.3	0.052	1.7	0.150	1.8	0.138	2.1	0.074	0.9	0.540	2.4	**0.046**
Sp. × Date	4	3.3	**0.028**	6.5	**0.001**	5.5	**0.003**	1.5	0.223	13.7	**<0.001**	6.1	**0.002**
T°C × Sp. × Date	8	1.0	0.438	0.6	0.800	0.4	0.917	0.6	0.786	0.9	0.560	1.0	0.490
Error C	24												

Error A: Tank (T°C)

Error B: Sp. × Tank (T°C)

Error C: Date × Tank (T°C), d.f.=12; Sp. × Date × Tank (T°C), d.f.=12

Independent variables were overwintering temperature (T°C; 0°C, 4°C and 9°C), bivalve species (Sp.; mussel and oyster) and Date (31 January, 14 February, 12 April, 4 May and 8 June).

Significant probabilities are in bold. Triglyceride data were log transformed and 18:4n-3 data were 1/square root(x) transformed.

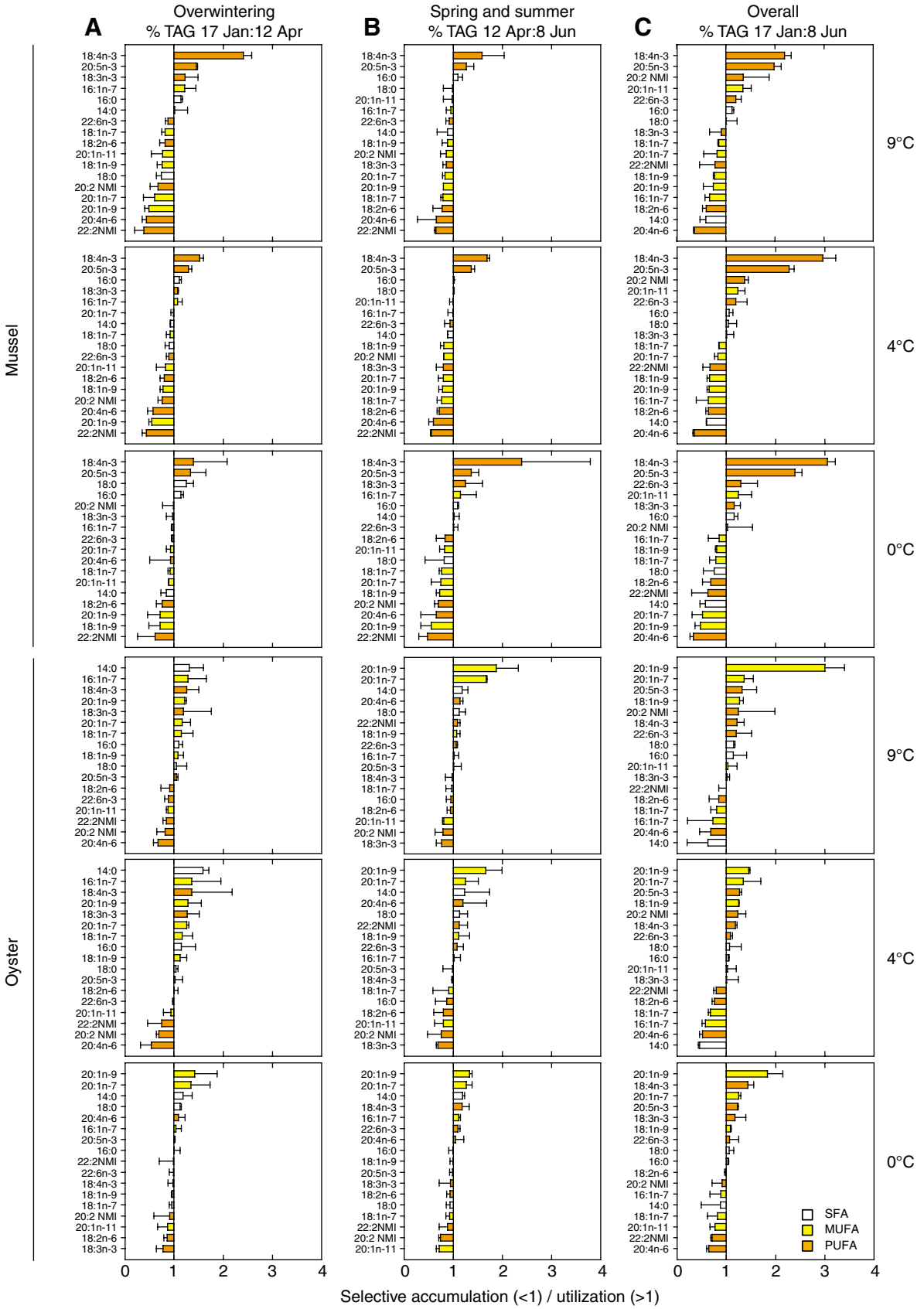

Fig. 4. See facing page for legend.

