Influences of Subzero Thermal Acclimation on Mitochondrial Membrane Composition of Temperate Zone Marine Bivalve Mollusks

Todd E. Gillis[a] and James S. Ballantyne[b,*]

[a]Cardiac Membrane Research Laboratory, School of Kinesiology, Simon Fraser University, Burnaby, British Columbia V5A 1S6, and [b]Department of Zoology, University of Guelph, Guelph, Ontario, N1G-2W1 Canada

ABSTRACT: The phospholipid and phospholipid fatty acid composition of gill mitochondrial membranes from two temperate zone marine bivalve mollusks, the quahog, *Mercenaria mercenaria,* and the American oyster, *Crassostrea virginica,* were examined after acclimation to 12 and −1°C. Cardiolipin (CL) was the only phospholipid with proportions altered upon acclimation to −1°C, increasing 188% in the mitochondrial membranes of *M. mercenaria.* Although the ratio of bilayer stabilizing to destabilizing lipids is frequently associated with cold acclimation in ectothermic species, no change was found in this ratio in either of the species. Polyunsaturated fatty acids (PUFA) were found only to increase in *C. virginica* with cold acclimation, with total n-3 PUFA increasing in the phospholipid phosphatidylethanolamine, total n-6 PUFA increasing in CL, and total PUFA increasing in phosphatidylinositol. Monounsaturated fatty acids, not PUFA, were found to have increased in *M. mercenaria,* with 18:1n-9 increasing by 150% in CL, and 20:1 increasing in both CL and phosphatidylcholine, by 146 and 192%, respectively. These manipulations of membrane phospholipid and fatty acid composition may represent an attempt by these species to help maintain membrane function at low temperatures.

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Ectothermic animals alter the phospholipid and fatty acid composition of their cellular membranes in order to defend membrane function from the effects of lowered environmental temperature. This is accomplished by strategies which include alterations in the proportions of membrane phospholipids and the fatty acid composition of membrane phospholipids (1,2).

While there are numerous studies of the effects of thermal acclimation on marine fishes, to date, no study has been made of membrane acclimation in ectothermic species below 5°C. Adaptation to subzero temperatures may demand previously unidentified changes to membrane composition. Changes in the physical properties of subzero water, such as pH, density, as well as the large impact of very low temperature on the kinetic properties of biological membranes may pose a significant challenge to the maintenance of membrane function. Many temperate zone species are exposed to subzero temperatures during the winter. Marine mollusks, owing to their limited mobility, are unable to migrate to warmer temperatures and are therefore seasonally exposed to such low temperature. Only limited study has been done of thermal effects on the fatty acid composition of bivalve mollusks (3–5).

To determine how marine mollusks utilize phospholipid composition to help maintain membrane function at subzero temperatures, we examined the mitochondrial membranes of two common species of temperate zone mollusk: the American oyster, *Crassostrea virginica*, and the quahog, *Mercenaria mercenaria* with acclimation to 12 and −1°C. These two species demonstrate tissue growth during the winter (4,6), suggesting that cellular activity and therefore membrane function are maintained during this time. The animals used in this study were obtained from waters which range in temperature from 12°C in the summer to −1.5°C in the winter. The acclimation temperatures used therefore resemble those to which the animals are accustomed.

MATERIALS AND METHODS

Quahogs, *M. mercenaria,* and American oysters, *C. virginica,* cultured in Prince Edward Island, were obtained from a local seafood supplier in September. The animals were held at 12.0 ± 0.7°C in a flow-through seawater system (32‰) at the University of Guelph for a period of 2 mon prior to any temperature manipulation in 250-L tanks. A blended mixture of frozen krill was fed to the animals three times a week prior to and during the experimental period. The photoperiod of the holding facilities in which the bivalves were kept was 12 h dark/12 h light. Individuals of each species were randomly assigned to one of two experimental tanks. One tank was maintained at 12.0°C for the entire experimental period, while the temperature of the other tank was lowered to −1.0°C over a period of 26 d by decreasing the temperature by 1°C every 2 d using a water chiller (Ramco Inc., Columbus, OH). Once −1.0°C was achieved in the cold tank, both tanks were kept at

*To whom correspondence should be addressed.
E-mail: jballant@uoguelph.ca

Abbreviations: CL, cardiolipin; monoene, monounsaturated fatty acid; PC, phosphatidylcholine; PC/(PE + CL), phosphatidylcholine to (phosphatidylethanolamine + cardiolipin) ratio; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SPH, sphingomyelin.

their respective temperatures for 65 d. Both acclimation tanks remained in the flow-through biological filtration system throughout the experimental period.

Mitochondrial isolation. The gills were excised and placed in ice-cold isolation medium consisting of 400 mM sucrose, 1 mM EDTA, 2 mM EGTA, 10 mM HEPES pH 7.4 at 20°C, and 1% bovine serum albumin (essentially fatty-acid free). The gills of two animals were pooled to obtain a sufficient mass of tissue, which was then weighed, minced, and suspended in ice-cold isolation medium in a ratio of 1 g of tissue to 5 mL of isolation medium. This mixture was homogenized with three passes of a loosely fitting Teflon pestle in a Potter-Elvehjem homogenizer. The homogenate was then centrifuged for 15 min at $1,200 \times g$. The resulting supernatant was centrifuged for 15 min at $13,600 \times g$; the pellet was removed and resuspended in 1 mL isolation medium, frozen in liquid nitrogen, and then stored at –80°C.

Analysis of mitochondrial membrane phospholipid composition. Mitochondrial lipids were extracted by a modification of the method of Bligh and Dyer (7) as described by Glemet and Ballantyne (8). The thin-layer chromatography method used for the separation of phospholipids from each other and from neutral lipids is essentially that of Holub and Skeaff (9). The lipid samples dissolved in 25 µL of a 2:1 mixture of chloroform/methanol were run on Merck silica gel 60 glass precoated 20×20 cm plates with a 0.25-mm layer thickness (British Drug House, Toronto, Canada). Phospholipid standards were run in separate lanes on the plates with the membrane lipids in separate lanes for reference. The solvent system used to develop the plates consisted of chloroform/methanol/acetic acid/water in a 50:37.5:3.5:2 (by vol) mixture. Once the solvent system had run within 0.5 cm of the top of the plate, the plate was removed and allowed to air dry then sprayed with a saturated solution of 2,7-dichlorofluorescein in methanol/water, 1:1 (vol/vol). The plate was then placed in a raised-bottom developing chamber containing 50–100 mL of 25% ammonium hydroxide for 5 min. The phospholipid bands were then visualized under ultraviolet light at a wavelength of 366 nm. This method is effective in completely separating the phospholipids: phosphatidylethanolamine (PE), phosphatidylcholine (PC), cardiolipin (CL), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SPH) (9).

