Lipid Insights

ORIGINAL RESEARCH

Nervonic Acid (24:1n-9) is a Dominant Unsaturated Fatty Acid in the Intestinal Brush Border of Atlantic Cod

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Abstract: Atlantic cod is a coldwater teleost of commercial importance. The intestinal epithelium is a large organ in vertebrates serving an important role in nutrient selection and uptake as well as an immunological barrier. Here, we perform lipid and fatty acid analysis of the plasma membrane from the cod intestinal enterocytes after separation of the brush border membrane and the basolateral membrane fractions. Our results show that both membrane fractions contain an unusually high amount of cholesterol and glycolipids but low levels of glycerophospholipids compared with other reported studies on fish. Sphingomyelin was the dominant lipid in the brush border fraction and was also prominent in the basolateral fraction where phosphatidylcholine was the dominant glycerophospholipid. Furthermore, our results show a distinct difference in fatty acids content, where monounsaturated fatty acids (MUFA) were more abundant than polyunsaturated fatty acid (PUFA). Nervonic acid (24:1n-9) was a prominent fatty acid in the BBM at ~50% of the total MUFA. We hypothesize that the high cholesterol content and the presence of this rare fatty acid may serve to maintain membrane fluidity in the cold environment.

Keywords: nervonic acid, brush border, fatty acids, lipids, cold adaptation, Atlantic cod, enterocytes, intestines

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Introduction

Biological membranes act as physical barriers to solute diffusion and, therefore, regulate the transport of metabolites and other events that must cross them. The ability of cells to utilize energy stored in transmembrane ion gradients is also dependent on membranes providing an organizing matrix for the assembly of protein complexes. Membranes are highly dependent on temperature to enable them to perform various biological roles.[1] At physiological temperature, the membrane bilayer is in a fluid-liquid state, whereas below its physiological temperature, the bilayer components lose their mobility, producing a progressive stiffness that can cause the rate of biochemical processes to decrease dramatically.[2] Conversely, above its physiological temperature, the bilayer loses its integrity and can dissolve. A proper degree of fluidity is, therefore, essential for the functionality of all biochemical membranes.[3,4]

The plasma membrane of eukaryotic cells contains three main lipid classes: glycerophospholipids, sphingolipids, and cholesterol. The glycerophospholipids consist of the glycerol-backbone with two ester-bonded fatty acids, usually of between 16 and 20 carbons. The sphingolipids, however, have only one fatty acid attached to the sphingosine backbone, which usually contains a longer and fully saturated acyl chain reaching up to 24 carbons. The relative proportions of these main lipid classes vary, not only between species but also among different cell types in vertebrates. The typical plasma membrane has the following relative ratios of these main lipid classes in addition to cholesterol at 30 to 40 mol%: glycerophospholipids at 40 to 60 mol% and sphingolipids at 10 to 40 mol%.[5] It has been shown for polarized cells that the lipid composition and fatty acid content vary significantly between the brush border and the basolateral membrane parts. This could be linked to their differential role in cellular processes.[2,4] Previously, lipid studies performed on the brush border and basolateral membrane parts of polarized intestinal enterocytes from rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) have shown that membranes exchange the phospholipid headgroups when adapting to different environmental temperatures. Variation in the fatty acid content of the glycerol-based lipids has also been related to cold adaptation, both in trout acclimated to 5 °C and 20 °C, and carp acclimated to 10 °C and 30 °C.[4,6] By this, the membrane is believed to maintain its viscosity at varying temperatures, which is essential for proper physiological function of the membrane proteins and enzymes.[3]

We have previously reported on the isolation of brush border and basolateral membranes from the intestinal enterocytes of Atlantic cod (*Gadus morhua*) with focus on proteins found in relatively high amounts in the intestinal lining.[7] Using ^{31}P-NMR, we found in that study that the brush border membrane contained an unusual amount of sphingomyelin at the level of 58% of the total phospholipids and a low amount of other phospholipids.[7] In view of this finding, we launched a more detailed analysis of the two membrane parts from the intestinal enterocytes, the brush border membrane (BBM) and the basolateral membrane (BLM), to evaluate both the difference in the main lipid classes and their fatty acid content.

Atlantic cod belongs to the group of animals that are dependent on their environmental temperature and has been shown to be able to adapt to a wide range of temperatures from −1 °C to 20 °C with optimal growth rate at 5.8 °C.[8] Therefore, Atlantic cod must have adapted its biological membranes to function at low temperatures. The transition between the fluid/crystalline state to gel state (because of a decrease in temperature) has been shown to affect plasma membrane function by (1) inducing clustering of integral membrane proteins, (2) slowing down lateral protein diffusion, and (3) reducing activity of enzymes connected to the membrane. These factors are thoroughly reviewed by Hazel et al[3] and articles cited therein as well as by Portner et al.[9] In order for species to adapt to frigid environments, the homeoviscous adaptation theory (HVA) states that the two most important factors for the membrane to maintain its functional integrity are firstly to increase the proportion of *cis* unsaturated fatty acids, especially polyunsaturated (PUFA), and secondly, to elevate the proportion of phosphatidylethanolamine over phosphatidylcholine.[3]

Here, we report a thorough lipidomic analysis of BBM and BLM fractions of the intestinal enterocytes from Atlantic cod (*Gadus morhua*), a cold temperature species, including the non-charged glycosphingolipids. The aim was primarily to find if these membrane fractions would show differences in the main lipid classes and fatty acid composition as well

as calculated indices by comparing them with results of previously reported studies from other fish types and vertebrates. We report that sphingolipids are more abundant than the phosphoglycerolipids and that phosphatidylethanolamine is not prominent. As far as the fatty acid content is concerned, a high proportion of 24:1n-9 was observed in the BBM, and this may help keep the membrane in a fluid state in addition to polyunsaturated fatty acids that were not particularly plentiful. This study lays the basis for our ultimate aim of studying the composition and functional aspects of lipid rafts in these membranes, including the role of alkaline phosphatase (and other proteins) in nutrient uptake[10] and immunity to pathogens.[11]

Materials and Methods

Materials

Sephadex G-25 fine, cholesterol, cholesteryl palmitate, glyceryl tri-palmitate, lyso-phosphatidylcholine, phosphatidylcholine, sphingomyelin, and galacto-cerebroside were analytical grade from Sigma-Aldrich (Steinheim, Germany). Silica gel (Kieselgel 60, 230–400 mesh) and silica gel 60 thin layer chromatography glass plates (20 × 20) were purchased from Merck (Darmstad, Germany). All other chemicals and solvents were of analytical grade from Sigma-Aldrich (Steinheim, Germany).

