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# Molecular and structural composition of phospholipid membranes in livers of marine and freshwater fish in relation to temperature

(fatty acids/molecular species/fluidity)

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ABSTRACT **The compositions and physical states of the liver phospholipids of marine and freshwater fish adapted to relatively constant but radically different temperatures were investigated. Fish adapted to low temperature (5–10°C) accumulated more unsaturated fatty acids than those in a warm (25–27°C) environment. There were no measurable differences in the gross fatty acid compositions of the total liver phospholipids from identical thermal environments. Docosahexaenoic acid (22:6) did not seem to participate in the process of adaptation. Cold adaptation was coincidental with oleic acid (18:1) accumulation, preferentially in the phosphatidylethanolamine. Determination of the molecular species composition of phosphatidylethanolamine revealed a 2- to 3-fold and 10-fold increase in the level of 18:1/22:6 and 18:1/20:5 species, respectively. ESR spectroscopy revealed a 7–10% compensation in the ordering state of native phospholipids with temperature. Combination of 16:0/22:6 phosphatidylcholine with phosphatidylethanolamines of cold-adapted marine fish showed a drastic fluidization near the C-2 segment of the bilayer, but not in the deeper regions. An appropriate combination (75:25) of phosphatidylcholines from warmth-adapted marine fish with phosphatidylethanolamines from cold-adapted marine fish mimicked a 100% adaptational efficacy in the C-2 segment as compared with the phosphatidylethanolamines of warmth-adapted marine fish. A specific role of 18:1/22:6 phosphatidylethanolamine in controlling membrane structure and physical state with thermal adaptation is proposed.**

Poikilotherms are often subjected to temperature stress or seasonal variation of temperature. These organisms exploit diversity in lipid structures to fashion membranes to prevailing ambient temperatures in such a manner that they become more fluid in a cold-acclimatized state and less fluid in a warmth-acclimatized state (1, 2). Sinensky (3) has termed this response to temperature "homeoviscous adaptation". The extent of homeoviscous efficacy with which cells compensate membrane fluidity in response to changes in ambient temperature is only partial (4, 5). However, this response is rather rapid in the carp liver endoplasmic reticulum (6) and erythrocytes (7). Restructuring of the polar headgroup composition of phospholipid classes, together with modification of the unsaturation of their fatty acyl chains, may ensure that the components present are best suited to function within the constraints imposed by a prevailing thermal environment. One rational explanation of these responses is that the melting points of the fatty acids decrease with an increasing number of double bonds in the molecule, resulting in a more fluid structure in membranes rich in polyunsaturated fatty acids. Accepting this hypothesis, one would expect a marked difference between fish species inhabiting regions of extremely different but relatively constant temperatures. Phospholipids from specific membranes of an Arctic marine fish proved more unsaturated than those of subtropical freshwater fish, and these differences were reflected in the microviscosity of these structures (8). The present article compares the liver phospholipid compositions of a number of marine and freshwater fish, and a feasible mechanism is proposed to explain the adaptational phenomena at a membrane structural level.

## MATERIALS AND METHODS

**Fish.** The following fish were involved in this study. Warm-adapted marine fish (WAMF) included *Carnax calla, Epinephelus bleekeri, Lutaganus sebeae, Mugil passia, Nemipterus hexodon, Nemipterus javonicus, Pomadysys hasta, Sardinella longiceps*, and *Tachisurus yella* from the South China Sea and the southwest coast of India (20–27°C). Cold-adapted marine fish (CAMF) included *Clupea harengus, Scorpaenichthys marmoratus, Hexaganus stelleri, Hipoglossus hipoglossus, Onchorhyncus kisutch, Onchorhyncus tshawsytscha, Ophioidon melanus*, and *Sebastosomus melanus* from the North Pacific, the North Atlantic, and the Baltic Sea (5–10°C). Cold-adapted freshwater fish (CAFF) included *Abramis brama, Acerina cernua, Coregonus loverostris*, and *Esox lucius* from North Finland (7–10°C). Warm-adapted freshwater fish (WAFF) included *Catla catla, Cirrhina mrigala, Clarias lasera*, and *Labeo rohita* from West Bengal, India (20–25°C).

