Very Long Chain Polyunsaturated Fatty Acids in the Blubber of Ringed Seals (*Phoca hispida* sp.) from Lake Saimaa, Lake Ladoga, the Baltic Sea, and Spitsbergen

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ABSTRACT: Blubbers of four ringed seal subspecies from Lake Saimaa, Lake Ladoga, the Baltic Sea, and Spitsbergen were analyzed for very long chain polyunsaturated fatty acids (VLCPUFA; $>C_{22}$) using gas–liquid chromatography and gas chromatography/mass spectrometry. The VLCPUFA of the blubber oils were mainly n-3 polyunsaturated fatty acids—23:5n-3, 24:3n-3, 24:4n-3, 24:5n-3, 24:6n-3, 26:5n-3, 26:6n-3, and 28:7n-3. The largest VLCPUFA components in all populations were 24:5n-3 (0.1–0.2 wt% of total fatty acids) and 24:6n-3 (0.1%), but 24:4n-3 (0.1%) was also prominent in the Baltic specimens. The blubber oils of the freshwater species contained considerably more 24:4n-6 and 24:5n-6 than the blubbers of the marine species. The differences among the VLCPUFA in these subspecies appear to be mainly due to different dietary VLCPUFA.

Lipids 30, 725–731 (1995).

Very long chain polyunsaturated fatty acids (VLCPUFA; $>C_{22}$) have been detected in aquatic invertebrates (1–4) and in fish (5–7), but no reports on VLCPUFA in marine mammals have been published. Seals deposit large amounts of polyunsaturated fatty acids (PUFA), mainly 22:6n-3, 20:5n-3, and 22:5n-3 (8–10) in their depot fats (blubbers). In addition, the blubbers of freshwater seals are known to contain considerable amounts of n-6 PUFA—20:4n-6, 22:4n-6, and 22:5n-6 (Ref. 10; Käkelä, R., unpublished results). Undoubtedly, diet is the major source of PUFA in seal depots, but it was not known if the VLCPUFA of the diet are incorporated into the blubber in the same way as the shorter chain PUFA. Among the latter, the 22:5n-3 in depot fats of seals and other marine mammals attracts attention, as it is at least one-half of the 22:6n-3 and sometimes equal to it (11), whereas in fish the proportion is normally one-tenth of 22:6n-3. It was felt that the VLCPUFA intermediates could reflect this difference and be of some help in assessing the origin of the VLCPUFA.

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Abbreviations: GC/MS, gas chromatography/mass spectrometry; PUFA, polyunsaturated fatty acids; VLCPUFA, very long chain polyunsaturated fatty acids.

The biosynthesis of 22:6n-3 and 22:5n-6 has recently been reevaluated (12,13). In rats, 20:5n-3 (in liver) and 20:4n-6 (in seminiferous tubules) are first elongated to 24:5n-3 and 24:4n-6, respectively, then Δ6-desaturated to 24:6n-3 and 24:5n-6 and, finally, chain-shortened to 22:6n-3 and 22:5n-6, respectively. Whether this could also occur in the tissues of marine mammals was not known. Alternatively, the levels and composition of the VLCPUFA of the seals could simply resemble those of their diet. Freshwater fish are known to contain large amounts of n-6 PUFA compared to marine fish (14–18). Accordingly, we have compared the VLCPUFA in the blubber oils of ringed seals from freshwater, brackish water, and ocean populations.

MATERIALS AND METHODS

Blubber samples. The blubbers of the ringed seals from Lake Saimaa (eastern Finland), Lake Ladoga (western Russia), the Bothnian Bay of the Baltic Sea, and Spitsbergen (the Arctic Ocean) were collected as described in Table 1. The blubber samples were stored and transferred frozen, except for those of four individuals from Lake Ladoga. These samples were sent to Joensuu in formalin. Blubber samples cut from the middle of the original sample blocks were homogenized and extracted with hexane using a mechanical blender. After alkali saponification of the recovered oils, the unsaponifiables were removed with diethyl ether, and the fatty acids were liberated with dilute H_2SO_4. The fatty acids were taken up in hexane, and their purity was improved by a thin-layer chromatography clean-up step.

Fractionation, hydrogenation, and methylation of the fatty acids. For qualitative studies, parts of the fatty acid samples were fractionated by urea complexing (19). The free fatty acids were dissolved in an ethanolic solution of urea by refluxing. Fractions were collected during two stages of cooling and filtration, first at room temperature and then in an ice bath. After addition of water and acidification, the fatty acids of nonurea-complexing fractions were extracted into hexane (19). However, only the original fatty acid samples were used for quantitation.