Table 4. *Characteristics of the membrane lipids in digestive glands (DG) and gills of mussels Mytilus edulis and oysters Crassostrea virginica sampled on 17 January before exposure to overwintering temperatures*

	Mussel		Oyster		
Variable	DG	Gills	DG	Gills	Significant effect
Lipid class					
PL/ST	8.7±0.9	6.7±2.4	8.0±0.6	6.0±0.7	Tissue
ST (mg g^{-1} AFDM)	4.0±0.9	7.8±2.1	4.8±1.4	7.6±2.3	Tissue
PL (mg g^{-1} AFDM)	34.7±7.8	48.8±10.8	38.3±12.0	45.6±14.5	NS
Fatty acid (mol %)					
14:0	2.4±0.4	0.8±0.1	1.1±0.1	0.8±0.1	Sp.×Tissue
15:0	0.8±0.0	0.9±0.1	0.9±0.0	1.2±0.0	Sp., Tissue
16:0	16.8±0.9	18.6±0.1	21.5±0.2	19.6±1.1	Sp.×Tissue
17:0	1.1±0.1	1.2±0.1	1.7±0.1	2.0±0.1	Sp.
18:0	4.0±0.3	3.9±0.0	4.0±0.2	4.0±0.2	NS
∑ SFA	25.0±1.7	25.5±0.1	29.5±0.4	28.3±0.9	Sp.
16:1n-7	4.0±0.7	1.4±0.2	1.7±0.2	1.4±0.2	Sp.×Tissue
16:1n-5	0.8±0.0	1.0±0.0	0.8±0.1	1.0±0.1	Tissue
18:1n-9	2.0±0.0	1.8±0.3	3.5±0.2	3.2±0.2	Sp.
18:1n-7	2.2±0.1	1.7±0.0	1.3±0.2	1.1±0.1	Sp., Tissue
20:1n-11	1.3±0.0	1.9±0.2	1.2±0.0	1.4±0.0	Sp.×Tissue
20:1n-9	2.1±0.5	3.2±0.2	0.9±0.1	1.2±0.2	Sp., Tissue
20:1n-7	1.0±0.0	1.1±0.2	4.5±0.0	4.9±0.3	Sp.
∑ MUFA	14.3±0.3	12.7±0.1	14.7±0.6	15.2±0.6	Sp.×Tissue
16:2n-6	2.2±0.1	2.7±0.0	0.8±0.0	0.9±0.0	Sp.×Tissue
18:2n-6	1.7±0.0	1.5±0.2	1.7±0.2	1.5±0.0	NS
$20{:}2NMI_{i,j}$	3.4±0.5	6.5±0.2	1.4±0.4	1.3±0.4	Sp.×Tissue
$22{:}2NMI_{i,j}$	4.7±0.4	6.4±0.1	6.8±0.0	8.4±0.1	Sp., Tissue
18:3n-3	1.2±0.1	0.4±0.0	1.3±0.4	0.8±0.1	Tissue
18:4n-3	2.2±0.4	1.0±0.7	2.1±0.5	1.2±0.3	Tissue
20:4n-6	3.8±0.2	5.0±0.2	4.8±0.3	6.9±0.3	Sp., Tissue
20:5n-3	18.8±1.0	13.5±0.7	13.8±0.3	12.5±0.2	Sp.×Tissue
21:5n-3	1.1±0.6	1.8±0.1	0.5±0.3	1.1±0.1	NS
22:5n-6	0.9±0.1	0.9±0.0	0.8±0.1	0.9±0.1	NS
22:5n-3	1.3±0.1	1.4±0.0	1.3±0.0	1.1±0.1	Sp.×Tissue
22:6n-3	15.4±1.2	15.1±0.2	16.3±0.5	15.7±0.8	NS
∑ PUFA	59.5±1.6	59.8±0.0	54.7±0.1	54.9±0.3	Sp.
Unsaturation index	276.3±6.9	261.5±0.9	257.0±0.9	254.5±5.2	Sp., Tissue

PL, phospholipids; ST, sterol.
All values represent the mean ± s.d., N=2 replicate tanks, 3 animals per tank were sampled.
Only fatty acids contributing >1% in at least one combination of treatment are reported.

respectively. Overall, the most readily mobilised fatty acids in mussels were 18:4n-3 and 20:5n-3, which ranged from 2.19–3.05 and 1.99–2.39, respectively. Interestingly, during overwintering, 18:4n-3 seemed to be mobilised less readily in mussels overwintered at 0°C and 4°C than in mussels maintained at 9°C whereas the inverse was true during the spring–summer simulation: 18:4n-3 seemed to be mobilised more readily in mussels overwintered at 0°C than in mussels maintained at higher temperatures. In oysters, the most readily mobilised fatty acid was 20:1n-9, which ranged between 1.34 and 2.00. Due to the low contribution of 20:1n-9 to the total TAG-fatty acids (<1%, Table 2), selective mobilisation of 20:1n-9 only had a marginal effect on the fatty acid composition of TAG.

Fig. 4. Relative mobilisation of fatty acids from the digestive glands of mussels and oysters overwintered at 0, 4 and 9°C during the overwintering period (A), the spring–summer simulation (B) and the overall period of study (C). The relative mobilisation of individual fatty acids was calculated as the ratio of initial to final levels in the TAG for each time interval. Values are means ± s.e.m, N=2. A ratio greater than, equal to, or lower than unity shows that the fatty acid is released more, equally, or less readily, respectively, than the total TAG-fatty acids. Values for fatty acids >2 mol % of the total are arranged in increasing order of relative mobilisation from bottom to top.