**Analytical Techniques.** Lipids in livers dissected from fresh animals were extracted, according to Folch *et al.* (9), in the presence of 0.01% butylated hydroxytoluene, and shipped by air to the laboratory in sealed vials under nitrogen. Phospholipids and neutral lipids were separated by silicic acid column chromatography by eluting the latter with chloroform and the former with methanol. Phospholipid subclasses were further separated on silica gel plates (Merck) according to Fine and Sprecher (10). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were methylated in the presence of silica gel with methanol containing 5% hydrochloric acid at 80°C for 2.5 hr. Fatty acids were separated on 10% FFAP coated onto Supelcoport 80–100 mesh (Supelco) in a 2-m column (2-mm i.d.) with a Hitachi 263-80 gas chromatograph.

**Molecular Species Determination.** The molecular species compositions of PC and PE were determined according to Takamura *et al.* (11). The dinitrobenzoyl derivatives of the diacylglycerols were separated by HPLC (Waters, model 440) on a Nucleosyl $C_{18}$ column (5-$\mu$m particle size; 4 mm i.d. × 250 mm), using acetonitrile/2-propanol (80:20, vol/vol) of HPLC grade (Carlo Erba, Milan, Italy) as mobile phase, with

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; WAMF, warm-adapted marine fish; CAMF, cold-adapted marine fish; WAFF, warm-adapted freshwater fish; CAFF, cold-adapted freshwater fish.

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detection at 254 nm. Identifications of peaks were based on the use of authentic standards (16:0/22:6, 18:0/22:6, 16:0/20:4, 18:0/20:4, 16:0/16:0, 18:0/18:0, 16:0/18:1, 18:1/18:1, 18:0/18:1) and on the relative elution times (12).

**Fluorescence Anisotropy Measurements.** To phospholipid (250 μg) dissolved in chloroform, 10 μl of 1 μM 2- and 12-(9-anthroyloxy)stearic acid and 16-(9-anthroyloxy)palmitic acid (Molecular Probes) dissolved in tetrahydrofuran was added with thorough mixing. The solvent was removed under high vacuum and the vesicles were prepared as described by Sen *et al.* (13). Measurements and calculations of steady-state anisotropy ($R_{ss}$) were performed according to Dey and Farkas (7). The SE in separate determinations was always <0.005. Fluorescence intensity and anisotropy were corrected for light scattering by measuring unlabeled samples under similar conditions (14).

**Fourier-Transform Infrared (FTIR) Spectroscopy.** Phospholipids (1 mg) were carefully hydrated in 0.2 M Tris·HCl buffer at pH 7.4 (15), and the samples were placed on a ZnS-Cleartran window (SpectraTech, Stamford, CT) in a hollow thermostatted cell in a flow of liquid nitrogen. The temperature stability was better than 0.1°C (16). Spectra were recorded with a Philips PU 9800 Fourier-transform spectrometer equipped with a deuterated triglycine sulfate detector. A pathlength of 4 mm was used in all experiments. A total of 300 scans were Fourier transformed to obtain a resolution of 2 $cm^{-1}$ over the spectral range. Buffer spectra obtained under exactly the same conditions were subtracted from the vesicle spectra to eliminate the $H_2O$ band. In the study of band contours, the method of Nilsson *et al.* (17) was followed.

**ESR Spectroscopy.** Phospholipid (0.5 μM) was mixed with 15 nmol of 5-(*N*-oxy-4′,4′-dimethyloxazolidino)stearic acid at room temperature for 5 min. The lipids were subsequently dried under high vacuum overnight to remove any traces of remaining solvent and then dispersed in 0.2 M Tris·HCl at pH 7.4 and sonicated for 20 min. Samples were contained in sealed glass capillaries (1-mm o.d.) accommodated within a standard quartz ESR tube that contained silicone oil for thermal stability. Spectra were recorded from 5°C to 30°C on an ECS-106 ESR spectrometer (Bruker). The order parameter ($S$) was calculated according to Seelig (18).