TABLE 1
The Ringed Seals (*Phoca hispida* sp.) Studied

Subspecies/origin	n	Cause of death (yr)	Males/females	Weight (kg)	Age
P. hispida saimensis Lake Saimaa, eastern Finland	5	Fishing net (1991–1993)	2/3	20–44	3 mon–2 yr
P. hispida ladogensis Lake Ladoga, western Russia	5	Fishing net (1991–1992)	not known	20–46	4 mon–4 yr
P. hispida botnica Bay of Bothnia, Baltic Sea	5	Shot (1992)	2/3	49–91	2–26 yr
P. hispida hispida Kongsfjorden, Spitsbergen, Arctic Ocean	5	Shot 1992	4/1	48–73	2–6 yr

To detect the proportions of the fatty acids of different chain lengths, samples of total fatty acids, and the nonurea-complexing fractions from urea complexation were hydrogenated. Fatty acids (5 mg) in methanol (30 mL) were stirred with platinum oxide catalyst (1 mg) under hydrogen for one hour. The solution was filtered and the filtrate was concentrated in a nitrogen stream. The hydrogenated fatty acids were recovered in hexane, washed with water, and concentrated under nitrogen.

Before gas–liquid chromatographic analysis, the free fatty acids were converted to methyl esters by heating at 100°C with 7% BF_3 in methanol plus hexane as cosolvent, in glass tubes with Teflon-lined caps for one hour under nitrogen. The esters were extracted into hexane, and the extract was dried and concentrated.

Gas–liquid chromatography. The fatty acid methyl esters were analyzed with a Perkin-Elmer 8420 gas chromatograph (Norwalk, CT) with flame-ionization detection on an Omegawax-320 capillary column (30 m × 0.32 mm i.d., film thickness 0.25 μm; Supelco Inc., Bellefonte, PA). Helium carrier gas was set at 12 psig. The injector and detector temperatures were 250 and 270°C, respectively. Both temperature-programmed (180°C/8 min, 3°C/min, 230°C to end) and isothermal (210 and 245°C) analyses were conducted. The peak areas were recorded on a Perkin-Elmer GP–100 graphic printer. The areas of the PUFA present in sufficiently large amounts were converted to weight percent using theoretical correction factors (20). The good reproducibility of the VLCPUFA analysis and the small deviations of that data from the whole blubber analyses showed that, in spite of small percentages, the accuracy of the quantitation was not significantly reduced.

Gas chromatography/mass spectrometry (GC/MS). The primary identifications of VLCPUFA were made using conventional techniques—the linear log plot procedures based on retention time (21), the occurrence of the components in different urea-complexing fractions, and chain-length studies after hydrogenation. The identifications were confirmed by electron impact MS [Hewlett Packard 5971 mass selective detector on a Hewlett Packard 5890 A gas chromatograph (Avondale, PA) equipped with an OV-1 methyl silicone capillary column (20 m × 0.32 mm i.d., film thickness 0.25 μm), isothermal and temperature programmed runs] after the method of Fellenberg *et al.* (22). These authors have discovered that the intensity of the fragment *m/z* 108 clearly exceeds that of the *m/z* 150 for n-3 PUFA, but *m/z* 150 is more considerable for n-6 PUFA.

Statistics. The fatty acid data (% w/w) were analyzed by one-way analyses of variance, and the means were compared by the Newman Keuls test (Statgraphics 5.0 software; STSC, Inc.). Prior to this analysis, the fatty acid percentages were normalized through arcsine-transformation (23).

RESULTS

The VLCPUFA in the blubber oils (hexane extract) of the ringed seals were mainly n-3 PUFA (Table 2). In GC/MS they produced intense fragments *m/z* 79 and 91, and the fragment *m/z* 108 was considerable, whereas *m/z* 150 was almost absent. The most important VLCPUFA in each subspecies were 24:5n-3 (0.10–0.24 wt%) and 24:6n-3 (0.06–0.13%), the ringed seal of Lake Ladoga having the highest amounts. The blubbers of the Lake Ladoga (Fig. 1) and Spitsbergen seals contained an amount of 24:5n-3 that was twice as high as 24:6n-3, but in the Lake Saimaa and the Baltic specimens these acids were almost equal. In the Baltic ringed seal, 24:4n-3 (0.11%) was also prominent, thus differing from the other subspecies (Fig. 2). The levels of 24:3n-3 and 23:5n-3 were low (0.02% or less) in each subspecies (Figs. 1 and 2), and traces of 25:5n-3 were also seen in some Baltic samples when examined by GC/MS.