The preparation of fatty acid methyl esters from the separated phospholipids followed the method of Holub and Skeaff (9). The individual phospholipid bands were scraped into glass Kimax tubes containing 2 mL of 6% H_2SO_4 in methanol and 10 µg of heptadecanoic acid (17:0) as an internal standard. The sealed tubes were then mixed and incubated at 80°C for 2 h. Upon completion, the tubes were cooled to room temperature, 2 mL of petroleum ether was added, and the tubes were then vortexed for 60 s. Double-distilled H_2O (1 mL) was added, and the tubes were vortexed for a further 30 s. The upper petroleum ether phase containing the eluted fatty acid methyl esters was removed and transferred to a glass vial and stored at –20°C until further processing. These samples were than analyzed as described by Glemet and Ballantyne (8), using a Hewlett-Packard (Mississauga, Ontario, Canada), HP5890 series II gas chromatograph fitted with a flame-ionization detector, an automatic injector (7673A; Hewlett-Packard) and an electronic pressure control program. Fatty acid methyl esters were analyzed on DB 225 megabore fused-silica column (Chromatographic Specialties Inc., Brockville, Ontario, Canada). Chain lengths shorter than C:14 were not resolved and therefore were not reported. Phospholipid content was calculated by summing the concentrations of fatty acids for each phospholipid, taking into account the number of fatty acids esterified to each phospholipid. The quantitative recovery of a known PI standard following these methods of thin-layer chromatography, methylation, and gas chromatography is 95% (9).

Extraction and quantification of total lipids from frozen krill. Total lipids of the frozen krill fed to the animals throughout the thermal acclimation study were extracted and quantified by the same methods used for the mitochondrial preparations.

Measurement of marker enzymes. Marker enzymes were measured in both the initial homogenate and in the mitochondrial preparation. The following marker enzymes were used: cytochrome C oxidase (mitochondrial membrane), glucose 6-phosphatase (endoplasmic reticulum), Na^+,K^+-ATPase (plasma membrane), and peroxidase (peroxisomes). A temperature-controlled Hewlett-Packard HP8452 diode array spectrophotometer maintained at 10°C by a Haake D8 circulating water bath (Haake Buchler Instruments Inc., Saddlebrook, NJ) was used for all enzyme assays. The methods for the specific enzyme measurements were as follows:

(i) Cytochrome C oxidase. The oxidation of fully reduced cytochrome C (50 µM) by the sample in 50 mM imidazole buffer (pH 7.5) at $\lambda = 550$ nm was measured by the method as described by Stuart and Ballantyne (10).

(ii) Glucose 6-phosphatase. The method used to measure the activity of the sample was modified from that of Aronson and Touster (11). The assay conditions were as follows: 50 mM imidazole (pH 6.5), 2 mM NAD, 20 mM glucose-6-phosphate, 9.6 units glutamate dehydrogenase, and 0.2 units malate dehydrogenase. Phosphorus production after 1 h incubation was measured at $\lambda = 820$ nm.

(iii) Na^+,K^+-ATPase. The method used to measure the activity of the sample was modified from that of Chen *et al*. (12). The sample was suspended in an environment of: 50 mM imidazole buffer (pH 7.5), 80 mM NaCl, 20 mM KCl, 6 mM $MgCl_2$, 1.5 mM EGTA, 10 mM NaF, and 3 mM ATP. Phosphorus production after 1 h of incubation was measured at $\lambda =$ 820 nm. Background ATPase activity was measured by comparing the rates of the enzyme in the presence to that in the absence of ouabain (10 mM). As ouabain acts to inhibit Na^+,K^+-ATPase, the difference in activity between measurement with and without ouabian equals the activity of Na^+,K^+-ATPase.

(iv) Peroxidase. Activity of the sample in 50 mM imidazole buffer (pH 7.5) was measured after the addition of 3 mM H_2O_2 at $\lambda = 240$ nm (10).

Protein determination. Protein content was determined by the method of Bradford (13) using bovine serum albumin as a standard. Mitochondrial and tissue protein was determined by measuring the difference between the protein concentration in the isolation medium and the mitochondria suspension.

Chemicals. The lipid standard used (Nu-Chek-Prep., Inc., Elysian, MN) was augmented by the addition of menhaden oil extract. Menhaden oil contains fatty acids not found in the commercial standard but which are present in the lipids of most marine and freshwater organisms. Solvents were obtained from Fisher Scientific Ltd. (Whitby, Ontario, Canada) and were of American Chemical Society-certified grade. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available.

Statistical analysis. Differences between the percentages of individual phospholipids and fatty acids from phospholipids were tested using a one-factor analysis of variance followed by Bonferroni's *posthoc* test. Statistical comparisons were only made with fatty acids which represented greater than 1.0% of the total. In all cases, the assumptions of parametric tests were verified and a $\log_{10}$ or square root transformation was used when necessary to normalize the data (14).

RESULTS

Characterization of mitochondrial membrane purity. The isolation procedure used resulted in a substantial enrichment of the mitochondrial membrane marker cytochrome-C-oxidase in relation to the other marker enzymes for plasma membrane, peroxisomes, and endoplasmic reticulum (Table 1), indicating little contamination of the mitochondria with these cellular membranes. A second indication that the membranes recovered were not significantly contaminated by other membranes was the almost complete absence of SPH (<1.0%). This phospholipid is found only in trace amounts in the mitochondrial membrane (1%) while it is prevalent in lysosomes, plasma membrane, and Golgi membrane, representing as much as 20, 16, and 8%, respectively, of total phospholipid in these membranes (15). The very low proportion of this phospholipid in these membranes is evidence that there was minimal contamination of the membrane preparations by these organelles in the present study.

Phospholipid composition. The most prevalent phospholipid in the mitochondrial membranes of both acclimation groups of *M. mercenaria* was PE equaling 41–42% of total membrane phospholipid (Table 2). In *C. virginica* PE and PC were the most prevalent phospholipids, equaling a cumulative 75% of membrane phospholipid in both acclimation groups (Table 2). The phospholipid SPH was present in trace amounts in the mitochondrial membranes of both species but in all preparations represented less than 1% of total membrane phospholipid. The proportion of CL increased in *M. mercenaria* with cold acclimation but not in *C. virginica* (Table 2). There was no change in the ratio of bilayer stabilizing- (PC) to bilayer-destabilizing phospholipids (PE, CL) in either of the two species with cold acclimation.