## RESULTS

Fig. 1 shows the average fatty acid compositions in the liver phospholipids of CAMF, CAFF, WAMF, and WAFF. The levels of saturated fatty acids, and their ratios to unsaturated fatty acids, were identical in fish from similar temperature conditions but were consistently higher in fish from warmer water (saturated/unsaturated ratios: 0.27 ± 0.03 and 0.41 ± 0.07, $P > 0.001$, in CAMF and WAMF, respectively, and 0.30 ± 0.03 and 0.41 ± 0.08, nonsignificant, in CAFF and WAFF, respectively). The level of oleic acid (18:1) was consistently higher in both CAMF and CAFF than in their warm-water partners. Arachidonic acid (20:4) exhibited significantly higher values in WAMF and WAFF than in CAMF and CAFF, but eicosapentaenoic acid (20:5) displayed an opposite trend. Docosahexaenoic acid (22:6) was invariant with the thermal adaptation.

Determination of the molecular species composition of PE revealed the accumulation of 1-oleoyl-2-docosahexanoyl (18:1/22:6) and 1-oleoyl-2-eicosapentaenoyl (18:1/20:5) species in CAFF and CAMF (Table 1). The sum of these species in CAMF and CAFF was 33.9 and 24.0% respectively, compared with 7.0 and 7.1%, respectively, in WAMF and WAFF. These data, together with earlier observations (19), permit the proposal that the specific differences in the molecular composition of PE are manifestations of the thermal adaptation of the lipid membranes at a structural level, rather than due to dietary effects.

Fourier-transform infrared spectra showed that the methylene stretchings ($\nu_s$-$CH_2$ at 2853 $cm^{-1}$ and $\nu_a$-$CH_2$ at 2923 $cm^{-1}$) were unaltered for CAMF, WAMF, CAFF, and WAFF measured at 5°C and 25°C (Fig. 2*A*), which suggests that in the hydrophobic core of the membrane there is no or negligible change due to higher unsaturation in ordering state of the membrane (17). The carbonyl stretching ($\nu$-C=O) at 1738 $cm^{-1}$ (16) exhibited a significant shift toward lower frequency in favor of CAFF and CAMF (Fig. 2*B*), suggesting higher unsaturation in the $\alpha$–$\beta$ position (20). This shift was 0.623 $cm^{-1}$ for CAFF and 4.155 $cm^{-1}$ for CAMF, which suggests the incorporation of unsaturation at the *sn*-1 position of the PEs (Table 1). The broadening of the C=O stretching band in cold water fish is due to the vibrational dephasing mechanism (21). The phosphate stretching in the frequency domain of 1000–1300 $cm^{-1}$ ($\nu_s$-$PO^{-2}$ at 1088 $cm^{-1}$ and $\nu_a$-$PO^{-2}$ at 1240 $cm^{-1}$) (17) also showed a shift of 3 $cm^{-1}$ for $\nu_s$-$PO^{-2}$ and a shift of 13 $cm^{-1}$ for $\nu_a$-$PO^{-2}$ toward higher frequency, in favor of CAMF and of 1.706 $cm^{-1}$ for $\nu_s$-$PO^{-2}$ and 9.809 $cm^{-1}$ for $\nu_a$-$PO^{-2}$ in CAFF (Fig. 2*C*).