Two hexacosapolyenoic acids, 26:5n-3 and 26:6n-3, were found (Fig. 2). The percentages for the ringed seals from Lake Ladoga and the Baltic were 0.02–0.04%, but the Lake Saimaa and Spitsbergen seals had even lower amounts (0.01% or less). No unambiguous interdependence between the n-3 ho-

TABLE 2

Very Long Chain Polyunsaturated Fatty Acid and Their Potential Precursors (wt%, mean ± SE) in the Blubbers of the Ringed Seals from Lake Saimaa (*Phoca hispida saimensis*), Lake Ladoga (*P. hispida ladogensis*), Bothnian Bay (*P. hispida botnica*), and Spitsbergen (*P. hispida hispida*)

Fatty acid	Freshwater		Marine		
	P. hispida saimensis n = 5	*P. hispida ladogensis* n = 5	*P. hispida botnica* n = 5	*P. hispida hispida* n = 5	*P*[a]
20:3n-3	0.41 ± 0.08 AB	0.61 ± 0.05 A	0.35 ± 0.08 B	0.11 ± 0.02 C	.0001
20:4n-3	1.0 ± 0.11 A	1.0 ± 0.08 A	1.1 ± 0.18 A	0.5 ± 0.04 B	.0082
20:5n-3	6.4 ± 0.19 A	7.5 ± 0.34 B	8.1 ± 0.46 BC	8.9 ± 0.43 C	.0015
21:5n-3	0.3 ± 0.01 A	0.4 ± 0.02 A	0.4 ± 0.04 A	0.5 ± 0.02 B	.0001
22:3n-3	0.07 ± 0.02 B	0.16 ± 0.01 A	0.14 ± 0.02 A	0.01 ± 0.003 C	<.0001
22:4n-3	0.2 ± 0.03 AB	0.3 ± 0.02 B	0.5 ± 0.13 B	0.1 ± 0.03 A	.0093
22:5n-3	6.8 ± 0.30	6.3 ± 0.28	5.8 ± 0.43	6.6 ± 0.16	.1466
22:6n-3	9.8 ± 0.18 A	12.3 ± 0.31 B	14.0 ± 0.80 B	13.7 ± 0.52 B	.0001
23:5n-3	0.008 ± 0.001 A	0.018 ± 0.002 B	0.016 ± 0.001B	0.008 ± 0.001 A	.0002
24:3n-3	0.009 ± 0.002 A	0.023 ± 0.005 B	0.023 ± 0.005B	0.006 ± 0.001 A	.0019
24:4n-3	0.023 ± 0.005 A	0.066 ± 0.012 B	0.112 ± 0.022 C	0.012 ± 0.002 A	<.0001
24:5n-3	0.104 ± 0.009 A	0.238 ± 0.013 B	0.096 ± 0.010 A	0.122 ± 0.012 A	<.0001
24:6n-3	0.085 ± 0.014 A	0.125 ± 0.015 B	0.079 ± 0.007 A	0.061 ± 0.004 A	.0052
26:5n-3	0.006 ± 0.001 A	0.030 ± 0.006 B	0.022 ± 0.004 B	0.011 ± 0.002 A	.0002
26:6n-3	0.009 ± 0.003 A	0.026 ± 0.004 B	0.035 ± 0.009 B	0.005 ± 0.001 A	.0003
28:7n-3	trace[c]	0.017 ± 0.004 A[b]	0.049 ± 0.009 B	0.020 ± 0.004 A	.0163
20:4n-6	3.5 ± 0.15 A	1.8 ± 0.06 B	0.8 ± 0.12 C	0.4 ± 0.03 D	<.0001
22:4n-6	0.9 ± 0.07 A	0.4 ± 0.02 B	0.2 ± 0.03 C	0.1 ± 0.02 C	<.0001
22:5n-6	1.2 ± 0.08 A	1.1 ± 0.07 A	0.4 ± 0.08 B	0.1 ± 0.01 C	<.0001
24:4n-6	0.024 ± 0.003 A	0.018 ± 0.002 A	0.006 ± 0.001 B	0.002 ± 0.001 C	<.0001
24:5n-6	0.019 ± 0.002 A	0.019 ± 0.003 A	0.005 ± 0.001 B	0.002 ± 0.000 C	<.0001