Gill mitochondrial phospholipid fatty acids. Polyunsaturated fatty acids (PUFA) were the predominant fatty acids in the mitochondrial membranes of both species from either acclimation group (Table 3), resulting from the high proportions of 22:2n-6 and 22:6n-3 in the membranes of *M. mercenaria* and 20:5n-3 and 22:6n-3 in *C. virginica* (Table 3). The saturated fatty acid (SFA) 16:0 represented the largest proportion of individual fatty acids in the membranes of both species (Table 3). The only effect of cold acclimation on the cumulative fatty profile of either species was a decrease in 22:4n-6 in *M. mercenaria* by 33.6% (Table 3).

Acclimation of *M. mercenaria* to −1°C caused alteration in the fatty acid composition of mitochondrial membrane PE, PC, and CL while PE, CL, PS, and PI were altered in *C. virginica.* In mitochondrial membrane PE of both species, PUFA were the dominant class of fatty acid constituting from 67.0–71.4% of the fatty acids in this phospholipid in both species (Table 4). With cold acclimation there was a decrease in the n-6 PUFA content in PE from the mitochondrial membranes of *M. mercenaria.* SFA in PE were found to increase with cold acclimation in *M. mercenaria* caused by a compositional increase in the fatty acid 16:0 (Fig. 1). In *C. virginica* there was an increase in n-3 PUFA content and unsaturation index in PE caused by increased proportions of the fatty acid 22:6n-3 (Fig. 1).

PUFA were the most prevalent fatty acids in mitochondrial membrane PC in both species ranging from 52.5–60.3% (Table 4). Cold acclimation of *M. mercenaria* increased the proportion of monounsaturated fatty acids (monoenes) due to an increase in the fatty acid 20:1 (Fig. 2); 22:4n-6 was found to decrease in this phospholipid. The fatty acid composition of PC from the mitochondrial membranes of *C. virginica* was not affected by cold acclimation.

TABLE 1
Assessment of Purity (fold purification) of Mitochondrial Membranes from Gill Tissue of *Crassostrea virginica* and *Mercenaria mercenaria* Acclimated to Either −1 or 12°C[a]

	C. virginica		*M. mercenaria*	
	−1°C	12°C	−1°C	12°C
Na^+,K^+-ATPase	0.054 ± 0.016	0.041 ± 0.017	0.069 ± 0.019	0.062 ± 0.015
Cytochrome C oxidase	24.84 ± 3.01	21.22 ± 4.56	30.09 ± 7.11	27.72 ± 4.35
Peroxidase	0.059 ± 0.007	0.046 ± 0.015	0.038 ± 0.012	0.030 ± 0.008
Glucose 6-phosphatase	0.29 ± 0.044	0.27 ± 0.076	0.16 ± 0.037	0.11 ± 0.046

[a]Values are means ± SE for $n = 4$. Fold purification = (activity in mitochondrial pellet/mg protein)/(activity in homogenate/mg protein).

TABLE 2
Percentage by mol of Phospholipids in Gill Mitochondria from *Mercenaria mercenaria* and *Crassostrea virginica* Acclimated to Either –1.0 or 12.0°C[a]

	M. mercenaria		*C. virginica*	
Phospholipid	–1.0 ± 0.5°C (n = 8)	12.0 ± 0.7°C (n = 8)	–1.0 ± 0.5°C (n = 8)	12.0 ± 0.7°C (n = 8)
Phosphatidylethanolamine	40.60 ± 1.22	41.80 ± 1.02	30.70 ± 0.59	32.58 ± 2.01
Phosphatidylcholine	20.11 ± 0.82	23.08 ± 1.09	44.10 ± 0.78	42.41 ± 1.92
Cardiolipin[b]	4.92 ± 1.42	1.70 ± 0.22	1.80 ± 0.39	3.14 ± 0.73
Phosphatidylserine	14.85 ± 1.12	12.77 ± 1.60	9.58 ± 0.58	8.61 ± 1.32
Phosphatidylinositol	19.50 ± 0.76	20.62 ± 0.84	13.70 ± 0.57	13.36 ± 0.73
PC/(PE + CL)[c]	0.44 ± 0.021	0.54 ± 0.030	1.35 ± 0.04	1.21 ± 0.15

[a]Values are presented as means ± SEM.
[b]Statistically significant difference between *M. mercenaria* acclimated to –1.0 and 12.0°C.
[c]PC = phosphatidylcholine, PE = phosphatidylethanolamine, CL = cardiolipin.

TABLE 3
Cumulative Percentages of Individual Fatty Acids in Gill Mitochondria from *Mercenaria mercenaria* and *Crassostrea virginica* Acclimated to Either –1.0 or 12.0°C[a]