Methods

Sample collecting and membrane fractionation

For a single typical extraction experiment, six two-year-old Atlantic cods (*Gadus morhua*) weighting ~1.0 kg cultured from the larval stage in seawater aquariums at constant 7 °C were obtained from the Marine Research Institute at Stadur, Grindavik, Iceland.[8] Daily feeding consisted of pellets of the regular diet "Sjófiskafóður" produced by the company Laxá in Akureyri, Iceland (http://www.laxa.is). Tissue fractionation and sub-cellular isolation were performed by adapting modified versions of the methods described by Booth and Kenny[12] and Danielsen,[13] a method previously described by Gylfason et al.[7] Briefly, the intestinal tube was cut longitudinally and rinsed in HBS buffer (25 mM HEPES, 150 mM NaCl, pH 7.1). The enterocytes were scraped from the inner side of the intestinal lumen into a cold homogenisation buffer (1:10 w/v) consisting of 2 mM Tris-HCl, 50 mM mannitol, pH 7.1 and homogenized manually using a Potter-Elevhjelm homogenizer for five minutes. DNAase was added to the homogenate to give a final concentration of 0.5 µg/mL and the homogenate incubated on ice for an hour. Homogenate was then cleared of unbroken cells and nuclear debris by centrifugation in a Hettich Universal 320R instrument at 150 xg (1100 rpm) for 5 min, followed by centrifugation at 2700 xg in the same centrifuge for 10 minutes to pellet the mitochondria. The supernatant was then adjusted to 10 mM $MgCl_2$ and incubated on ice for 10 minutes and centrifuged in a Sorvall SS-34 rotor at 1150 xg (~3000 rpm) for 10 minutes. The pellet, containing some intracellular membranes together with the basolateral membrane, was saved. The supernatant was then centrifuged again in the Sorvall SS-34 rotor at 48,000 xg (~20,000 rpm) for 30 min to pellet the BBM. All pellets were suspended in HBS buffer to give a protein concentration of ~5 mg/mL, flash frozen in liquid nitrogen, and stored at –20 °C until used in further studies.

Total lipid extraction and fractionation of lipid classes

Lipids, including polar gangliosides, were extracted from isolated membrane fractions using the previously described method of Folch[14] with later modification by Suzuki.[15] Briefly, 0.5 mL of cellular homogenate (5 mg/mL protein) in HBS buffer were mixed with 5 mL of chloroform (C):methanol (M) (1:1 v/v) and incubated for two hours. After removing insoluble material from the homogenate, three further extractions were performed: first with 3 mL of the same solvent, second with 3 mL of C:M (1:2 v/v), and finally with C:M:H_2O (48:35:10 v/v/v). The four extracts were then combined and dried under a stream of nitrogen and dissolved in 2.5 mL of C:M:H_2O (60:30:4.5 v/v/v) and applied onto a Sephadex G25 superfine column (diameter 1.0 cm, height 3.5 cm) and the sample eluted with 5 mL of the same solvent mixture followed by 2.5 mL of C:M (2:1 v/v) and finally 2.5 mL C:M:H_2O (48:35:10 v/v/v). The total lipid was then dried under nitrogen and dissolved in 0.5 mL chloroform for isolation of different lipid classes.

The total lipid was further separated into the five major lipid classes: neutral lipids, non-sialylated glycolipids, non-sialylated phospholipids, mono-sialylated

gangliosides, and poly-sialylated gangliosides using column chromatography on silica gel (Kieselgel 60, 230–400 mesh, diameter 0.6 cm, height 2.5 cm). The column chromatography was performed by adapting and modifying combinations of solvents as described earlier by Drayfus et al[16] for the separation of neutral lipids from a combined fraction of non-sialylated glycolipids (NSL) and total phospholipids (PL).[16] Briefly, 0.5 mL samples of total lipid dissolved in chloroform were applied to the column and eluted with 12 mL of chloroform for recovery of neutral lipids. Next, a combined fraction of NSL and PL was first eluted with 1.5 mL of chloroform:methanol:acetone:acetic acid:H_2O (52:8:8:18:4 v/v/v/v/v) followed by 8 mL of C:M (4:1 v/v). The last two lipid classes were then eluted from the column by 2.5 mL of C:M (2:3 v/v) eluting the mono-gangliosides followed by three different solvent combinations for eluting the poly-sialogangliosides: first 2.5 mL C:M:H_2O (65:25:4 v/v/v), then 2.5 mL C:M:H_2O (60:35:8 v/v/v), and finally 2.5 mL C:M:H_2O (48:35:10 v/v/v). Further separations of the combined fraction of non-sialylated glycolipids and phospholipids fractions were performed by applying these fractions to 300 mg of silica gel slurry placed in the same column as described earlier by Pernet et al.[17] Briefly, the combined sample transferred to the column was first eluted with 10 mL of chloroform, eluting any traces of neutral lipids, followed by 15 mL of acetone:methanol (9/1 v/v), which elutes the non-sialylated glycolipids. Finally, the phospholipids were eluted by 10 mL of methanol mixed with 200 µL of acetic acid.

Each lipid class fraction was then dried under stream of nitrogen and dissolved in 1:1, by vol. chloroform/methanol containing butylated hydroxytoluene (BHT) at 50 µg/mL and stored at –20 °C until further analysis.