The temperature dependence of the order parameter ($S$) of 5-(*N*-oxy-4′,4′-dimethyloxazolidino)stearic acid incorporated into the phospholipid vesicles of representative CAFF, CAMF, WAFF and WAMF is shown in Fig. 3. It is evident that the compensation of the ordering state is far from complete. From Fig. 3 it can be taken that, for perfect compensation for temperature, the order parameter should have been around 0.635 and 0.610 in the phospholipids of CAFF and CAMF, respectively, at 25°C, whereas the mea-

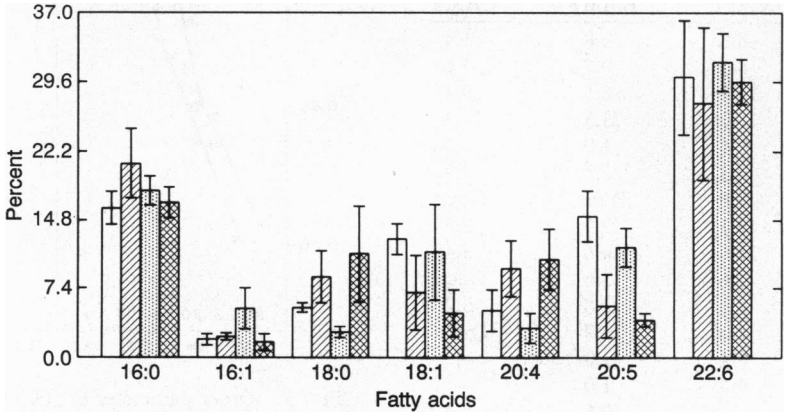


FIG. 1. Average fatty acid composition (mol %) of total phospholipids from CAMF (open bars; $n = 8$), WAMF (hatched bars; $n = 9$), CAFF (stippled bars; $n = 4$), and WAFF (cross-hatched bars; $n = 4$). Only the major fatty acids are shown.