	M. mercenaria		*C. virginica*	
Fatty acid	–1.0 ± 0.5°C (n = 8)	12.0 ± 0.7°C (n = 8)	–1.0 ± 0.5°C (n = 8)	12.0 ± 0.7°C (n = 8)
14:0	0.4 ± 0.1	0.2 ± 0.1	1.1 ± 0.1	0.9 ± 0.1
14:1	0.5 ± 0.2	0.3 ± 0.2	0.6 ± 0.2	0.7 ± 0.4
16:0	22.6 ± 0.8	23.9 ± 1.6	20.6 ± 0.5	20.5 ± 1.0
16:1	4.6 ± 0.8	6.6 ± 0.7	3.7 ± 0.3	5.0 ± 0.5
18:0	8.1 ± 1.5	6.5 ± 0.4	5.2 ± 0.2	5.2 ± 0.3
18:1n-9	3.9 ± 0.4	3.1 ± 0.4	6.2 ± 0.3	6.1 ± 0.5
18:2n-6	n.d.[b]	n.d.	n.d.	n.d.
18:3n-3	1.0 ± 0.2	0.9 ± 0.3	1.0 ± 0.1	1.6 ± 0.3
18:4n-3	0.9 ± 0.3	0.4 ± 0.2	0.1 ± 0.1c	1.1 ± 0.4
20:0	1.6 ± 0.2	1.5 ± 0.2	1.7 ± 0.2	2.0 ± 0.3
20:1	7.3 ± 0.6	6.6 ± 0.7	10.6 ± 0.5	10.2 ± 0.9
20:2n-6	0.9 ± 0.1	1.0 ± 0.1	0.1 ± 0.02[c]	0.1 ± 0.03[c]
20:3n-6	2.4 ± 0.1	3.3 ± 0.2	5.2 ± 0.4	5.2 ± 0.3
20:4n-6	1.6 ± 0.4	0.7 ± 0.1	1.6 ± 0.3	1.0 ± 0.2
20:3n-3	0.2 ± 0.1[c]	n.d.	0.1 ± 0.02[c]	0.2 ± 0.2[c]
20:4n-3	0.2 ± 0.1	0.1 ± 0.1[c]	0.3 ± 0.1	0.2 ± 0.1[c]
20:5n-3	6.7 ± 0.5	6.2 ± 0.5	13.4 ± 0.3	12.0 ± 0.6
22:0	0.3 ± 0.1	n.d.	0.4 ± 0.2	0.4 ± 0.3[c]
22:1*iso*	0.1 ± 0.1[c]	0.1 ± 0.2[c]	0.02 ± 0.02[c]	0.2 ± 0.1
22:2n-6	14.7 ± 0.6	15.2 ± 1.2	8.2 ± 0.2	9.9 ± 0.6
23:0	1.1 ± 0.1	1.8± 0.1	0.5 ± 0.03	0.6 ± 0.1
22:4n-6	3.4 ± 0.1	4.5 ± 0.3[d]	1.2 ± 0.1	1.2 ± 0.1
22:5n-6	n.d.	n.d.	n.d.	n.d.
22:5n-3	4.0 ± 0.3	3.4 ± 0.3	2.0 ± 0.1	2.3 ± 0.4
22:6n-3	13.1 ± 0.8	13.7 ± 0.8	16.3 ± 0.7	13.7 ± 0.5
24:0	n.d.	n.d.	n.d.	n.d.
24:1	n.d.	n.d.	n.d.	n.d.
Total	100	100	100	100
Saturated fatty acids	34.3 ± 1.3	33.9 ± 1.2	29.5 ±0.7	29.7 ± 0.9
Monounsaturated fatty acids	16.5 ± 1.1	16.7 ± 0.9	21.1 ± 0.8	22.3 ± 1.5
Polyunsaturated fatty acids	49.2 ± 1.1	49.4 ± 1.4	49.5 ± 0.9	48.0 ± 1.1
n-3 Polyunsaturated fatty acids	26.2 ± 1.0	24.8 ± 1.1	33.2 ± 0.8	30.7 ± 0.6
n-6 Polyunsaturated fatty acids	23.0 ± 0.5	24.7 ± 1.4	16.3 ± 0.5	17.3 ± 0.8
n-3/n-6 Polyunsaturated fatty acids	1.1 ± 0.1	1.0 ± 0.1	2.0 ± 0.1	1.8 ± 0.1
Unsaturation index[e]	215.0 ± 5.8	214.5 ± 6.1	243.4 ± 4.3	229.3 ± 3.7

[a]Values are presented as means ± SEM.
[b]Not detectable.
[c]Three or more values expressed in mean were not detectable, therefore included as zeroes.
[d]Statistically significant difference between treatment groups of the species in this column.
[e]Unsaturation index = $\sum m_i \cdot n_i$, where m_i is the mole percentage and n_i is the number of C–C double bonds of the fatty acid *i*.

TABLE 4
Percentages of Fatty Acid Classes in the Membrane Phospholipids from the Gill Mitochondria of *Mercenaria mercenaria* and *Crassostrea virginica* Acclimated to Either 12.0 or −1.0°C[a]

		PE		PC		CL		PS		PI	
Species	Class	12°C	−1°C	12°C	−1°C	12°C	−1°C	12°C	−1°C	12°C	−1°C
M. mercenaria	SFA	11.7 ± 0.6[b]	16.0 ± 0.7	24.4 ± 1.8	20.3 ± 1.1	32.1 ± 8.7	39.5 ± 4.6	44.9 ± 8.6	28.8 ± 3.3	86.9 ± 2.3	90.9 ± 1.5
(*n* = 8)	Monoenes	18.5 ± 2.1	14.4 ± 1.7	15.3 ± 1.1[b]	21.1 ± 1.9	7.3 ± 3.2[b]	13.5 ± 1.1	19.4 ± 2.8	17.4 ± 3.4	9.2 ± 2.1	5.3 ± 1.5
	PUFA	69.8 ± 1.7	69.5 ± 1.2	60.3 ± 2.2	58.6 ± 1.9	60.6 ± 8.8	47.0 ± 5.0	35.7 ± 9.2	53.8 ± 4.8	3.9 ± 0.4	3.8 ± 0.5
	n-3 PUFA	27.6 ± 1.6	33.7 ± 1.8	44.2 ± 1.9	45.0 ± 1.3	43.0 ± 7.8	35.6 ± 4.1	14.2 ± 4.0	14.5 ± 1.2	1.1 ± 0.2	1.5 ± 0.2
	n-6 PUFA	42.2 ± 1.5[b]	35.9 ± 0.9	16.1 ± 0.8	13.6 ± 1.0	17.6 ± 5.1[b]	11.4 ± 1.0	21.5 ± 8.2	39.3 ± 5.3	2.8 ± 0.3	2.4 ± 0.3
	Unsat. ind.[c]	273.0 ± 7.3	288.0 ± 8.2	308.4 ± 10.5	301.7 ± 8.1	289.6 ± 42.2	238.4 ± 24.5	148.9 ± 29.4	181.9 ± 6.8	23.0 ± 3.1	19.4 ± 2.1
C. virginica	SFA	17.4 ± 0.8	16.3 ± 0.6	24.8 ± 1.0	26.1 ± 0.8	24.8 ± 1.8	26.2 ± 4.0	28.5 ± 1.4	29.2 ± 1.5	83.9 ± 4.2[b]	70.1 ± 1.1
(*n* = 8)	Monoenes	15.6 ± 2.4	12.2 ± 0.7	22.8 ± 1.6	20.8 ± 0.3	26.9 ± 4.3	25.4 ± 7.7	20.9 ± 0.9	20.1 ± 2.8	12.8 ± 3.0	17.6 ± 1.0
	PUFA	67.0 ± 2.8	71.5 ± 1.0	52.5 ± 1.0	53.0 ± 0.9	48.3 ± 4.9	48.4 ± 9.4	50.7 ± 1.5	50.8 ± 2.7	3.3 ± 1.4[b]	12.4 ± 0.9
	n-3 PUFA	36.7 ± 1.3[b]	43.6 ± 1.4	39.3 ± 0.5	40.1 ± 0.7	43.0 ± 4.2	37.3 ± 8.6	15.3 ± 0.9	17.4 ± 0.9	2.1 ± 1.2[b]	5.8 ± 0.4
	n-6 PUFA	30.3 ±1.6	27.9 ± 0.8	13.2 ± 0.6	12.9 ± 0.5	5.4 ± 1.3[b]	11.1 ± 1.5	35.3 ± 1.1	33.3 ± 2.2	1.2 ± 0.3[b]	6.6 ± 0.6
	Unsat. ind.[c]	286.2 ± 8.3[b]	321.4 ± 7.4	269.8 ± 4.8	275.0 ± 4.3	286.6 ± 25.6	273.6 ± 50.2	178.5 ± 5.8	187.5 ± 7.0	24.8 ± 7.8[b]	67.7 ± 3.4