Biochemical assays

Protein concentrations of the membrane fractions were determined by use of a dye-binding assay with Coomassie Blue G-250 using a commercial human serum albumin/globulin mixture (Sigma-Aldrich, Steinheim, Germany) as standard.[18] Cholesterol concentrations were determined by a conjugated enzyme assay from Randox Laboratories Ltd (Crumlin, UK), CHOD-PAP regent kit. Briefly, a sample, previously dried down under nitrogen was dissolved in 15 µL of 20% (w/v) Triton X-100, 75 µL ethanol and 700 µL of CHOD-PAP reagent, and incubated at 37 °C for 30 minutes before the absorbance at 500 nm was read. Phospholipid concentrations in membrane fractions were determined as total inorganic phosphate (from $HClO_4$ hydrolysis of phospholipids) using the colorimetric method of Rouser et al.[19] Glycolipid concentrations were determined by a colorimetric assay according to the procedure described by Kushwaha et al,[20] using galactose as standard. For calculation purposes, it was assumed that the molar ratio of hexose to glycolipid was 1:1.

Thin layer chromatography

Thin layer chromatography (TLC) was performed on Merck TLC silica gel 60 glass plates cut to suitable size. Extracted lipids (~15 nmol) were spotted on the plates from each fraction in line with known standards. For phospholipids, the silica plates were developed with chloroform:methanol:acetic acid:H_2O (60:50:1:4 v/v/v/v), for non-sialylated glycolipids with chlorof orm:methanol:acetone;acetic acid;H_2O (52:8:8:18:4 v/v/v/v/v), and for neutral lipids and sterols with hexane:diethyl ether:acetic acid (60:40:2 v/v/v). After development, the plates were dried and charred by immersing them in a solution of 10% (w/v) copper sulphate in 8% (v/v) aqueous phosphoric acid for 20 seconds and then dried for 10 minutes and placed in an oven at 130 °C for 10 minutes. Plates containing phospholipids were frequently stained with iodine vapour, but this proved insensitive to spot minor lipids and lipids with saturated fatty acids. Amine containing lipids (serine, ethanolamine) were stained with ninhydrin by spraying and subsequent baking in an oven at 130 °C for 10 min. Proportional content analysis from each lane of the TLC plates was performed by densitometry of digital photographs with the ImageJ software (v. 1.42q, http://rsb.info.nih.gov/ij/, USA).

Preparation of fatty acid methyl esters and gas chromatography

Fatty acid methyl esters (FAME) were prepared from each lipid class using a 14% boron trifluoride in methanol (BTM) from Sigma-Aldrich (Steinheim, Germany) following the method described by Metcalfe et al.[21] FAME were separated using a Hewlett-Packard 6890 GC system (Waldbronn, Germany) fitted with a Megabore DB-225 column (30 m × 0.25 mm i.d. × 0.25 µm film)

(J & W Scientific, California, USA) with a helium carrier gas linear flow rate of 0.6 mL min^{-1}. The injector and detector manifold were at 250 °C and 180 °C respectively. The oven temperature was then increased at a rate of 3 °C min^{-1} from 150 °C to 220 °C and held at this temperature for 10 minutes for a total runtime of 33.3 minutes. The separated FAME were detected using a Hewlett-Packard 5973 mass selective detector (Waldbronn, Germany) with a mass range between 50 and 550 m/z. FAME was identified by retention time compared against authentic standards (FAME 37 mix #47885U, Pennsylvania, USA) and a fragmentation pattern library (Whiley 275 K, 1996) in a mass spectral search program (National Institute of Standards and Technology, Gaithersburg, MD, USA). The relative composition of each fatty acid was done by peak integration and expressed for individual FAME as percentages of the total in each run.

Statistics

For statistical analysis, values are expressed as the mean of three independent experiments starting from live fish ± SD (n = 3), each based on triplicate analyses. Group differences were assessed using the unpaired *t* test. A *P* value less than 0.05 was considered to indicate statistical significance. All statistical analyses were conducted using the statistical package GraphPad InStat (GraphPad Software, Inc, La Jolla, CA, USA). The FA unsaturation index (UI) was calculated as the number of double bonds per 100 FA (percentage of each FA times number of double bonds, summed for all FA).

Results

Lipid composition: TLC of main lipid classes

Figure 1 shows the percentage mole fractions of the three groups of total lipid (cholesterol, phospholipid, and glycolipids) from both the brush border membranes (BBM) and the basolateral membranes (BLM). Comparison of the membrane fractions showed that cholesterol was higher in the BBM fraction at 37.8 mol% compared with 28.0 mol% in the BLM fraction. The content of phospholipids in these two membrane fractions showed a small but significant difference at 16.8 mol% of the total lipids in the BLM compared with 13.8 mol% in the BBM. Also, the glycolipids were less abundant in the BBM fraction than the BLM fraction, at 55.1 mol% and 48.5 mol% respectively.

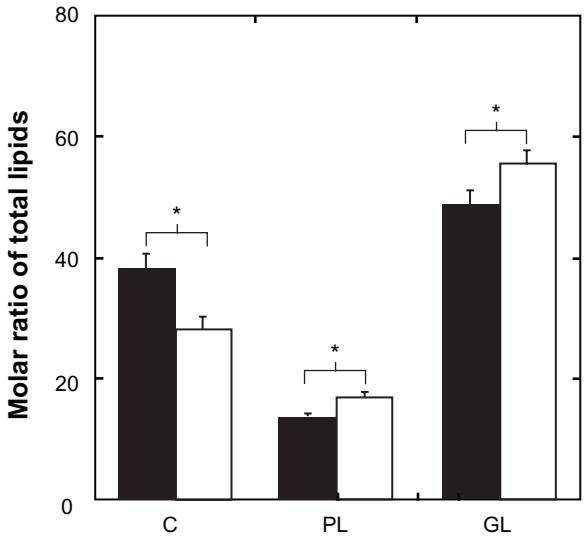

Figure 1. Comparison of main lipid classes of the brush border (BBM) and basolateral (BLM) membrane fractions from Atlantic cod (*Gadus morhua*) intestinal enterocytes.
Notes: Data are presented as means ± standard deviation (n = 9). A difference was taken as significant when $P < 0.05$ and is marked with asterisks.
Abbreviations: BBM, black columns; BLM, white columns; C, cholesterol; PL, phospholipids; GL, glycolipids.