Membrane lipids

The phospholipid to sterol (ST) ratio varied as a function of time, tissue and species (Fig. 5, Table 4, Table 5). Mussel and oyster tissues showed a 1.2-fold increase in their phospholipid to sterol ratio between 12 April and 4 May, when temperature increased from 0°C, 4°C or 9°C to 20°C. The phospholipid to

sterol ratio of digestive glands was 1.4× higher than that of gills (11.9 and 8.4, respectively; Fig. 5), mainly due to the higher ST content in gills (Table 4). Interestingly, mussels were characterised by a phospholipid to sterol ratio 1.5× higher than in oysters, irrespective of time or tissue (Fig. 5).

The unsaturation index was initially 5.2% higher in mussels than in oysters and 3.3% higher in digestive glands compared to gills (Table 4). The unsaturation index varied as a function of tissue×date. In gills, the unsaturation index increased slightly (by 3.6%) during early overwintering, remained elevated during overwintering, and decreased markedly during the spring–summer simulation at 20°C. In digestive glands, the unsaturation index remained constant during overwintering and decreased markedly during the spring–summer simulation. The unsaturation index also varied as a function of species×tissue (Fig. 5, Table 5). As observed for TAG, the unsaturation index of polar lipids was positively correlated with PUFA (y=4.4×PUFA–6.4; r^2=0.867, N=120, P<0.001; Fig. 5) and more particularly with 22:6n-3 (y=7.8×22:6n-3+144.4; r^2=0.633, N=120, P<0.001; Fig. 5) and 20:5n-3 (y=4.6×22:6n-3+192.8; r^2=0.601, N=120, P<0.001; Fig. 5). The fatty acid 22:6n-3 remained constant during overwintering and decreased by 35.5% during the spring–summer simulation, irrespective of species or tissue. The fatty acid 20:5n-3 varied as a function of tissue×date, species×date and species×tissue (Fig. 5, Table 5). Although some minor differences occurred in the dynamics of 20:5n-3 during overwintering between species and tissues, 20:5n-3 decreased during the spring–summer simulation in digestive glands and gills for both mussels and oysters (Fig. 5).

Overall, there was no significant effect of overwintering temperature on the fatty acid composition of membrane lipids. However, regression models using temperature as an explanatory variable and the unsaturation index of animals acclimated at 0°C, 4°C, 9°C (April 12) and 20°C (June 8) as the response variable showed that the unsaturation index in gills was negatively correlated with temperature (Fig. 6A). Interestingly, the unsaturation index of mussels was higher than that of oysters, irrespective of acclimation temperature. In mussel gills, 20:5n-3 showed a wider range of variation (8.0–16.5%) than 22:6n-3 (12.7–16.7%) in response to acclimation temperature, whereas in oyster gills, 20:5n-3 and 22:6n-3 varied to the same extent (Fig. 6B). In contrast to gills, the unsaturation index in digestive glands was not significantly correlated with acclimation temperature: a threshold value