[a]One-way analysis of variance. The means are compared by the Newman-Keuls test; values with no common capital letter differ at $P < 0.05$ (the accurate figures for 20:3n-3 and 22:3n-3 support statistics).
[b]n = 3.
[c]Trace amount, <0.001 wt%.

mologues from eicosapolyenoic to hexacosapolyenoic fatty acids was found in the comparison of the subspecies (Table 2). The possible octacosapolyenoic homologues of 26:5n-3 and 26:6n-3 were too small to be detected. However, 28:7n-3 was found (Fig. 2). Although the lipids from the other subspecies contained 0.02–0.05% of 28:7n-3, only trace amounts of this fatty acid were observed in the gas–liquid chromatography analyses of lipids from seals of Lake Saimaa.

The blubbers of the freshwater seals contained two n-6 VLCPUFA, 24:4n-6 and 24:5n-6, both subspecies having approximately 0.02% (Table 2, Fig. 1). These fatty acids were present in negligible proportions in the marine ringed seals. Thus, the contents of 24:4n-6 and 24:5n-6 clearly reflected those of the shorter chain homologues in the blubbers of the seals. The identification of n-6 VLCPUFA was based on retention times and occurrence in fractionated samples, despite their coincidence (24) with much larger n-3 VLCPUFA on the OV-1 column used in GC/MS. Unfortunately, the accuracy of the quantitation of 24:4n-6 was diminished by a plasticizer, dioctyl (di-2-ethylhexyl) phthalate (25), eluting just before it and causing inaccuracies in the integration of the peak area.

The analyses of the hydrogenated fatty acids gave the proportions of the different chain lengths in the fatty acids of the blubbers (Table 3, Fig. 3). The results for fatty acids $>C_{20}$ paralleled those of the PUFA of the unhydrogenated samples, but gave a little larger total for each chain length. When the amounts of 24:1n-9 and 24:0 were added to the sum of tetracosapolyenoic acids, the agreement with hydrogenated 24:0 was satisfactory. In addition, traces of 26:0, 26:1, 28:0, and 28:1 could be seen in the chromatograms of the nonhydrogenated fatty acids, and these contributed to the sums of hexacosanoic and octacosanoic acids. However, these fatty acids failed to totally account for the differences observed, suggesting that there were probably several trace polyunsaturated components that could not be detected individually.

DISCUSSION

Although the VLCPUFA found in the blubbers of the ringed seal subspecies studied were qualitatively the same, clear differences in the abundance of several VLCPUFA were detected (Table 2). The Baltic ringed seal had high proportions of 24:4n-3 and 28:7n-3 compared to other subspecies, and 24:5n-3 was particularly high in the Lake Ladoga specimens. As anticipated, the blubbers of the freshwater seals contained more n-6 VLCPUFA than those of the marine individuals. The proportion of total VLCPUFA in the blubbers also varied; the ringed seal of Lake Ladoga had the largest amount (0.58%), followed by the Baltic (0.44%), Lake Saimaa