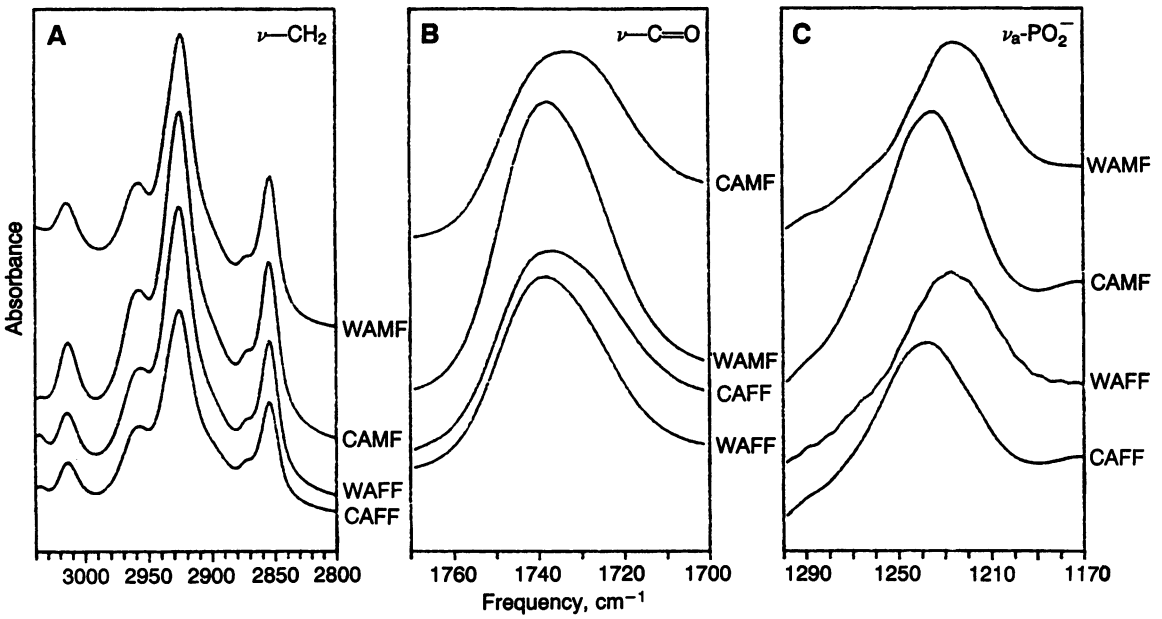


FIG. 2. Fourier-transform infrared spectra of phospholipids from representative CAMF (*Scorpaenichthys marmoratus*), WAMF (*Pomadysys hasta*), CAFF (*Coregonus loverostris*), and WAFF (*Catla catla*); (*A*) $\nu$-$CH_2$ (methylene) stretch. (*B*) $\nu$-C=O (carbonyl) stretch. (*C*) $\nu_a$-$PO_2^-$ (phosphate antisymmetric) stretch, $\nu_s$-$PO_2^-$ (phosphate symmetric) stretch.

sured values were 0.740 and 0.690, respectively. The calculated homeoviscous efficacy is 7–10%.

To reveal the effect of 18:1/22:6 PE species accumulating at lower temperature, a series of model experiments was carried out with PC and PE differing in molecular composition, using 2- and 12-(9-anthroyloxy)stearic acid and 16-(9-anthroyloxy)palmitic acid. PEs from WAMF and CAMF were incorporated into 16:0/22:6 PC in a molar ratio resembling that for native phospholipids (i.e., 75% 16:0/22:6 PC with 25% native PE). The incorporation of PE from WAMF into 16:0/22:6 PC vesicles resulted in a more rigid structure than that of 16:0/22:6 PC alone in each segment (C-2, C-12, and C-16) and at each measured temperature from 5°C to 30°C (Fig. 4 *A* and *B*). A rigidifying effect of PE on phospholipid bilayers was described earlier (22). When PE from CAMF was used instead, this rigidifying effect was not observed; in contrast, the vesicles become more fluid in the C-2 segment, but not in the deeper (C-12 and C-16) regions. PCs isolated from both CAMF and WAMF were also cross-combined with PEs of both origins (Fig. 4*C*). Again, PE from WAMF rendered the combined vesicles more ordered than PE from CAMF (Fig. 4*C*). The difference was drastic in the C-2 segment of the vesicle but also persisted in the deeper regions, albeit in a less pronounced way. From these model experiments, we conclude that 18:1/22:6 and 18:1/20:5 PE species accumulating at lower temperature might be involved in the regulation of membrane fluidity in response to temperature and that this effect is most pronounced in the upper half of the bilayer, while in the deeper region the physical properties of 22:6 (present also in PC) predominate.

Table 1. Major molecular species in liver PE

| Molecular species | % (wt/wt) | | | |
|---|---|---|---|---|
| | WAFF | CAFF | WAMF | CAMF |
| 22:6/22:6 | 0.2 | 0.8 | 1.8 | 1.4 |
| 18:1/20:5 | 0.3 | 8.7 | 0.5 | 3.6 |
| 18:1/22:6 | 6.8 | 25.2 | 6.5 | 20.4 |
| 16:0/22:6 | 16.0 | 19.7 | 23.3 | 21.3 |
| 16:0/20:4 | 1.7 | 1.6 | 1.0 | 4.0 |
| 18:0/20:5 | Trace | 4.3 | 5.8 | 4.9 |
| 18:0/16:1 | 3.1 | 1.6 | Trace | Trace |
| 18:0/22:6 | 11.0 | 10.3 | 7.5 | 2.5 |
| 18:0/20:4 | 20.4 | 12.6 | 26.6 | 3.4 |
| 18:1/18:1 | 3.4 | 0.6 | 2.6 | Trace |
| 16:0/18:1 | 17.6 | 5.8 | 7.8 | 3.2 |
| 16:0/16:0 | 10.1 | Trace | 3.2 | Trace |
| 16:0/18:0 | 4.6 | 0.4 | 1.0 | — |
| 18:0/18:0 | 2.9 | 0.5 | 1.0 | — |
| 18:0/18:1 | 1.0 | — | 0.6 | 0.4 |

WAFF, *Labeo rohita*; CAFF, *Coregonus loverostris*; WAMF, *Pomadysys hasta*; CAMF, *Scorpaenichthys marmoratus*.