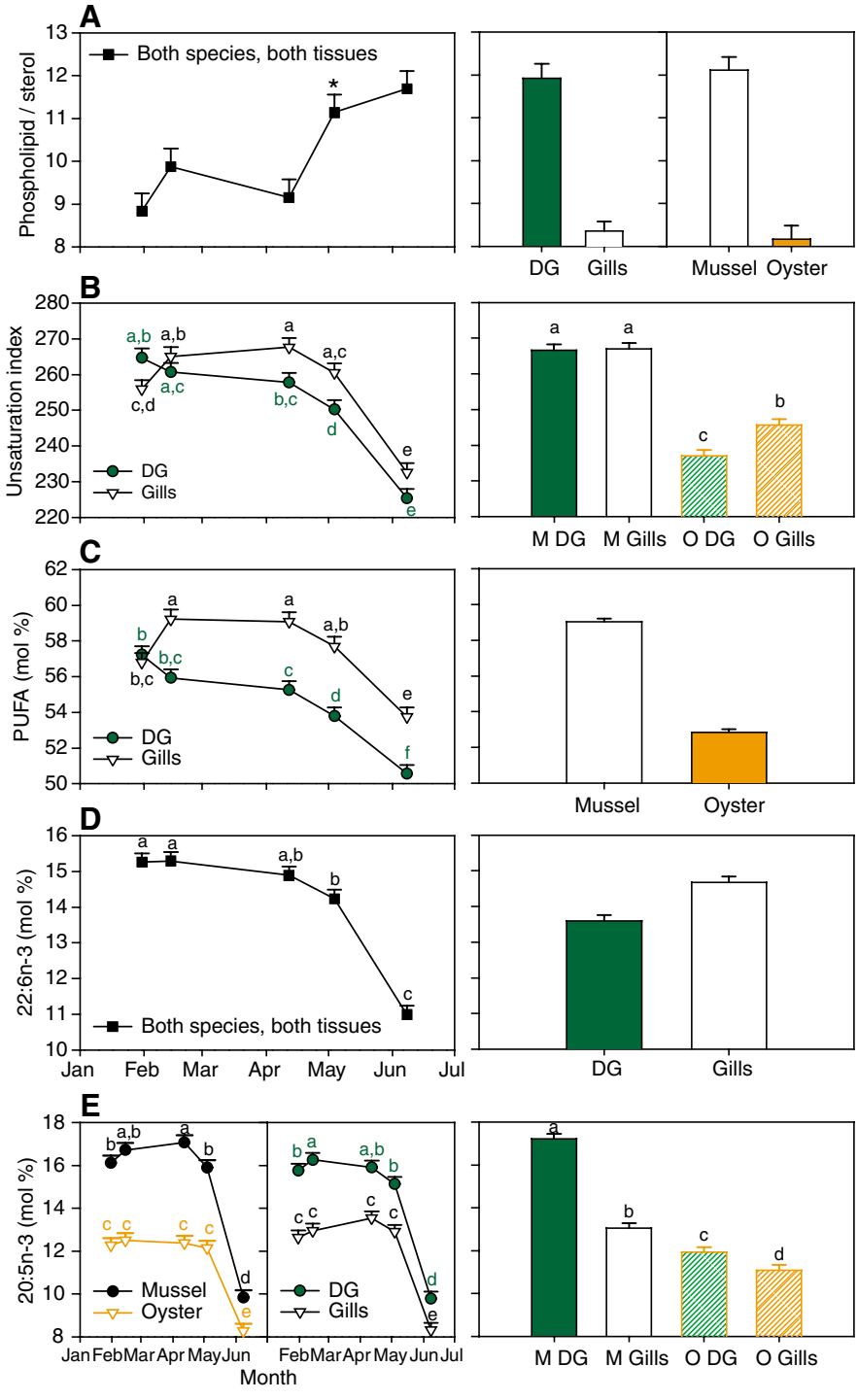

Fig. 5. Characteristics of the membrane lipids in mussel (M) and oyster (O) digestive glands (DG) and gills, as a function of time (left) and species and tissues (right). (A) The phospholipid to sterol ratio, (B) the unsaturation index and (C–E) the mol % of polyunsaturated fatty acids (PUFA; C), 22:6n-3 (D) and 20:5n-3 (E) in the polar lipids. Values are means ± s.e.m, N=2–6 tanks. The unsaturation index is calculated as the sum of the molar percentage of each unsaturated fatty acid multiplied by the number of double bonds within that fatty acid. Data from different overwintering temperatures were pooled as this effect was not significant. Different letters indicate significant differences.

Table 5. *Summary of the split-split plot four-way ANOVAs on the effect of overwintering temperature, bivalve species, tissue and sampling date on the phospholipid to sterol ratio, unsaturation index, total PUFA and major individual PUFA of polar lipids*

		PL/ST		Unsaturation		PUFA		22:6n-3		20:5n-3		20:4n-6		22:2 NMI		20:2 NMI	
Source of variation	d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Main plot analysis																	
T°C	2	1.4	0.373	1.1	0.446	0.4	0.677	2.6	0.225	2.1	0.264	1.6	0.343	1.4	0.372	2.6	0.222
Error A	3																
Subplot analysis																	
Sp.	1	73.2	**0.003**	238.8	**0.001**	625.4	**0.001**	6.0	0.090	161.6	**0.001**	0.4	0.588	470.7	**<0.001**	1522.1	**<0.001**
T°C×Sp.	2	0.0	0.967	0.5	0.644	0.5	0.652	0.6	0.621	1.5	0.356	0.8	0.517	0.8	0.517	2.0	0.280
Error B	3																
Sub-subplot analysis																	
Tissue	1	103.2	**<0.001**	7.6	**0.008**	151.7	**<0.001**	24.6	**<0.001**	165.4	**<0.001**	221.9	**<0.001**	162.0	**<0.001**	150.5	**<0.001**
T°C×Tissue	2	1.2	0.313	0.3	0.710	0.3	0.723	0.4	0.673	1.0	0.376	0.1	0.934	0.4	0.668	0.9	0.432
Species×Tissue	1	0.1	0.733	6.2	**0.016**	0.0	0.973	1.0	0.311	73.3	**<0.001**	2.0	0.161	2.2	0.140	224.0	**<0.001**
T°C×Species×Tissue	2	0.6	0.537	0.2	0.807	0.4	0.652	0.1	0.932	1.0	0.375	0.3	0.758	0.5	0.585	0.6	0.573
Date	4	10.0	**<0.001**	60.6	**<0.001**	31.4	**<0.001**	55.6	**<0.001**	122.4	**<0.001**	88.4	**<0.001**	4.1	**0.006**	1.2	0.313
T°C×Date	8	0.3	0.965	1.1	0.379	0.5	0.845	1.1	0.356	2.1	**0.049**	0.6	0.778	1.0	0.464	1.0	0.460
Species×Date	4	1.1	0.348	0.8	0.550	0.1	0.973	2.1	0.095	7.7	**<0.001**	26.6	**<0.001**	5.1	**0.002**	1.8	0.136
T°C×Species×Date	8	0.4	0.921	0.7	0.715	0.5	0.823	1.1	0.383	1.4	0.209	2.6	**0.017**	1.9	0.083	1.4	0.234
Tissue×Date	4	1.0	0.402	4.5	**0.003**	6.4	**0.001**	2.1	0.100	3.0	**0.029**	2.5	0.053	2.3	0.075	1.0	0.431
T°C×Tissue×Date	8	1.8	0.107	1.0	0.448	1.1	0.437	1.0	0.479	0.7	0.681	1.2	0.348	1.6	0.139	1.1	0.358
Speceis×Tissue×Date	4	1.0	0.412	0.9	0.492	0.6	0.639	0.4	0.803	1.0	0.413	1.3	0.295	0.3	0.869	2.4	0.064
T°C×Species×Tissue ×Date	8	1.6	0.157	0.7	0.662	0.8	0.576	0.7	0.721	1.3	0.246	1.3	0.268	1.0	0.433	0.2	1.340
Error C	54																

Error A: Tank (T°C)

Error B: Species×Tank (T°C)

Error C: Tissue×Tank (T°C), d.f.=3; Species×Tissue×Tank (T°C), d.f.=3; Date×Tank (T°C), d.f.= 12; Species×Date×Tank (T°C), d.f.=12; Tissue×Date×Tank (T°C), d.f.=12; Species×Tissue×Date×Tank (T°C), d.f.=12

PL/ST, phospholipid to sterol ratio.