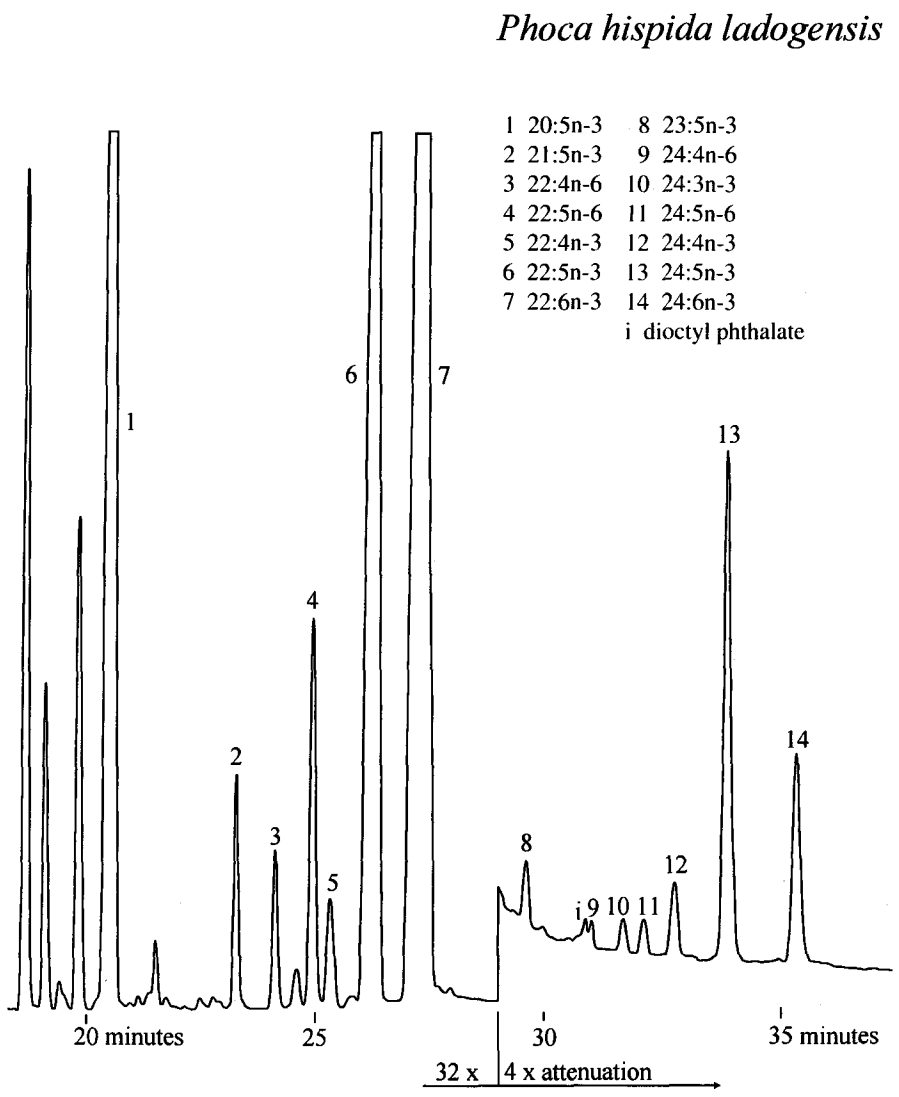

FIG. 1. Partial gas–liquid chromatogram of the total fatty acids from the blubber of the ringed seal of Lake Ladoga showing the area of C_{22-24} PUFA (180°C/8 min, 3°C/min, 230°C to end, Omegawax-320 capillary column; Supelco, Bellefonte, PA).

(0.29%), and Spitsbergen (0.25%) seals. These differences seem to be too large to be due to metabolic divergence only and suggest a dietary explanation.

It is known that the fatty acid composition of seal blubber reflects the dietary fatty acids to a great extent. Especially, the freshwater and marine seals have very different compositions, the former having more of the n-6 PUFA that are abundant in freshwater fish (14–18), and less 20:1n-9 and 22:1n-11 derived from copepoda (10,26). The n-6 PUFA contents of freshwater fish are almost five times those of marine fish, a ratio also found in the long-chain PUFA and VLCPUFA of blubbers from lacustrine and marine seals (Table 2). As far as we know, freshwater fish have not been analyzed for VLCPUFA, and analyses of marine fish for these fatty acids are few. The Baltic herring (*Clupea harengus membras*), an important prey species for the Baltic ringed seal, has been found to contain VLCPUFA of 24–32 carbons in the chain, but the percentages of the total fatty acids have been reported only for 24:4n-3, 24:5n-3, 24:6n-3, 26:5n-3, 26:6n-3, and 28:7n-6 (5). The compositions of the VLCPUFA in the total flesh lipids of the Baltic herring and the ringed seal blubber differ in two ways (Table 4). First, the levels of VLCPUFA in the blubber oils were only one-tenth or less of the herring lipid levels. Second, the 28:7 of the ringed seal was identified to be a n-3 isomer and that of the herring is reported as a n-6 isomer (5). Řezanka (6,27) has used herring (*C. harengus*, unknown origin) as a source of VLCPUFA and, although reporting 28:7n-3 as the main isomer, he also found 28:7n-6 (Table 4). Recently Ota *et al.* (7) found as much as 8 mol% of 24:6n-3 in the flesh triglycerides of flathead flounders from the Sea of Japan.