[a]Values are presented as means ± SEM. PS, phosphatidylserine; PI, phosphatidylinositol; SFA, saturated fatty acids; monoenes, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. See Table 2 for other abbreviations.
[b]Statistically significant difference between treatment groups of the species in this column.
[c]Unsaturation index (unsat. ind.) = $\sum m_i \cdot n_i$, where m_i is the mole percentage and n_i is the number of C–C double bonds of the fatty acid *i*.

In mitochondrial CL, PUFA were the predominant fatty acids in both species, representing from 47.0–60.6% of the fatty acids in this phospholipid (Table 4). Cold acclimation of *M. mercenaria* increased the proportion of monoenes in CL (Table 4) due to increased levels of 18:1n-9 and 20:1 (Fig. 3). Additionally, there was a decrease in n-6 PUFA in this phospholipid (Table 4). Cold acclimation of *C. virginica* caused an increase in the content of n-6 PUFA as well as in 20:5n-3 while the level of the fatty acid 16:1 was found to decrease (Fig. 3).

PUFA were the most prevalent fatty acids in mitochondrial membrane PS in both species, ranging from 35.7–53.8% of total fatty acid content. This phospholipid was not affected by cold acclimation in *M. mercenaria*; however, there was an increase in the content of 20:4n-6 in this mitochondrial membrane phospholipid in *C. virginica* increasing from 0.57 ± 0.16 to 2.48 ± 0.96%.

In mitochondrial membrane PI, SFA were the predominant fatty acids in both species equaling 70–90%. This result is due to the 16:0 content in this phospholipid (Fig. 4). Cold acclimation of *M. mercenaria* did not affect the fatty acid content of this phospholipid. In *C. virginica*, cold acclimation caused a decrease in SFA content due to a decrease in 16:0. Total PUFA content of PI as well as n-3 PUFA content, n-6 PUFA content, and the unsaturation index were found to increase in

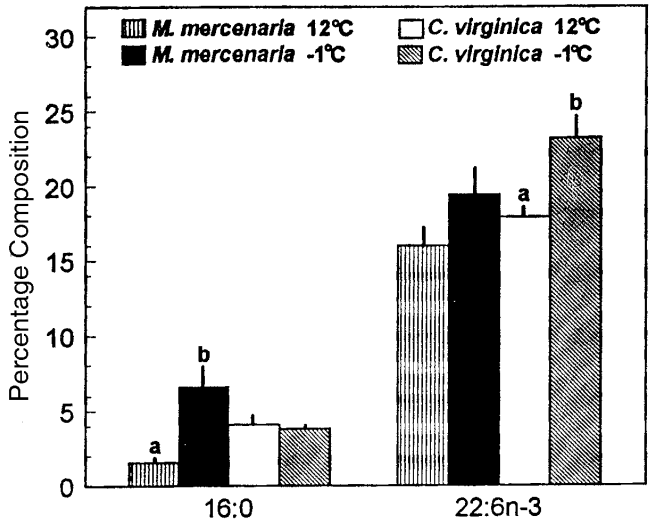

FIG. 1. Percentages of the fatty acids 16:0 and 22:6n-3 in the mitochondrial membrane phospholipid phosphatidylethanolamine of the bivalve mollusks, quahog clam, *Mercenaria mercenaria,* and American oyster, *Crassostrea virginica,* acclimated to either 12 or −1°C. Values, within species, are statistically different if they are marked with the letters a and b. Values presented as means ± SEM.

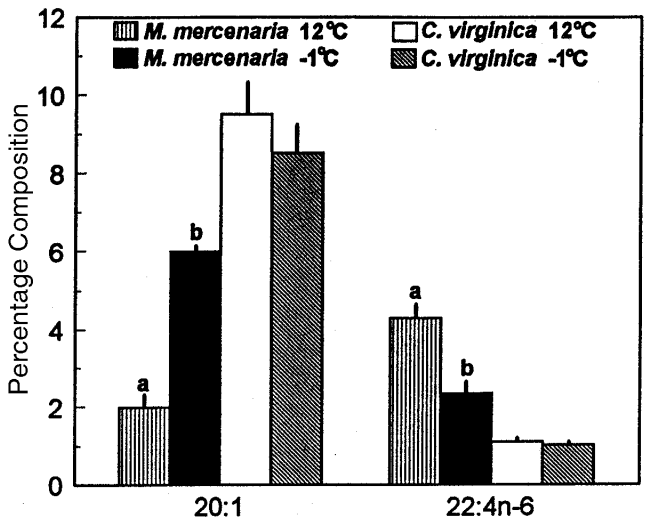

FIG. 2. Percentages of the fatty acids 20:1 and 22:4n-6 in the mitochondrial membrane phospholipid phosphatidylcholine of the bivalve mollusks, quahog clam, *Mercenaria mercenaria,* and American oyster, *Crassostrea virginica,* acclimated to either 12 or −1°C. Values, within species, are statistically different if they are marked with the letters a and b. Values presented as means ± SEM.