Independent variables were overwintering temperature (T°C; 0°C, 4°C and 9°C), bivalve species (mussel and oyster), tissue (gills and digestive glands) and sampling date (31 January, 14 February, 12 April, 4 May and 8 June).

Significant probabilities are in bold. Data of PL/ST, 20:2 and 22:2 NMI were log+1 transformed.

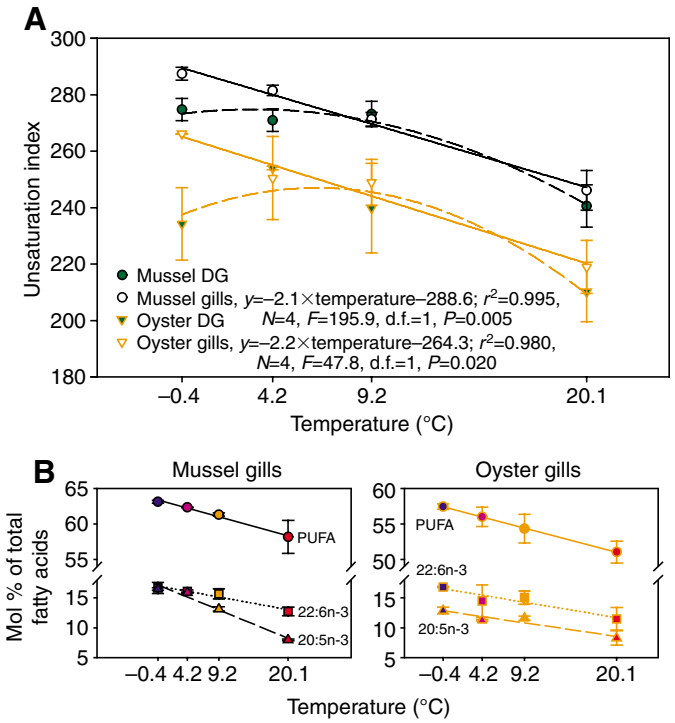

Fig. 6. (A) Regression models using temperature as the explanatory variable and the unsaturation index of animals acclimated at 0°C, 4°C, 9°C (April 12) and 20°C (June 8) as the response variable in digestive glands (DG) and gills of mussels and oysters. (B) Mol % of polyunsaturated fatty acids (PUFA), 22:6n-3, and 20:5n-3 in the gills of mussels and oysters acclimated at 0°C, 4°C, 9°C (April 12) and 20°C (June 8) as a function of temperature.

was attained at 9°C and did not increase further with a decrease in temperature.

The fatty acid 20:4n-6, an eicosanoid precursor of many biologically active lipids, showed species- and tissue-specific patterns (Table 4). Indeed, 20:4n-6 was initially lower in mussels (4.4%) than in oysters (5.9%) and lower in digestive glands (4.3%) compared to gills (6.0%, Table 4). The fatty acid 20:4n-6 varied as a function of overwintering temperature×species×date (Fig. 7, Table 5). During the overwintering period, 20:4n-6 increased in mussels whereas it remained stable in oysters. Therefore, at the end of the overwintering period, mussels and oysters showed similar levels of 20:4n-6. During the spring simulation period, 20:4n-6 increased markedly in mussels, increased moderately in oysters overwintered at 0°C and 9°C, and did not increase significantly in oysters overwintered at 4°C.

Finally, the non-methylene-interrupted dienoic fatty acids (NMI), a group of long-chain PUFA naturally biosynthesised by molluscs, showed species- and tissue-specific patterns (Table 4). Significant levels of both 20:2 and 22:2 NMI characterised mussels, whereas 22:2 NMI was the predominant NMI fatty acid in oysters. Additionally, 22:2 NMI was lower in mussels than in oysters and lower in digestive glands compared to gills (Table 4, Fig. 8). Levels of 20:2 NMI did not vary during the experiment, whereas 22:2 NMI varied as a function of species×date and tissue×date (Fig. 8, Table 5). In oysters, 22:2

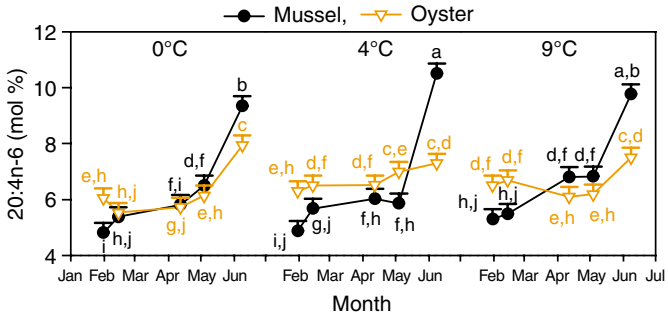

Fig. 7. Mol % of arachidonic acid (20:4n-6) in the polar lipids as a function of temperature×species×date. Values are means ± s.e.m, N=2 tanks). Data from different overwintering groups and tissues were pooled as these effects were not significant. Different letters indicate significant differences.