Figure 2A, C, and E show the separation of the BBM and BLM lipids with reference to standards, and the results are further described in the following sections. The thin layer chromatography (TLC) was performed on the phospholipids (PL), neutral lipids (NL), and non-sialylated glycolipids (NSL) fractions to visualize their compositional pattern.

Phospholipids

The proportion of individual phospholipids (PL) was clearly different in the brush border membrane (BBM) compared with BLM as shown in Figure 2A and B. The comparison of sphingomyelin (CerPCho), a dominant lipid in both membrane fractions, showed a much higher ratio in the BBM, at 66.3% compared with 35.5% in BLM. On the other hand, the ratio of phosphatidylcholine (PhtCho) was higher in the BLM fraction than in the BBM, at 49.4% compared with 24.5% in the BBM. The ratio of the other phospholipids, phosphatidylserine (PhtSer), phosphatidylethanolamine (PhtEtn), phosphatidyl inositol (PtdIns), were relatively low in both membrane fractions, but the BLM had greater

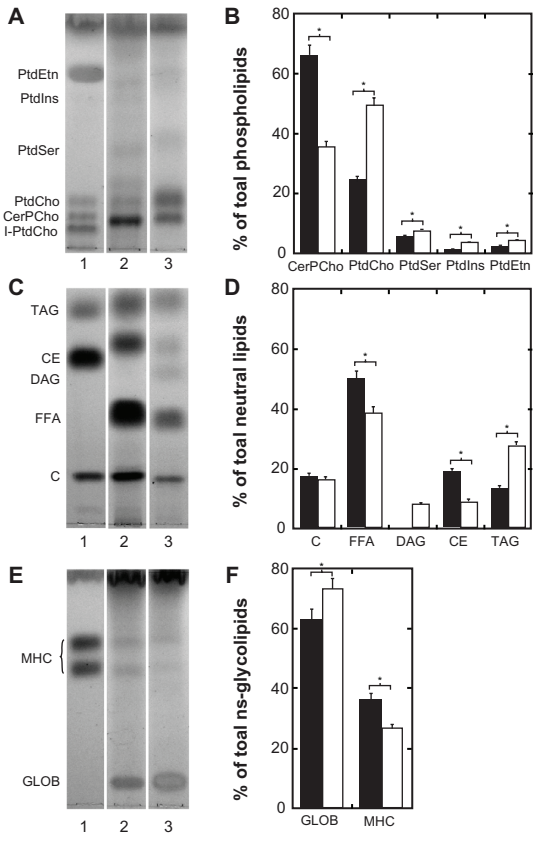

Figure 2. Thin layer chromatography (TLC) of sample from the three main lipid classes extracted from brush border (BBM) and basolateral membrane (BLM) parts of Atlantic cod intestinal enterocytes. (**A**) Separation of phospholipids by TLC. (**B**) Proportion of each phospholipid. (**C**) Separation of neutral and sterol lipids by TLC. (**D**) Proportion of each neutral and sterol lipids from BBM (black columns) and BLM (white columns). (**e**) Separation of non-sialylated glycolipids by TLC. (**F**) Proportion of each non-sialylated glycolipid.
Notes: Lanes on TLC plates: (1) standards, (2) BBM, (3) BLM. Data are presented as means ± standard deviation (n = 9). A difference was taken as significant when $P < 0.05$ and is marked with asterisks.
Abbreviations: BBM, black columns; BLM, white columns; PhtEtn, phosphatidylethanolamine; PhtIns, phosphatidylinositol; PhtCho, phosphatidylcholine; CerPCho, sphingomyelin; l-PhtCho, lyso-phosphatidylcholine; TAG, triacylglycerol; CE, cholesterol ester; DAG, diacylglycerol; FFA, unesterified fatty acids; C, cholesterol; MHC, monohexosyl ceramide; GLOB, globosides.

abundance overall. In the BBM, PhtSer was at 5.6%, PhtEtn was at 2.4%, and PhtIns was 1.1% of the phospholipids, compared with 7.5%, 4.2%, and 3.3% in the BLM fraction respectively.

Neutral lipids and sterols

The comparison of the TLC patterns of neutral lipids and sterols is shown in Figure 2C and D. The proportion of cholesterol (C) was almost identical in the BBM and BLM samples, at 17.5% versus 16.5% respectively. Another spot traveled on the TLC plates where unesterified fatty acids (FFA) would be expected and was clearly dominant in both neutral lipid membrane fractions. This brought the proportion of the neutral lipids to 86.6% in the BBM fraction (FFA 50.1%, CE 19.0%, C 17.5%), which is considerably higher than in the BLM fraction at 64.1% (FFA 38.7%, CE 8.9%, C 16.5%). Diacylglycerol (DAG) was only detected in the BLM fraction (8.1%) and the amount of triacylglycerols (TAG) was evidently higher for the BLM sample than the BBM fraction, at 27.7% and 13.5% respectively.

Non-sialylated glycolipids

As noted above, non-sialylated glycolipids were 50% to 60% of the total lipid (Fig. 1). A comparison of the different non-sialylated glycolipids is shown in Figure 2E and F. Globosides (GLOB) with more than one sugar were the dominant lipids in both the BBM and the BLM fractions, at 63.5% versus 73.3% respectively. Monohexosyl ceramide (MHC) was found in both membranes but was proportionally more abundant in the BBM fraction, at 36.5% versus 26.7% in the BLM fraction.

Calculated indices

To make comparison with previously reported studies easier, we calculated from the analytical data some indices as shown in Table 1. The ratios of each lipid class to protein showed that cholesterol over protein (C/Pr) was significantly higher in the BBM part in comparison with the BLM part, at 1.14 and

Table 1. Indices calculated using lipid analysis data from the brush border membrane (BBM) and the basolateral membrane (BLM) fractions isolated from Atlantic cod (*Gadus morhua*) intestinal enterocytes.