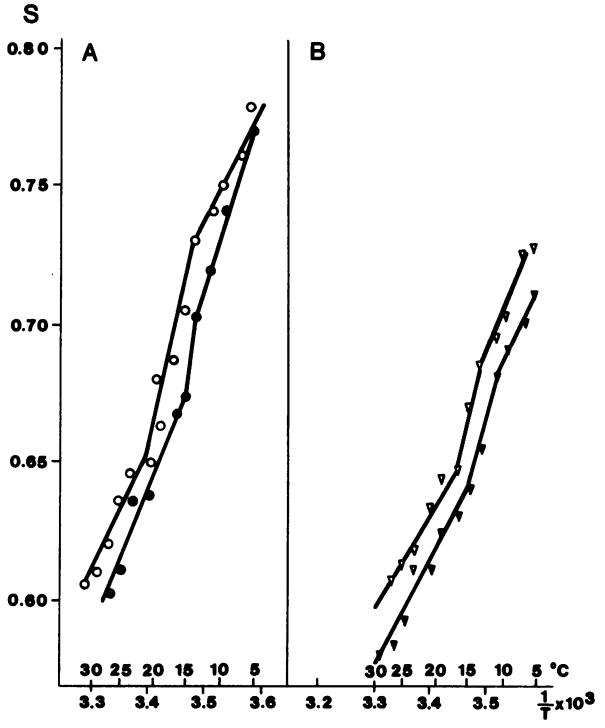


FIG. 3. Order parameter (*S*) of 5-(*N*-oxy-4',4'-dimethyloxazolidino)stearic acid in phospholipids from livers of representative WAMF (*Pomadysys hasta*) (○) and CAMF (*Lutganus sebeae*) (●) (*A*) and WAFF (*Labeo rohita*) (▽) and CAFF (*Abramis brama*) (▼) (*B*).