Could endogenous metabolism (from shorter chain PUFA obtained in the diet) produce VLCPUFA in the seal tissues? Voss *et al.* (28) and Aveldaño *et al.* (13) have shown that

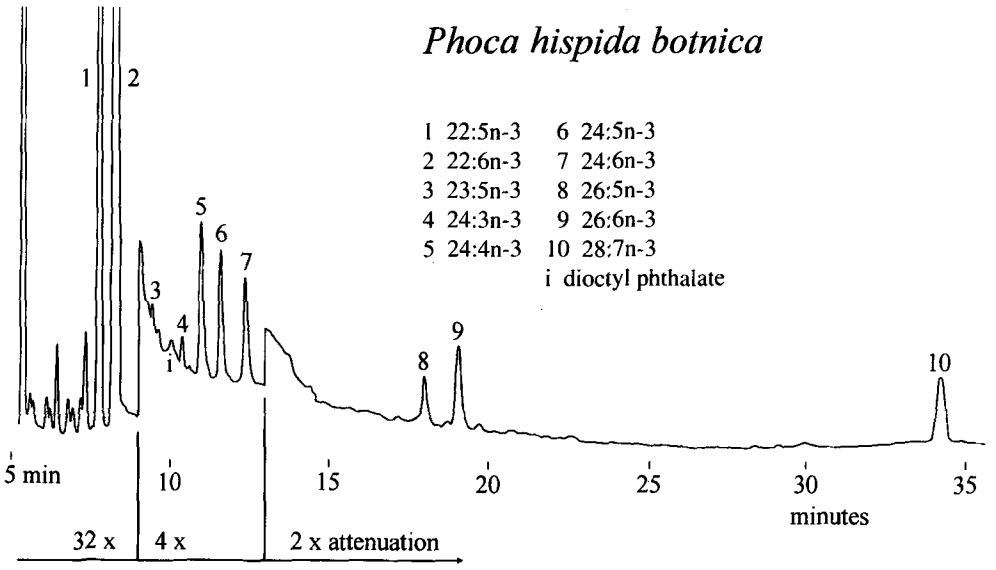

FIG. 2. Partial gas–liquid chromatogram of the total fatty acids from the blubber of the Baltic ringed seal (isothermal 245°C, Omegawax-320 capillary column; Supelco, Bellefonte, PA).

24:5n-3, 24:6n-3, 24:4n-6, and 24:5n-6 are intermediates in the metabolism of 22:6n-3 and 22:5n-6 in the rat, but radiolabel experiments are needed to find out if, or on what scale, these processes occur in seal liver or blubber. Whatever the organisms in which the VLCPUFA of the ringed seal blubbers have been biosynthesized, the biochemistry of these fatty acids seems to follow the pathways proposed in the rat studies. Theoretically, all the VLCPUFA of the ringed seal blubbers could be produced by chain elongation, β- and α-oxidation, Δ5-desaturase and Δ6-desaturase. Even the 28:7n-3 could be seen as being derived from the Δ5-desaturation and subsequent elongation of 26:6n-3.

The occurrence of odd-chain VLCPUFA, 23:5n-3, and trace 25:5n-3 suggest either elongation of 21:5n-3 or α-oxidation. Aveldaño *et al.* (13) have proposed that α-oxidation is an important mechanism in the shortening of fatty acids and that this produces odd-chain PUFA as metabolic intermediates. However, 21:5n-3 was first identified in seal oils (29), where it is more obvious than in fish oils. Possibly the retroconversion of VLCPUFA by β-oxidation is not totally specific, and α-oxidation takes place instead.

Although our knowledge of the biochemistry of PUFA and VLCPUFA has recently expanded, the biological importance of VLCPUFA is not yet understood (30). VLCPUFA have been detected in large amounts in mammalian spermatozoa/testes (1,31–33), brain (34,35), and retina (36–39), which suggests essentiality for at least some specific membrane functions. The tissues known to be rich in VLCPUFA in terrestrial mammals, and also worth analyzing, are those of diving mammals. These obtain large amounts of PUFA from their diets and have special requirements in sensory physiology.