	BBM	BLM	*P*-value
C/Pr[a]	1.14 ± 0.06	0.79 ± 0.04	0.0001
PL/Pr[a]	0.41 ± 0.01	0.45 ± 0.01	0.0080
GL/Pr[a]	1.46 ± 0.02	1.48 ± 0.09	0.7262
PhtEtn/PhtCho[b]	0.14 ± 0.01	0.08 ± 0.01	0.0018
CerPCho/PhtCho[b]	3.81 ± 0.19	0.71 ± 0.04	0.0001
C/PL[c]	2.77 ± 0.13	1.74 ± 0.09	0.0004
C/GL[c]	0.78 ± 0.02	0.53 ± 0.02	0.0001
PL/GL[c]	0.29 ± 0.01	0.31 ± 0.02	0.1963

Notes: $P < 0.05$ significant. [a]nmol/µg protein; [b]relative abundance. [c]Mole ratios. Results are expressed as the mean from three different extraction experiments (n = 3) performed in triplicate. ± indicates standard deviation.
Abbreviations: C, cholesterol; Pr, protein; PL, phospholipids; GL, glycolipids; PhtEtn, phosphatidylethanolamine; PhtCho, phosphatidylcholine; CerPCho, sphingomyelin.

0.79 respectively. The phospholipid over the protein ratio (PL/Pr) showed, on the other hand, not such a large difference where PL/Pr was 0.41 in the BBM compared with 0.45 in the BLM. The glycolipid over the protein ratio (GL/Pr) was not significantly different. However, the PhtEtn/PhtCho ratio proved to be significantly higher for the BBM compared with the BLM, at 0.14 versus 0.08 respectively. The most striking difference was observed for the CerPCho/PhtCho ratio, being fourfold higher in the BBM than in the BLM fraction.

The cholesterol over phospholipids (C/PL) molar ratio for the BBM was significantly higher compared with the BLM fraction, at 2.77 and 1.74 respectively. The same pattern was evidenced for the C/GL ratio, where molar ratio in the cod BBM was higher compared with BLM, at 0.78 and 0.53, respectively. Nonetheless, the phospholipid over glycolipids (PL/GL) was not substantially different for BBM and BLM membrane fractions, at 0.29 and 0.31 respectively.

Fatty Acid Analyses

Fatty acids (FA) were isolated from phospholipids (PL), non-sialylated glycolipids (NSL), monosialylated gangliosides (MG), and polysialylated gangliosides (PG) from the BBM and the BLM fractions, as described in Material and Methods. The results from the GC/MS analyses are tabulated in Table 2 and will be summarized in the following sections. Calculated indices from these results are plotted in Figure 3A through D for both of the membrane fractions. The sum of ratios of the saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and the polyunsaturated fatty acids (PUFA) from Table 2 are also plotted in Figure 4A through C that accompany the discussion.

Fatty acid analyses of phospholipids

Table 2 shows the distribution of fatty acids into three main groups, namely saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids. In the BLM phospholipids fraction, the saturated fatty acids (SFA) were at a much higher proportion than in the BBM, at 55.3% and 25.5% respectively. The difference was mainly because of the high proportion of palmitic acid (16:0) and stearic acids (18:0) in the BLM fraction. Both were in significantly lower amounts in the BBM membrane fraction, in particular 16:0. In contrast, the monounsaturated fatty acids (MUFA) were significantly more abundant in the BBM than in BLM membrane fraction, at 67.6% and 35.4% respectively. The main MUFA in both membrane fractions was nervonic acid (24:1n-9), contributing over ~59% to the total FA of phospholipids in the BBM and ~17% in the BLM fraction. The second most abundant MUFA in the BLM fraction was oleic acid (18:1n-9), giving ~10% of the total phospholipids compared with ~3% in the BBM fraction. The polyunsaturated fatty acids (PUFA) proved to be more prevalent in the BLM than in the BBM fraction, at ~10% and ~7% respectively. Both membrane fractions contained low amounts of the well-known fish oil PUFA, with docosahexaenoic acid (22:6n-3) ~2.6% in both membrane fractions and eicosahexaenoic acid (20:5n-3) only detected in the BBM fraction of 1.0%. The second most abundant PUFA in the BBM fraction was 18:2n-6 at 1.6% of the total fatty acids analyzed in the phospholipid sample. The main PUFA in the BLM fraction proved to be 18:2n-6 and 18:3n-6, at 4% and 3.3% respectively. Despite the fact that more types of PUFA were detected in the BBM fraction, they were mostly in low quantity at ~1.0% or less, and the total PUFA content was proportionally less than in BLM.

Fatty acid analyses of non-sialylated glycosphingolipids

The glycosphingolipids were separated into non-sialylated glycolipids and gangliosides with one or more sialic acids. The proportion of saturated fatty acids (SFA) in the non-sialylated glycolipids (NSL) was significantly higher in the BLM fraction than in the BBM fraction, at 58.1% compared with 43.5% in the BBM fraction (Table 2). In the BLM fraction, palmitic acid (16:0) and stearic acid (18:0) contributed ~19% and ~29% of the total fatty acid content. The reverse was found in the BBM membrane fraction, where palmitic acid was more abundant than steric acid. As for MUFA, the BLM part contained a slightly higher proportion than the BBM fraction, 26.7% and 23.6% respectively. The main FA in both membrane fractions was 18:1n-9 (11%–14%). Specifically, the BLM part contained a much higher proportion of 22:1n-9 (~6%) than BBM (~0.4%). Finally, the PUFA content was twice as high in the BBM fraction at 32.9% compared with 16.0% in BLM fraction. The most

Table 2. Comparison of the fatty acid content of the intestinal brush border (BBM) and basolateral (BLM) membranes from the intestinal enterocytes of Atlantic cod (*Gadus morhua*).