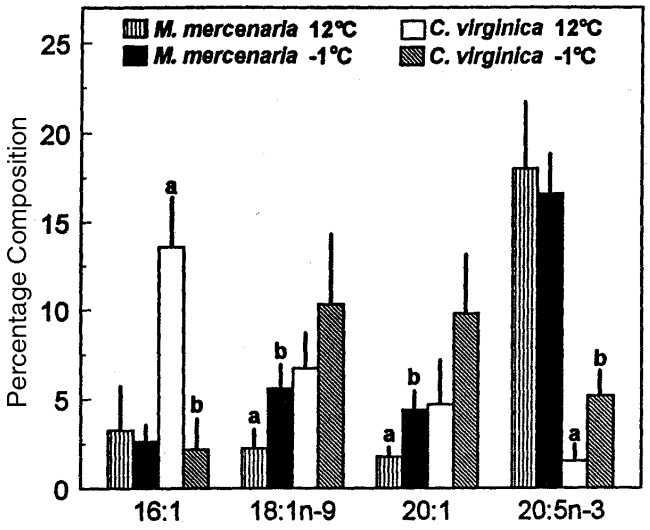

FIG. 3. Percentages of the fatty acids 16:1, 18:1n-9, 20:1, and 20:5n-3 in the mitochondrial membrane phospholipid cardiolipin of the bivalve mollusks, quahog clam, *Mercenaria mercenaria,* and American oyster, *Crassostrea virginica,* acclimated to either 12 or −1°C. Values, within species, are statistically different if they are marked with the letters a and b. Values presented as means ± SEM.

C. virginica with cold acclimation (Table 4). These results were due to increases in the fatty acids 20:2n-6 and 20:5n-3 (Fig. 4). The monoene 20:1 was also found to increase with cold acclimation in this phospholipid with cold acclimation (Fig. 4).

Fatty acid composition of krill. SFA were the most abundant fatty acids in the total lipid equaling 52%, PUFA composed primarily of 20:5n-3 and 22:6n-3 represented 32% of total fatty acid content (Table 5).

Mitochondrial protein. No change occurred in the concentration of mitochondrial protein with cold acclimation in either of the two species. In *M. mercenaria* mitochondrial protein was 6.43 ± 0.81 mg/g gill in animals acclimated to 12°C and 7.02 ± 0.92 mg/g gill in animals acclimated to −1°C. In *C. virginica*, mitochondrial protein was 10.14 ± 1.37 mg/g gill and 11.03 ± 1.52 mg/g gill in animals acclimated to 12 and −1°C, respectively. All protein values presented are from an *n* of 4.

TABLE 5
Percentages of Individual Fatty Acids in the Total Lipids Extracted from the Frozen Krill Used to Feed Both Species of Mollusk During the Thermal Acclimation Study

Fatty acid	mol%
14:0	13.70
14:1	0.97
16:0	22.08
16:1	8.86
18:0	16.00
18:1n-9	4.66
18:3n-3	1.27
18:4n-3	3.24
20:0	0.02
20:1	1.33
20:3n-3	0.07
20:4n-3	0.21
20:5n-3	17.68
22:0	0.068
22:1	0.77
22:2n-6	0.51
Saturated fatty acids	52.00
Monounsaturated fatty acids	16.84
Polyunsaturated fatty acids	31.38
n-3 Polyunsaturated fatty acids	30.7
n-6 Polyunsaturated fatty acids	0.67
n-3/n-6	45.57
Unsaturation index[a]	173.05

[a]Unsaturation index = $\Sigma m_i \cdot n_i$, where m_i is the mole percentage and n_i is the number of C–C double bonds of the fatty acid *i.*

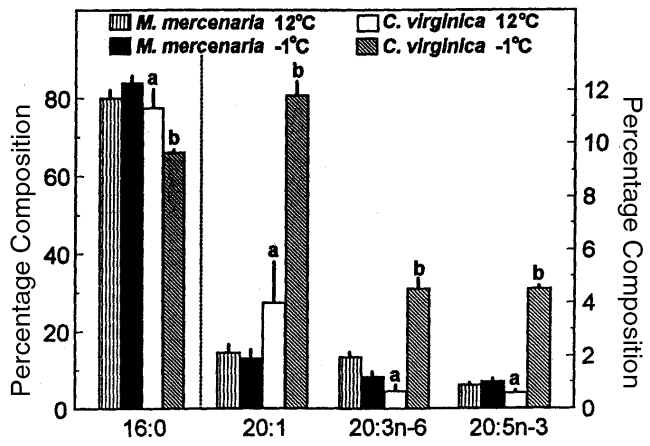

FIG. 4. Percentages of the fatty acids 16:0, 20:1, 20:3n-6, and 20:5n-3 in the mitochondrial membrane phospholipid phosphatidylinositol of the bivalve mollusks, quahog clam, *Mercenaria mercenaria,* and American oyster, *Crassostrea virginica,* acclimated to either 12 or −1°C. Values, within species, are statistically different if they are marked with the letters a and b. Values presented as means ± SEM.

DISCUSSION

Many of the observed changes in the composition of the mitochondrial membranes in the marine mollusks in the present study differ from what was previously reported in other ectothermic species during thermal acclimation. Previous laboratory studies demonstrated that the ratio of bilayer-stabilizing to bilayer-destabilizing lipids decreases in the cellular membranes of ectothermic animals with cold acclimation (16–18). As in other cellular membranes, PC stabilizes the bilayer in mitochondrial membranes as it favors the formation of a laminar bilayer, while PE destabilizes the bilayer by keeping the membrane close to the phase transition between laminar and hexagonal (H_{II}) phase conformations (16,19). Mitochondria contain a second bilayer-destabilizing phospholipid, CL. This phospholipid adopts the H_{II} phase conformation in the presence of Ca^{2+} (20,21) and the membrane protein cytochrome C oxidase (22), both of which are associated with the inner mitochondrial membrane. Cold acclimation of either species of mollusk in the present study did not alter the ratio of PC/(PE + CL), indicating that alterations of membrane-stabilizing to

-destabilizing lipids are not used by either of these animals to maintain membrane function at low temperatures.

The increase in the proportion of CL in the mitochondrial membranes of *M. mercenaria* with cold acclimation represents an acclimatory response to lowered environmental temperature. No change occurred in the ratio of PC/(PE + CL), therefore the likelihood that this alteration would affect bilayer stability is slight. Since CL is a required component for the function of several mitochondrial proteins (15), the observed changes may relate to shifts in metabolic organization. Previous studies demonstrated that the proportion of PUFA increases at the expense of SFA in the tissue lipids upon the acclimation of ectothermic animals to low temperatures (1,16,18,23–25). Compared to SFA, PUFA have lower melting points and, when included in a membrane, disrupt the monolayer due to their permanently "kinked" acyl chain (16,23,26). These two characteristics of PUFA increase the fluidity of a biological membrane and, it is suggested, enable the membranes of cold-acclimated animals to remain fluid and functioning (1,16,23,). In the present study no increase of PUFA took place in the mitochondrial membranes of *M. mercenaria* with cold acclimation, while the fatty acid 22:4n-6 actually decreased, suggesting that this species of marine mollusk is not utilizing PUFA to maintain membrane function at low temperatures and therefore may be utilizing a different strategy. Only in *C. virginica* were PUFA found to increase with cold acclimation, with n-3 PUFA increasing in PE, n-6 PUFA increasing in CL, and total PUFA increasing in PI. This response indicates that these animals are actively manipulating membrane composition and that PUFA may be important to membrane function at low temperatures at least in this mollusk.