NMI increased from 7.9 to 9.3% during the acclimation period at 20°C whereas it remained low in mussels (Fig. 8). In gills, 22:2 NMI increased from 7.3 to 8.4% during the acclimation period at 20°C, whereas it was maintained constant in digestive glands.

Discussion

Storage lipids: acquisition and mobilisation

Mussels and oysters differed in their lipid metabolism during overwintering. In *M. edulis*, a species adapted to the harsh winters of Atlantic Canada, high initial TAG concentrations in the digestive gland declined markedly during the study, suggesting a reliance on TAG for energy metabolism or reproductive processes (De Zwaan and Mathieu, 1992). In contrast, *C. virginica*, which generally occurs in warmer habitats, showed low and constant TAG concentrations during overwintering. This mirrors biogeographical patterns of reliance on lipids for energy metabolism among marine species. For example, herbivorous copepods from polar and temperate areas use lipid stores for energy and reproductive needs during the fall

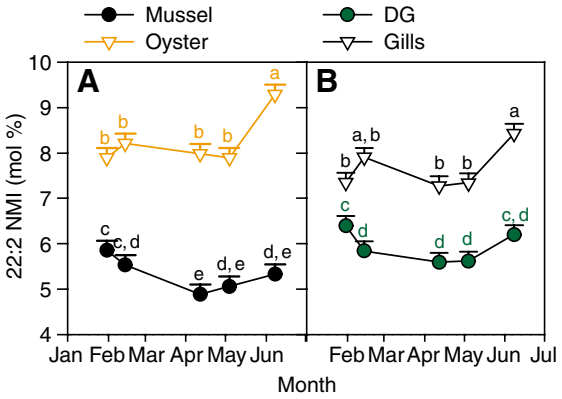

Fig. 8. Mol % of non-methylene interrupted dienoic fatty acids (22:2 NMI) in the polar lipids as a function of species×date (A; data from different tissues pooled) and tissue×date (B; data from different species pooled). Values are measn ± s.e.m, N=2–6 tanks). Data from different overwintering temperatures were pooled as this effect was not significant. Different letters indicate significant differences.

and winter, presumably in response to the cyclic annual productivity typical of northern food webs, whereas zooplankton from tropical biomes generally do not accumulate large lipid stores (Lee et al., 2006). Likewise, the capacity for lipid storage increases in cold-adapted and cold-acclimated fish (Pörtner, 2002; Guderley, 2004). Although mussels and oysters overlap in their distribution, the discrepancy in their TAG metabolism may reflect the fact that mussels grow better in colder habitats than oysters do.

Alternatively, differences in TAG metabolism between the two species may reflect differences in their reproductive cycle at low temperatures. The synthesis, storage and use of TAG often show pronounced seasonal cycles in bivalves: TAG are sequestered during periods of high food availability in late summer and fall, and are subsequently used for maintenance metabolism during periods of reduced feeding in the winter and for initiation of gametogenesis (De Zwaan and Mathieu, 1992; Thompson et al., 1996; Barber and Blake, 2006 and references therein). It is therefore likely that the TAG stored in digestive glands of mussels were transferred to the developing gonads during overwintering. By contrast, oysters that showed low and constant levels of TAG during overwintering may be quiescent, waiting for the spring revival to initiate gametogenesis. According to their specific environment, bivalves may support gametogenesis using recently ingested food, stored reserves or a combination of the two (Barber and Blake, 2006).

Discrepancies in TAG dynamics between mussels and oysters during overwintering partly reflect differences in feeding rate. At 9°C, the temperature at which animals were maintained for 8 weeks before the experiment began, the CR of mussels was higher (~2.8 $l\,h^{-1}\,g^{-1}$ dry mass) than that of oysters, where the CR was well below <0.5 $l\,h^{-1}\,g^{-1}$ dry mass (Fig. 2A). Therefore, mussels likely accumulated more lipids from phytoplankton prior to overwintering than did oysters, explaining the higher TAG levels in mussel digestive glands at the onset of the experiment. While the CR of mussels increased with overwintering temperature, as found by Cusson et al. (Cusson et al., 2005) and references therein, that of oysters maintained at 0°C, 4°C and 9°C was similar. Although few studies have specifically examined the effect of temperature on feeding in *C. virginica*, one paper reports that this species does not feed below 5°C (Loosanoff, 1958), in agreement with our results.

Not only were mussels characterised by a greater capacity for TAG accumulation compared to oysters, but they also seemed better able to selectively incorporate 20:5n-3. This essential fatty acid was markedly higher in mussel than in oyster TAG and largely exceeded dietary levels. Although the fatty acid composition of the TAG in bivalve digestive glands generally reflects that of their diet (Soudant et al., 1999), scallop larvae fed a diet rich in 20:5n-3 preferentially accumulate 22:6n-3 at the expense of 20:5n-3 in their TAG, suggesting regulation of the incorporation/utilisation of essential PUFA in TAG (Delaunay et al., 1993). *M. edulis* may deposit more 20:5n-3 into TAG than *C. virginica*, thus increasing the availability of storage fats at low temperatures (Florant, 1998).