	Phospholipids %			Non-sialylated glycolipid %			Mono sialylated ganglioside %			Poly sialylated ganglioside %		
	BBM	**BLM**	***P***	**BBM**	**BLM**	***P***	**BBM**	**BLM**	***P***	**BBM**	**BLM**	***P***
14:0	5.9 ± 1.0	2.2 ± 0.7	0.0063	5.1 ± 0.8	–	–	–	–	–	4.9 ± 2.1	1.7 ± 1.1	0.0796
15:0	0.3 ± 0.1	–	–	1.1 ± 0.3	–	–	–	–	–	–	1.0 ± 0.4	–
16:0	5.7 ± 1.5	23.5 ± 6.5	0.0099	26.3 ± 1.8	19.4 ± 5.0	0.0877	12.6 ± 0.04	19.9 ± 5.8	0.0023	12.5 ± 2.4	19.0 ± 3.6	0.0599
17:0	0.2 ± 0.1	–	–	0.7 ± 0.1	1.1 ± 0.1	0.0088	–	–	–	–	0.8 ± 0.1	–
18:0	10.5 ± 1.1	25.0 ± 2.7	0.0010	9.2 ± 0.2	28.5 ± 0.9	0.0001	18.2 ± 0.1	20.2 ± 1.7	0.1117	14.5 ± 2.0	16.2 ± 1.1	0.2666
20:0	1.3 ± 0.5	1.9 ± 0.7	0.2956	0.4 ± 0.1	2.8 ± 0.4	0.0005	8.4 ± 0.1	3.8 ± 0.7	0.0004	2.1 ± 0.5	4.2 ± 1.0	0.0313
21:0	0.1 ± 0.2	1.8 ± 0.4	0.0028	0.4 ± 0.1	3.3 ± 0.5	0.0001	–	1.0 ± 0.2	–	–	3.1 ± 0.6	–
22:0	1.0 ± 0.1	0.8 ± 0.1	0.0705	0.2 ± 0.1	1.2 ± 0.1	0.0003	7.3 ± 0.1	–	–	1.3 ± 1.1	0.6 ± 0.2	0.3392
23:0	0.3 ± 0.03	–	–	–	–	–	–	–	–	–	–	–
24:0	0.3 ± 0.2	–	–	0.2 ± 0.03	1.7 ± 1.2	0.0969	3.5 ± 0.1	–	–	1.3 ± 1.2	–	–
∑SFA	**25.5 ± 2.4**	**55.3 ± 4.4**	**0.0005**	**43.5 ± 2.4**	**58.1 ± 2.3**	**0.0002**	**50.0 ± 0.1**	**44.4 ± 5.3**	**0.0059**	**36.7 ± 2.8**	**46.4 ± 5.2**	**0.0002**
16:1n-7	0.4 ± 0.3	2.0 ± 0.9	0.0432	2.4 ± 0.2	3.0 ± 2.8	0.7300	–	4.1 ± 0.6	–	–	3.7 ± 1.1	–
18:1n-9	2.6 ± 1.0	10.4 ± 3.9	0.0284	13.5 ± 0.9	11.3 ± 0.9	0.0402	4.2 ± 0.2	8.4 ± 2.1	0.0261	4.5 ± 0.3	7.8 ± 1.2	0.0099
20:1n-9	1.9 ± 0.6	2.8 ± 0.1	0.0625	3.7 ± 0.3	3.0 ± 1.6	0.4978	–	4.8 ± 1.6	–	–	4.6 ± 1.3	–
22:1n-9	3.4 ± 0.3	3.0 ± 1.0	05432	0.4 ± 0.1	6.1 ± 0.5	0.0001	2.2 ± 0.3	21.4 ± 5.7	0.0043	2.8 ± 0.7	19.9 ± 4.5	0.0029
24:1n-9	59.2 ± 2.8	17.2 ± 3.4	0.0001	3.5 ± 0.5	3.3 ± 1.0	0.7722	34.7 ± 0.6	7.3 ± 4.1	0.0003	51.7 ± 2.1	9.9 ± 1.5	0.0001
∑MUFA	**67.6 ± 3.6**	**35.4 ± 6.3**	**0.0015**	**23.6 ± 0.5**	**26.7 ± 1.9**	**0.0197**	**41.1 ± 0.8**	**46.2 ± 4.0**	**0.0017**	**59.6 ± 2.1**	**46.2 ± 2.7**	**0.0025**
18:2n-6	1.6 ± 0.4	4.0 ± 1.6	0.0653	3.1 ± 0.3	3.4 ± 0.7	0.5325	–	1.9 ± 0.5	–	2.4 ± 0.9	1.9 ± 0.9	0.5336
18:3n-3	0.6 ± 0.1	–	–	1.0 ± 0.1	2.2 ± 0.2	0.0007	4.7 ± 0.5	4.1 ± 1.5	0.5469	1.4 ± 1.2	2.8 ± 0.7	0.1558
18:3n-6	–	3.3 ± 1.6	–	0.3 ± 0.04	–	–	–	–	–	–	–	–
20:2n-6	0.2 ± 0.2	–	–	0.6 ± 0.1	–	–	–	–	–	–	–	–
20:3n-6	0.2 ± 0.4	–	–	0.1 ± 0.02	–	–	–	–	–	–	–	–
20:4n-6	0.5 ± 0.3	–	–	1.5 ± 0.1	2.4 ± 0.5	0.0378	2.9 ± 0.3	2.9 ± 1.2	1.0000	–	2.1 ± 0.5	–
20:5n-3	1.0 ± 0.1	–	–	9.5 ± 0.2	3.1 ± 0.8	0.0002	1.4 ± 0.01	–	–	–	–	–
22:2n-6	0.1 ± 0.1	–	–	–	–	–	–	–	–	–	–	–
22:6n-3	2.6 ± 0.6	2.5 ± 0.3	0.1000	16.7 ± 2.6	4.9 ± 1.0	0.0018	–	2.0 ± 1.1	–	–	0.8 ± 0.2	–4.0
∑PUFA	**6.9 ± 1.4**	**9.8 ± 2.0**	**0.1088**	**32.9 ± 2.5**	**16.0 ± 3.6**	**0.0026**	**8.9 ± 2.3**	**9.5 ± 1.7**	**0.5380**	**3.7 ± 0.9**	**7.4 ± 1.3**	**0.0001**
	100	100		100	100		100	100		100	100	

Note: Results are shown relative to total fatty acid analyzed expressed as the mean from three different extraction experiments performed in triplicate with standard deviation shown.
–, Not detected; ∑SFA, Sum of saturated fatty acids; ∑MUFA, Sum of monounsaturated fatty acids; ∑PUFA, Sum of polyunsaturated fatty acids.