While PUFA levels in the membranes of *M. mercenaria* did not increase with cold acclimation, monoene levels did. The fatty acid 18:1n-9 increased in CL while 20:1 was found to increase in PC and CL. Monoenes also were demonstrated to increase in tissue phospholipids and cellular membranes of other ectothermic animals with cold acclimation (18,25,27, 28). However, in these previous studies, PUFA increased in parallel with monoenes. Only in PI from the gills of *C. virginica* in the present study was such a result noted. An increase in monoenes in animals acclimated to cold temperatures may be related to the maintenance of membrane fluidity. The presence of the monoene 18:1 in the *sn*-1 position of PE of cold-acclimated or adapted fish was suggested to increase the influence of that phospholipid on the membrane (27). This would act to increase the fluidity of the membrane and therefore help maintain membrane function at low temperatures. The results of the present study suggest that *M. mercenaria* is increasing monoene content, not PUFA, to help maintain membrane function at low temperatures.

In both marine bivalves, the SFA 16:0 constitutes 66–84% of the fatty acids in PI. High levels of 16:0 were previously demonstrated to occur in gill mitochondrial membranes of *C. virginica* (8); therefore, the levels reported in the current study are not unexpected. The increase in 16:0 found in PE from the membranes of cold-acclimated *M. mercenaria* would act to stabilize the membrane at low temperature. The significance of this particular finding is not known at this time.

Limited study has been made of the metabolism of dietary fatty acids in bivalve mollusks. It was demonstrated that the oyster, *C. virginica*, and the yellow clam, *Mesodesma matroides*, are capable of elongation and desaturation of fatty acids (29–31). The fatty acid composition of the diet (krill) fed to these animals throughout the experiment has a higher proportion of SFA and lower proportion of PUFA and monoenes than the fatty acid composition of the membrane phospholipids of both species used in this study. Both mollusks therefore accumulated these fatty acids in their membranes in a higher proportion to what is found in the diet. The long-chain PUFA 22:6n-3 is lower in the krill-based diet than in the mitochondrial membranes. Long-chain n-3 fatty acids such as 22:6n-3 can only be made from other shorter n-3 fatty acids which are all synthesized from the precursor 18:3n-3. This fatty acid must be obtained from the diet. The diet used contained sufficient concentrations of all precursors for the synthesis of longer-chained PUFA.

The ratio of n-3 to n-6 fatty acids (n-3/n-6) of the mitochondrial membrane phospholipids of the marine mollusks in the present study is lower compared to those of previously published values for marine teleost fish. This ratio is 1.0 in *M. mercenaria* and 2.0 in *C. virginica* at 12°C, while it is 16 in the winter flounder *Pseudopleuronectes americanus*, acclimated to 10°C (32), and 76 in Arctic char, *Salvelinus alpinus*, caught from 4°C seawater (33). This is due to the lower levels of n-6 fatty acids in the phospholipids in the marine teleosts compared to that of the marine bivalves. Within wild populations of marine organisms, low levels of n-6 fatty acids are common when compared to similar freshwater species. This difference was attributed to fatty acid precursors available in the diet (34). However, the diet fed the mollusks throughout the current study had an n-3/n-6 ratio of 45. This is much higher than that of the marine bivalves due to the low content of n-6 fatty acids This indicates that n-6 fatty acids are being concentrated in the mitochondrial membrane, suggesting a functional significance of these fatty acids in the membrane.

The seasonal temperatures experienced by most temperate zone marine organisms in northern latitudes range from −1.5°C in winter to 12°C in summer. The large alterations of membrane phospholipid composition seen in previous thermal acclimation studies were in response to correspondingly large changes in environmental temperature for example: 5 vs. 30°C (35), 7 vs. 27°C (24), and 10 vs. 32°C (25). The results of the present study indicate that during thermal shifts such as those occurring during seasonal changes in environmental temperature changes in membrane phospholipids do occur in mollusks.

Unlike thermal acclimation in fishes and some other organisms, the ratio of membrane-stabilizing phospholipids to membrane-destabilizing phospholipids was not altered. Most of the acclimation response is due to changes in phospholipid fatty acids such as alterations in PUFA content (*C. virginica*) or monoene levels (*M. mercenaria*).