TABLE 3
Hydrogenated Fatty Acids (wt%) of Longer Chain Lengths from a Blubber Sample from the Ringed Seals from Lake Saimaa (*Phoca hispida saimensis*), Lake Ladoga (*P. hispida ladogensis*), Bothnian Bay (*P. hispida botnica*), and Spitsbergen (*P. hispida hispida*)

	Freshwater		Marine	
Fatty acid	*P. hispida saimensis*	*P. hispida ladogensis*	*P. hispida botnica*	*P. hispida hispida*
20:0	13.78	12.77	12.67	19.96
21:0	0.41	0.61	0.78	0.71
22:0	20.21	24.59	25.18	22.35
23:0	0.05	0.07	0.04	0.05
24:0	0.42	0.73	0.49	0.34
25:0	trace	trace	0.01	0.01
26:0	0.02	0.11	0.10	0.03
27:0	ND[a]	ND	trace	ND
28:0	trace[b]	0.02	0.04	0.02

[a]Not detected. [b]Trace amount, <0.005 wt%.

TABLE 4
Comparison of the Main Very Long Chain Polyunsaturated Fatty Acids (VLCPUFA) (wt%) of the Blubber from the Baltic Ringed Seal (*Phoca hispida botnica*) with the VLCPUFA of the Baltic Herring (*Clupea harengus membras*) Flesh and the VLCPUFA of a Fraction of Silver-Ion Exchange High-Performance Liquid Chromatography (HPLC) from Herring (*C. harengus*, unknown origin)

Fatty acid	Baltic ringed seal	Baltic herring[a]	Herring, HPLC fraction[b]
24:4n-3	0.11	1.3	5.65
24:5n-3	0.10	1.4	6.09
24:6n-3	0.08	0.9	3.91
26:5n-3	0.02	0.5	2.16
26:6n-3	0.04	0.7	3.04
28:7n-3	0.05	—	1.97
28:7n-6	—	0.4	0.43

[a]From Reference 5. [b]From Reference 6.

ACKNOWLEDGMENTS

We are grateful to Drs. E. Helle (Finnish Game and Fisheries Research Institute, Helsinki), C. Lydersen (University of Oslo), and M.O. Hammill (Institut Maurice Lamontagne, Quebec) for donating the Baltic and Spitsbergen blubbers. Dr. O. Lavrantieva (Goshniorsk, St. Petersburg) and Ph. Lic T. Sipilä provided us with the Lake Ladoga samples. Dr. R. Julkunen-Tiitto assisted in GC/MS. This study was supported by a grant (to R.K. and H.H.) from the Academy of Finland.

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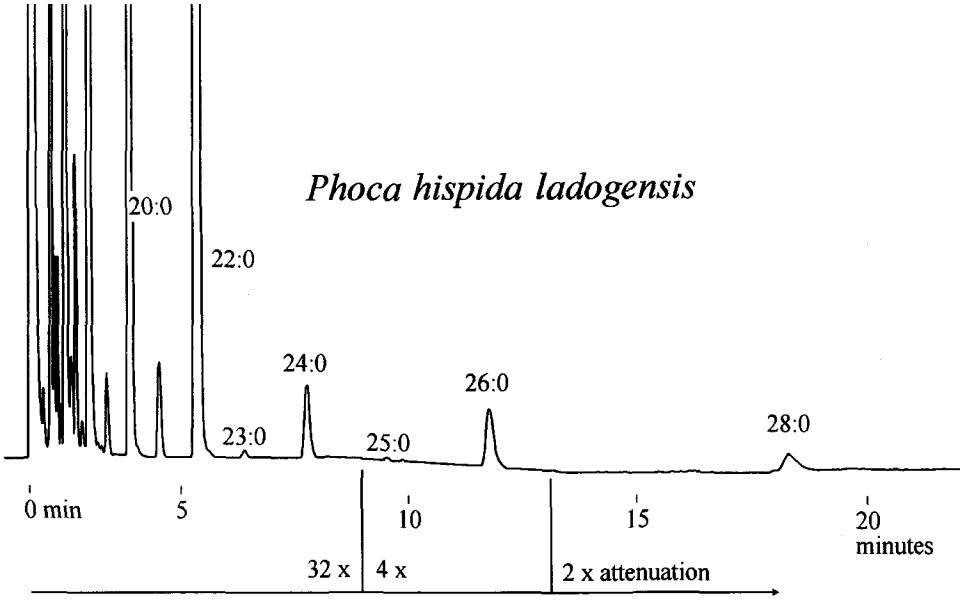

FIG. 3. Gas–liquid chromatogram of the hydrogenated total fatty acids from the blubber of the ringed seal of Lake Ladoga (isothermal 245°C, Omegawax-320 capillary column; Supelco, Bellefonte, PA).

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[Received February 7, 1995, and in revised form June 5, 1995; Revision accepted June 6, 1995]