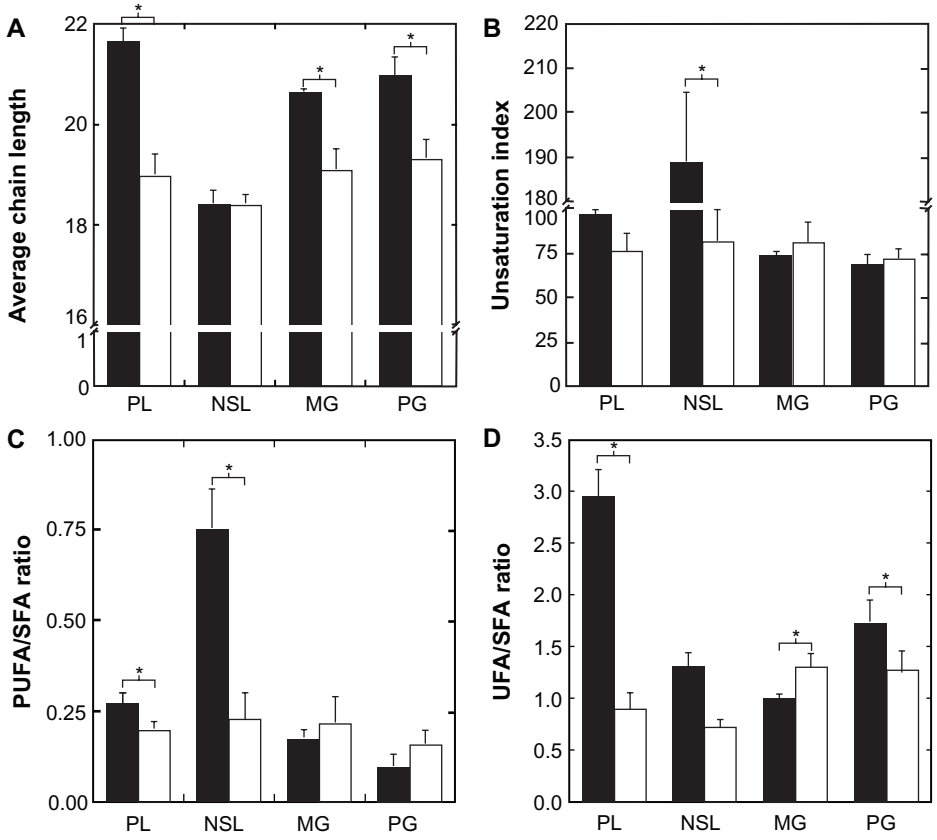

Figure 3. Calculated indices from fatty acids analysis of different lipid classes extracted from brush border membranes (BBM, black columns) and basolateral membranes (BLM, white columns) from Atlantic cod (*Gadus morhua*). (**A**) Average carbon chain length. (**B**) Unsaturation index of the fatty acids (percentage of each FA times number of double bonds, summed for all FA). (**C**) Polyunsaturated fatty acids over saturated fatty acids (PUFA/SFA). (**D**) Unsaturated fatty acids over saturated fatty acids (UFA/SFA, where UFA is the sum MUFA+PUFA).
Notes: Data are presented as means ± standard deviation from three separate experiments, each performed in triplicate.
Abbreviations: PL, phospholipids; NSL, non-sialylated glycolipids; MG, mono-sialylated gangliosides; PG, poly-sialylated gangliosides.

common PUFA was docosahexaenoic acid (22:6n-3) in both membrane fractions, at ~17% in the BBM but ~5% in the BLM fraction. The second most abundant PUFA was either eicosahexaenoic acid (20:5n-3) at ~10% in the BBM or 18:2n-6 at ~3% in the BLM fraction.

Fatty acid analyses of mono-sialylated gangliosides

Mono-sialylated gangliosides (MG) contained more saturated fatty acids (SFA) in the BBM than in the BLM fraction, at 50.0% and 44.4% respectively (Table 2). This higher proportion was mainly due to the relatively high amount of arachidic acid 20:0 and behenolic acid (22:0) at ~7%–8% in BBM compared with ~0%–4% in BLM). However, palmitic acid (16:0) and stearic acid (18:0) were in higher amounts in the BLM fraction, although the difference was not statistically significant for the latter. The MG lipids had more MUFA and PUFA in BLM compared with BBM. In the MUFA lipids, the difference was mainly due to the presence of nervonic acid (24:1n-9) at ~35% in the BBM fraction compared with 7% in the BLM fraction. On the other hand, the BLM membrane fraction showed a very high content of 22:1n-9 at ~21% of the total fatty acid content, but this fatty acid was only found at ~2% in the BBM fraction. The PUFA proportion in BBM and the BLM fraction were similar at ~9%. The two FA dominating in both membrane fractions were 18:3n-3 and 20:4n-6, at ~4% and ~3% respectively.

Fatty acid analyses of poly-sialylated gangliosides

In the poly-sialylated gangliosides (PG) fractions, a significantly higher proportion of SFA was found in the BLM than in the BBM fraction, at 46.4% and 36.7% respectively. This was mainly due to 16:0 that