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REFERENCES

1. Hazel, J.R. (1983) The Incorporation of Unsaturated Fatty Acids of the n-9, n-6, and n-3 Families into Individual Phospholipids by Isolated Hepatocytes of Thermally-Acclimated Rainbow Trout, *Salmo gairdneri, J. Exp. Zool. 227,* 167–176.
2. Hazel, J.R., and Carpenter, R. (1985) Rapid Changes in the Phospholipid Composition of Gill Membranes During Thermal Acclimation of the Rainbow Trout, *Salmo gairdneri J. Comp. Physiol. 155B,* 597–602.
3. Pollero, R.J., and Brenner, R.R. (1981) Effect of the Environment and Fasting on the Lipid and Fatty Acid Composition of *Diplodon patagonicus, Lipids 16,* 685–690.
4. Chu, F.E., Webb, K.L., and Chen, J. (1990) Seasonal Changes of Lipids and Fatty Acids in Oyster Tissues, *Crassostrea virginica*, and Estuarine Particulate Matter*, Comp. Biochem. Physiol. 95A,* 385–391.
5. Napolotano, G.E., MacDonald, B.A., Thompson, R.J., and Ackman, R.G. (1992) Lipid Composition of Eggs and Adductor Muscle in Giant Scallops, *Placopecten magellanicus*, from Different Habitats, *Mar. Biol. 223,* 71–76.
6. Peterson, C.H., and Fegley, S.R. (1986) Seasonal Allocation of Resources to Growth of Shell, Soma, and Gonads in *Mercenaria mercenaria, Biol. Bull. 171,* 587–610.
7. Bligh, E.G., and Dyer, W.J. (1959) A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol. 37,* 911–917.
8. Glemet, H.C., and Ballantyne, J.S. (1995) Influences of Environmental Salinity on the Structure and Function of Gill Mitochondrial Membranes of an Osmoconforming Invertebrate, *Crassostrea virginica, Mar. Biol. 121,* 673–683.
9. Holub, B.J., and Skeaff, C.M. (1987) Nutritional Regulation of Cellular Phosphatidylinositol, *Methods Enzym. 141,* 234–244.
10. Stuart, J.A., and Ballantyne, J.S. (1996) Subcellular Organization of Intermediary Metabolism in the Hepatopancreas of the Terrestrial Snail, *Cepaea nemoralis*—A Cytosolic β-Hydroxybutyrate Dehydrogenase, *J. Exp. Zool. 274,* 291–299.
11. Aronson, N., and Touster, O. (1974) Isolation of Rat Liver Plasma Membrane Fragments in Isotonic Sucrose, *Methods Enzym. 31,* 90–102.
12. Chen, P.S., Toribara, T.Y., and Warner, H. (1956) Microdetermination of Phosphorus, *Anal. Chem. 28,* 1756–1758.
13. Bradford, M.M. (1976) A Rapid Sensitive Method for Quantification of Microgram Quantities of Protein Using the Principle of Protein Dye Binding, *Anal. Biochem. 72,* 248–254.
14. Steele, R.G.D., and Torrie, J.H. (1980) *Principles and Procedures of Statistics: A Biometrical Approach*, 633 pp., McGraw-Hill Inc., New York.
15. Daum, G. (1985) Lipids of Mitochondria, *Biochim. Biophys. Acta 822,* 1–42.
16. Hazel, J.R. (1995) Thermal Adaptation in Biological Membranes: Is Homeoviscous Adaptation the Explanation? *Annu. Rev. Physiol. 57,* 19–42.
17. Hazel, J.R., and Zerba, E. (1986) Adaptation of Biological Membranes to Temperature: Molecular Species Compositions of Phosphatidylcholine and Phosphatidylethanolamine in Mitochondrial and Microsomal Membranes of Liver from Thermally-Acclimated Rainbow Trout, *J. Comp. Physiol. 156B,* 665–674.
18. Pruitt, N.L. (1988) Membrane Lipid Composition and Overwintering Strategy in Thermally Acclimated Crayfish, *Am. J. Physiol. 254,* R870–R876.
19. Thurmond, R.L., and Lindblom, G. (1997) NMR Studies of Membrane Lipid Properties, in *Current Topics in Membranes* (Epand, R., ed.) Vol. 44, pp. 103–166, Academic Press, New York.
20. Rand, R.P., and Sengupta, S. (1972) Cardiolipin Forms Hexagonal Structures with Divalent Cations, *Biochim. Biophys. Acta 255,* 484–492.
21. Cullis, P.R., Verkleij, A.J., and Ververgaert, P.H.J.Th. (1978) Polymorphic Phase Behaviour of Cardiolipin as Detected by ^{31}P NMR and Freeze-Fracture Techniques. *Biochim. Biophys. Acta 513,* 11–20.
22. De Kruijff, B., and Cullis, P.R. (1980) Cytochrome c Specifically Induces Non-Bilayer Structures in Cardiolipin-Containing Model Membranes, *Biochim. Biophys. Acta 602,* 477–490.
23. Sinensky, M. (1974) Homeoviscous Adaptation—A Homeostatic Process That Regulates the Viscosity of Membrane Lipids in *Escherichia coli*, *Proc. Natl. Acad. Sci. USA 71,* 522–525.
24. Chapelle, S. (1978) The Influence of Acclimation Temperature on the Fatty Acid Composition of an Aquatic Crustacean, *Carcinus maenas*, *J. Exp. Zool. 204,* 337–346.
25. Wodtke, E. (1981) The Effects of Acclimation Temperature on the Unsaturation of the Main Neutral and Charged Phospholipids in Mitochondrial Membranes of the Carp, *Cyprinus carpio*, *Biochim. Biophys. Acta 640,* 698–709.
26. Coolbear, K.P., Berde, C.B., and Keough, K.M.W. (1983) Gel to Liquid-Crystalline Phase Transitions of Aqueous Dispersions of Polyunsaturated Mixed-Acid Phosphatidylcholines, *Biochemistry 22,* 1466–1473.
27. Dey, I., Buda, C., Wiik, T., Halver, J.E., and Farkas, T. (1993) Molecular and Structural Composition of Phospholipid Membranes in Livers of Marine and Freshwater Fish in Relation to Temperature, *Proc. Natl. Acad.Sci. USA 90,* 7498–7502.
28. Fodor, E., Jones, R.H., Buda, C., Kitajka, K., Dey, I., and Farkas, T. (1995) Molecular Architecture and Biophysical Properties of Phospholipids During Thermal Adaptation in Fish: An Experimental and Model Study, *Lipids 30,* 1119–1126.
29. DeMoreno, J.E.A., Moreno, V.J., and Brenner, K.R. (1976) Lipid Metabolism of the Yellow Clam, *Mesodesma matroides*, 2. Polyunsaturated Fatty Acid Metabolism, *Lipids 11,* 561–566.
30. Waldock, M.J., and Holland, D.L. (1984) Fatty Acid Metabolism in Young Oysters *Crassostrea virginica*, Polyunsaturated Fatty Acids, *Lipids 19,* 332–336.
31. Chu, F.-L.E., and Greaves, J. (1991) Metabolism of Palmitic, Linoleic, and Linolenic Acids in Adult Oysters, *Crassostrea virginica*, *Mar. Biol. 110,* 229–236.
32. Glemet, H.C., and Ballantyne, J.S. (1997) Comparisons of Liver Mitochondrial Membranes from an Agnathan, *Myxine glutinosa*, an Elasmobranch, *Raja erinacea*, and a Teleost Fish, *Pleuronectes americanus*, *Mar. Biol. 124,* 509–518.
33. Glemet, H.C., Gerrits, M.F., and Ballantyne, J.S., Membrane Lipids of Red Muscle Mitochondria from Land-Locked and Sea Run Arctic Char, *Salvelinus alpinus, Mar. Biol. 129,* 673–679.
34. Sargent, J.R., Bell, M.V., Henderson, R.J., and Tocher, D.R. (1990) Polyunsaturated Fatty Acids in Marine and Terrestrial Food Webs, in *Comparative Physiology* (Kinne, R.K.H., Kinne-Saffran, E., and Beyenbach, K.W., eds.) Vol. 6, pp. 11–23, S. Karger, Basel.
35. Van Den Thillart, G., and De Bruin, G. (1981) Influence of Environmental Temperature on Mitochondrial Membranes, *Biochim. Biophys. Acta 640,* 439–447.

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