During overwintering, mussels decreased the proportion of certain unsaturated fatty acids in TAG, with 20:5n-3 and 18:4n-3 declining markedly during the study, whereas oysters showed fewer changes (Figs 3 and 4). The Antarctic fish *Trematomus newnesi* preferentially oxidises unsaturated fatty acids (Lund and Sidell, 1992), with the hormone-sensitive lipase (HSL) of its adipose tissue preferring substrates in the order PUFA>monoenes>saturates (Hazel and Sidell, 2004). Mammalian HSL shows similar substrate preferences (Raclot, 2003). Although this lipase has not been studied in bivalves, the decrease in 20:5n-3 and 18:4n-3 from the TAG in mussel digestive glands suggests a similar selectivity. The minimal changes of oyster TAG during overwintering may reflect the limited TAG deposition at the start of the study more than differences in the HSL properties. Despite the fact that 20:5n-3 and 18:4n-3 decreased markedly in mussel TAG during overwintering, the more constant levels of 22:6n-3, 20:4n-6 and NMI fatty acids and the decrease of 16:0 maintained the % PUFA and the unsaturation index constant during overwintering.

The marked decrease in 20:5n-3 in TAG of mussel digestive gland during overwintering could reflect utilisation of this PUFA for maintenance during periods of reduced feeding or for initiation of gametogenesis. In support of the latter, a recent study showed that the female gonad of the scallop *Nodipecten subnodosus* specifically accumulates 20:5n-3 during gametogenesis (Palacios et al., 2005). If 20:5n-3 is also accumulated in the developing gonads of mussels, it must come from dietary sources or from stored lipids since bivalves have a limited capacity for *de novo* synthesis of long-chain PUFA (DeMoreno et al., 1976; Langdon and Waldock, 1981; Delaunay et al., 1993; Caers et al., 2003).

The rate of change of 18:4n-3 levels in digestive gland TAG fell as a function of overwintering temperature and then rose during the spring–summer simulation (Fig. 4). At first glance, a reduced mobilisation of 18:4n-3 at low temperatures would seem to help maintain fluidity, and thus the availability of the TAG, at low temperatures. However, the unsaturation index of the TAG, which is a good indicator of the melting point, was similar among mussels overwintered at 0°C, 4°C and 9°C. Therefore, it is unlikely that reduced mobilisation of 18:4n-3 counteracted an ordering effect of low temperatures on the TAG. The functional role of reduced mobilisation of 18:4n-3 at low temperatures remains unclear.

Membrane lipids: HVA and metabolism

The increase in the phospholipid to sterol ratio during the early stage of warming in mussels and oysters (Fig. 5) suggests that sterols were not used for HVA (Robertson and Hazel, 1995; Crockett, 1998; Zehmer and Hazel, 2003). If the distribution of sterols in cellular membranes is similar to that in vertebrates (Lange et al., 1989; Lange and Steck, 1996) changes in the relative importance of plasma and organelle membranes could be the cause of this pattern. HVA may have been accomplished by the decreased unsaturation of phospholipid acyl chains with increasing temperature. Furthermore, the sterols of marine bivalves include a complex mixture of phytosterols (Knauer et al., 1998) that have less of an ordering effect on membranes than cholesterol (Suckling et al., 1979).

The higher phospholipid to sterol ratio of digestive glands compared to gills agrees with patterns in rainbow trout, in which the ratio of cholesterol to phospholipid is markedly higher in

gills than in other tissues (including liver) (Robertson and Hazel, 1995). The high cholesterol levels in gills may enhance their permeability. The higher phospholipid to sterol ratio in digestive glands could reflect a greater relative importance of organelles. Accordingly, in *C. virginica*, the mitochondrial volume fraction was twice as high in digestive gland as in gills (Cherkasov et al., 2006).

The unsaturation index decreased during the beginning of warming in mussel and oyster gills and digestive glands, principally due to 22:6n-3 and 20:5n-3 (Fig. 5). Although membrane fluidity was not measured in our study, these temporal variations in 22:6n-3 and 20:5n-3 may counteract the disordering effect of rising temperatures. Accordingly, 22:6n-3 is thought to be important in controlling membrane fluidity during cold acclimation of fish (Dey et al., 1993; Buda et al., 1994; Tiku et al., 1996; Logue et al., 2000). Furthermore, a 45% increase in 20:5n-3 in gill membranes of the sea scallop *P. magellanicus* after 21 days of acclimation at 5°C correlates with an increase in membrane fluidity (Hall et al., 2002).

For both mussels and oysters, the unsaturation index of digestive gland phospholipids was not correlated with acclimation temperature, whereas a perfect negative relationship was observed in gills as predicted by HVA. Thus the unsaturation index attained a threshold value at 9°C in digestive glands while it continued to increase with falling temperatures in gills. The microenvironments of gills and digestive glands may require different physical responses by the membranes. In support of this hypothesis, the basolateral and brush border membranes from intestinal epithelia of cold- and warm-acclimated rainbow trout respond to thermal acclimation in opposite fashions (Crockett and Hazel, 1995). Basolateral membranes exhibit perfect homeoviscous efficiency due to an increase in the unsaturation index, whereas brush border membranes from cold-acclimated fish are more ordered than those from warm-acclimated fish. These authors suggest that bile and digestive lipase, two constituents of the brush border membrane microenvironment, may impose unusual physical requirements. The fact that the unsaturation index of digestive gland phospholipids did not increase beyond a threshold value at 9°C in both oysters and mussels suggests a need for stability of the membrane to counteract regular exposure to digestive enzymes and acids.