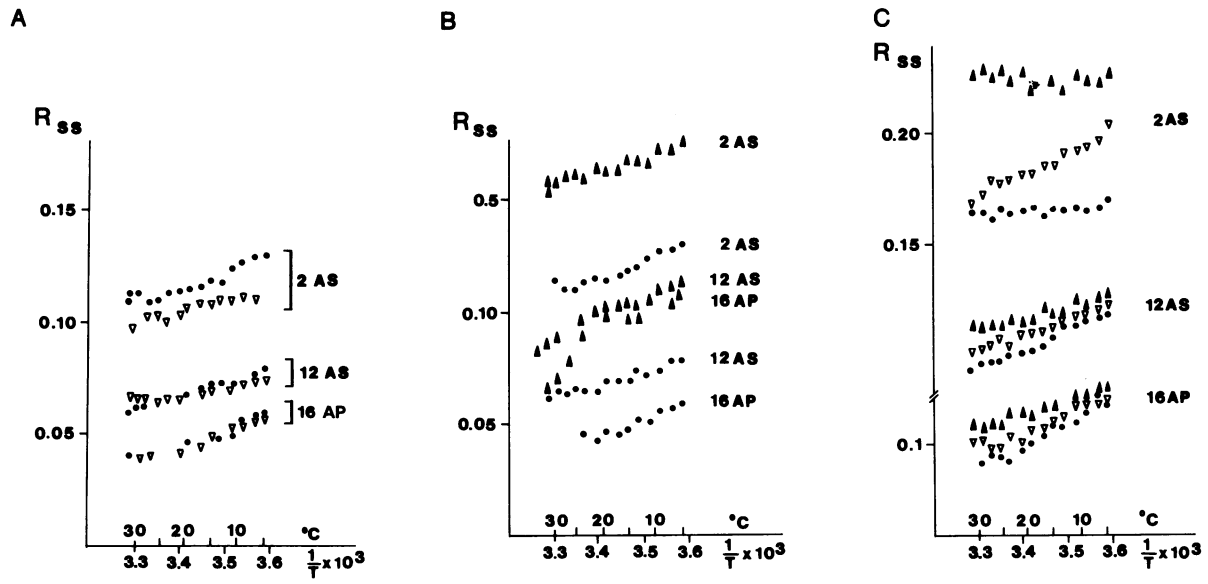


FIG. 4. Effect of PEs on fluorescence anisotropy ($R_{ss}$) of 2- and 12-(9-anthroyloxy)stearic acid (2AS and 12AS) and 16-(9-anthroyloxy)palmitic acid (16AP) in PC vesicles. (*A*) ●, 16:0/22:6 PC alone; ▽, 16:0/22:6 PC (75%) plus CAMF PE (25%). (*B*) ●, 16:0/22:6 PC alone; ▲, 16:0/22:6 PC (75%) plus WAMF PE (25%). (*C*) ●, Isolated fish PC (from both warm- and cold-adapted) vesicles; as there is no difference in the PCs from warm- and cold-adapted species, average values are represented here; ▽, 75% PC plus 25% CAMF PE; ▲, 75% PC + 25% WAMF PE.

## DISCUSSION

Although the number of species investigated was limited, we suggest that the data are representative of fish adapted to cold and warm environments. The differences in the fatty acid compositions of the liver phospholipids of marine and freshwater fish (evolutionary) adapted to contrasting temperatures resemble those achieved during the seasonal acclimatization of several freshwater fish. The similarity in fatty acid composition of phospholipids from all habitats can be interpreted as an overall effect of temperature on the lipid metabolism in the fish liver. However, these responses do not seem to be sufficient to provide perfect compensation for the thermal differences in the hydrophobic core of the membranes as demonstrated by ESR (Fig. 3) and Fourier-transform infrared (Fig. 2*A*) measurements. For an effective control of membrane fluidity, provided that the lipids are the only compounds involved, a proper balance of unsaturated versus saturated phospholipid molecular species is anticipated. The thermotropic phase-transition temperatures of 16:0/22:6 PC and 16:0/18:1 PC are rather close (23). On the other hand, the disaturated species such as 16:0/16:0 PC with a high transition temperature (+41°C) have proved to be low in all the fish phospholipids investigated so far (24–26). The reported decrement and increment in 16:0/18:1 PC and 16:0/22:6 PC, respectively, in the individual membranes of some freshwater fish (25, 26) are probably insufficient for control of the membrane physical state. The accumulation of 16:0/22:6 PC might likewise not have an immense impact on the packing properties of the bilayer, since its molecular area is only about 15% greater than that of 16:0/18:1 PC (27).

Polyunsaturated fatty acids in the *sn*-2 position of phospholipids may favor the formation of more condensed structures than those of phospholipids containing 18:1 in the same position (28). The apparent difference between PC and PE was a higher proportion of 18:1 in the latter, which was directed mostly into the *sn*-1 position in cold-adapted fish from both habitats. PE and PC differ in molecular shape: the former are conical and the latter are cylindrical (29). The substitution of 16:0 by 18:1 in the *sn*-1 position may further accentuate the conicity of PE, due to the presence of the *cis* double bond. Thus the replacement of 16:0 by 18:1 in the *sn*-1 position may conceivably favorably affect both the phase state and the packing properties of membranes during cold adaptation. An elevated level of conical molecules is required to maintain the functional integrity of biomembranes in the cold (30), and the accumulation of PE during cold adaptation has been demonstrated (31, 32). The present results indicate that fish can increase not only the number of conic shaped molecules but also the conicity of the existing conical molecules during cold adaptation. Thus, it can be proposed that molecular species such as 18:1/22:6 and 18:1/20:5 play an important role in adapting the membrane physical state to temperature and, in agreement with Cevec (33), that fatty acyl chains in position *sn*-1 are more important than those in *sn*-2, whereas 22:6 in position *sn*-2 has only a mechanical role in maintaining membrane integrity.

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