The inverse relationship between the unsaturation index of gill phospholipids and acclimation temperature was principally due to changes in 22:6n-3 and 20:5n-3, but the magnitude of the response of these fatty acids varied between oysters and mussels (Fig. 6B). The decrease of 20:5n-3 with a rise in temperature was much stronger in mussels than oysters. Reliance on 20:5n-3 for remodelling membrane phospholipids in mussels goes hand in hand with the importance of this fatty acid in TAG metabolism. As the melting point of 20:5n-3 is 10°C lower than that of 22:6n-3, efficiency of HVA in mussels is likely higher than that of oysters. HVA efficiency varies among species, ranging from 20 to 100% for thermal acclimation and averaging 70% for thermal adaptation (Hazel, 1995).

Both oxygen uptake and membrane unsaturation were higher in mussels than oysters, irrespective of temperature, time or tissue (Fig. 2B). Similarly, standard $\dot{V}_{O_2}$ and phospholipid unsaturation show a positive correlation in comparisons of wild and selectively bred hard clams (Pernet et al., 2006b). The unsaturation of membrane phospholipids is positively correlated with basal metabolic rate (Hulbert and Else, 1999; Hulbert and Else, 2005) in allometric comparisons of mammals and birds. The positive relationship between standard $\dot{V}_{O_2}$ and phospholipid unsaturation in mussels and oysters suggests that Hulbert's theory of membranes as metabolic pacemakers may be applicable to invertebrates.

Membrane lipids: why do 20:4n-6 and NMI fatty acids not follow HVA?

One of the most important functions of C_{20} PUFA is as precursors of eicosanoids, a group of hormones that includes prostaglandins, leukotrienes and hydroxyeicosatetraenoic acids (Smith and Murphy, 2003). The increase in 20:4n-6 in mussels during the spring–summer simulation period (Fig. 7) may compensate for the concomitant decrease in 20:5n-3. Eicosanoid production is associated with stressful or energetically expensive situations, such as gametogenesis and spawning in bivalves or stimulation of immune function in other invertebrates (Osada et al., 1989; Stanley and Howard, 1998). Since eicosanoids produced from 20:4n-6 are generally more active than those produced from 20:5n-3, the replacement of 20:5n-3 by 20:4n-6 in mussels may have significant impacts. Levels of 20:4n-6 in mussels may have risen during the spring–summer simulation through the stimulation of immune function due to increased bacterial loads in the seawater as temperature rose. Similarly, an increase in 20:4n-6 in scallop and haddock larvae coincided with elevated mortality and exposure to pathogenic and opportunistic microbes (Pernet et al., 2005; Plante et al., 2007). Bivalve haemocyte membrane lipids contain elevated amounts of 20:4n-6, presumably to regulate immune responses (Delaporte et al., 2003). We suggest that the increase in 20:4n-6 levels during the spring–summer simulation may reflect an increased demand for 20:4n-6-rich immune cells to control bacterial proliferation in mussels (Delaporte et al., 2006).

In contrast to our previous work on juvenile hard clams, where we observed an increase in 22:2 NMI during a decrease in temperature (Pernet et al., 2006b), NMI fatty acids in mussel and oyster tissues showed no consistent response to temperature change (Fig. 8). In contrast to essential PUFA, NMI fatty acids are synthesised *de novo* by bivalves (Zhukova, 1991). Therefore, the synthesis of NMI fatty acids was suggested as an alternative to the selective incorporation of essential PUFA at low temperatures, particularly when phytoplankton concentrations are low or when feeding has ceased (Pernet et al., 2006b). When extended, this argument would suggest that the synthesis of NMI fatty acids could be important in animals that do not have any source of PUFA, either from exogenous feeding or endogenous reserves. Although endogenous TAGs are generally considered as energy reserves, they also constitute a PUFA reservoir that could be used for remodelling membrane lipids as the need arises. This hypothesis suggests that oysters had more changes in NMI than mussels because they had fewer TAG reserves at the onset of overwintering.

In conclusion, we found major differences between species in lipid dynamics that correlate with the species' thermal habitats, with mussels apparently maintaining the fluidity of

digestive gland TAG during over-wintering, presumably to facilitate their use at cold temperatures. The exploitation of cold habitats by oysters may be limited by reduced feeding at low temperatures and their limited changes in TAG fluidity during over-wintering at cold temperatures. On the other hand, over-wintering led to marked membrane remodelling in both oysters and mussels. In both species, the unsaturation of gill phospholipids perfectly followed HVA, whereas in digestive glands, unsaturation and temperature were only related down to 9°C. By comparing mussels and oysters before and during acclimation to three over-wintering temperatures, and after the spring revival, we obtained a comprehensive view of their lipid dynamics and physiological responses. More broadly, this study suggests that lipids can be used as biochemical indicators of condition to gain mechanistic insight into the effects of climate change on economically important poikilotherms that occur in stressful habitats or at the edge of their distribution range.

List of abbreviations

AFDM	ash-free dry mass
CHGRA	*Chaetoceros muelleri*
CR	clearance rate
FAME	fatty acid methyl esters
HSL	hormone-sensitive lipase
HVA	homeoviscous adaptation
NMI	non-methylene-interrupted dienoic fatty acids
PUFA	polyunsaturated fatty acid
ST	sterol
TAG	triglyceride
TISO	*Isochrysis galbana*
$\dot{V}_{O_2}$	routine metabolic rate
$\dot{V}_{O_2min}$	standard metabolic rate

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