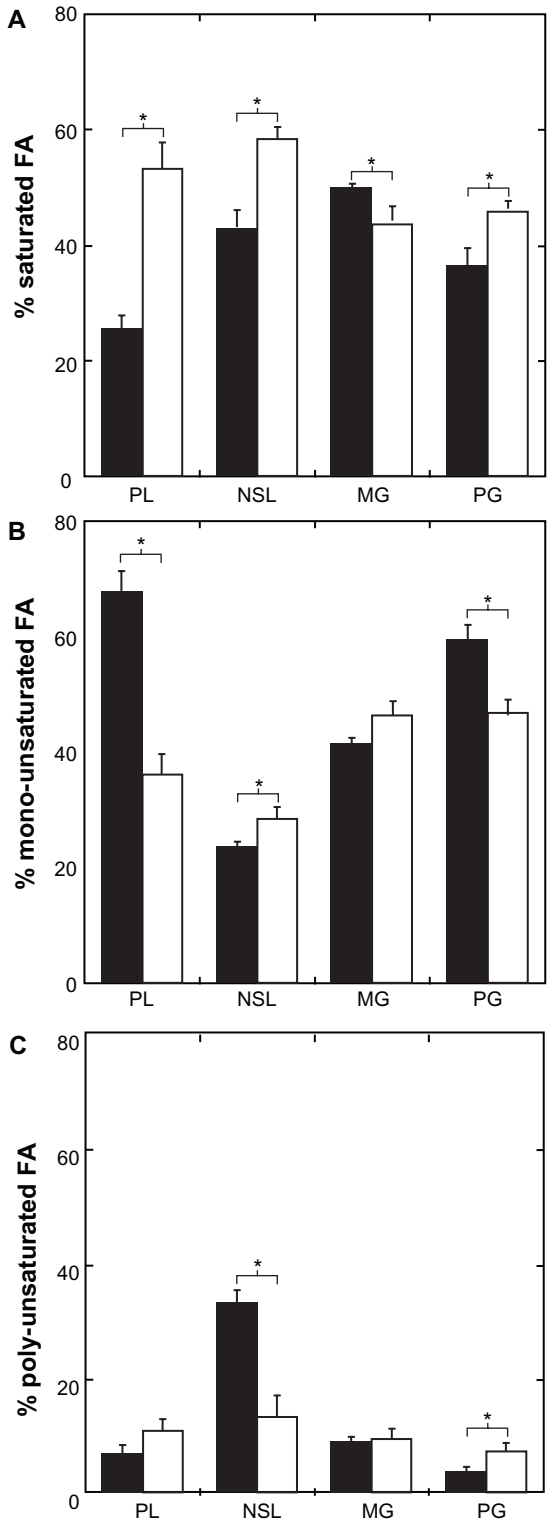

Figure 4. Proportional saturation of fatty acids in lipids isolated from the intestinal enterocytes of Atlantic cod (*Gadus morhua)*. Data taken from Table 2. (**A**) Ratio of saturated fatty acids against total fatty acids. (**B**) Ratio of monounsaturated fatty acids against total fatty acids. (**C**) Ratio of polyunsaturated fatty acids against total fatty acids.
Note: Data are presented as means ± standard deviation (n = 3).
Abbreviations: BBM, brush border membrane (black columns); BLM, basolateral membranes (white columns); PL, phospholipids; NSL, non-sialylated glycolipids; MG, mono-sialylated gangliosides; PG, poly-sialylated gangliosides.

was higher in BLM than in the BBM fraction (19.0% vs. 12.5%). Both 18:0 and 20:0 were also more prominent in the BLM fraction. On the other hand, the proportional amount of MUFA proved to be significantly much higher in the BBM, at 59.6% compared with the 46.2% in the BLM fraction because of the high proportion of 24:1n-9 in the BBM (51.7%) compared with the BLM (9.9%). However, the main FA in the MUFA lipids of the BLM fraction was 22:1n-9 at 19.9%, with ~3% being found in the BBM sample. The content of PUFA in BBM fraction was 3.7% of the total fatty acids, compared with 7.4% in the BLM fraction. This was mainly due to a higher proportion of 20:4n-6, at ~2% in BLM.

Indices from the fatty acid analyses of BBM and BLM

The average carbon chain length (AC) was calculated and plotted and is shown in Figure 3A. All lipid classes, excluding the NSL fraction, showed significantly increasing acyl chain length in the BBM fraction compared with the BLM. Overall, the average chain length was 18 to 19 carbons in the BLM fractions, but more fluctuations were found in the average chain length in the BBM fractions, varying from 19 to 22 carbons.

The unsaturation index (UI) was also calculated for each lipid class. In Figure 3B, these indices are shown for both the BBM and BLM fractions. The UI in the PL class was higher in the BBM fraction than in the BLM fraction at 96.9 and 74.8 respectively. The difference in UI was particularly large in the NSL fraction, where it was 188.9 in BBM part compared with 81.2 in the BLM. On the other hand, the UI was not significantly different for the two ganglioside fractions. The mono-ganglioside fractions had a UI of 80.6 in the BLM and 73.4 in the BBM, and for the poly-ganglioside fraction, the UI was at 71.1 for BLM and 68.5 for the BBM fraction.

We also calculated the amount of PUFA over SFA as a ratio and plotted these, as shown in Figure 3C. The largest PUFA/SFA ratio was present in the NSL fraction, where the difference was over fourfold in the BBM part of the cells compared with the BLM fraction, 0.76 and 0.23 respectively. The PUFA/SFA ratio for PL was also significantly higher in BBM (BBM 0.27 and BLM 0.20). However, neither of the ganglioside fractions showed significant differences in the PUFA/SFA ratios. As both the membrane fractions

contained a high amount of MUFA, we also calculated the ratio of unsaturated fatty acids (UFA as the sum of MUFA and PUFA) over SFA as shown in Figure 3D. This index was highest for the BBM phospholipids, threefold higher in BBM than in the BLM, at 2.9 and 0.9 respectively. The NSL and PG lipids also had a higher ratio in the BBM cell part than in the BLM part, whereas for the MG fraction, the index was higher in the BLM fraction than in the BBM fraction.

Discussion

In this study, we analysed the lipid content of the intestinal enterocytes from Atlantic cod, both the glycerophospholipids and the two other major lipid classes, cholesterol and glycolipids. Glycolipids are frequently omitted in the total analysis of lipids as van Meer and colleagues have pointed out,[5] despite being a relatively large membrane component. Information concerning this coldwater fish has been sorely wanting, notwithstanding the important role the intestines play in digestion, nutrient absorption, and as an immunological barrier to the environment. Our main objective was to study and understand the functional environment that proteins enjoy in this organ, especially the differences in the polarized enterocyte´s brush border and basolateral membrane parts, and how low temperature may shape the molecular content as related to the bilayer membrane.[7]

Lipid Analyses

Cholesterol is a major part of the plasma membrane in vertebrate cells. In cold acclimated fish, the level of cholesterol in intestinal enterocytes has previously been reported to be higher in the outer BBM part than in the internal BLM part, and up to equimolar to the phospholipids.[3,5,6,22] Our results confirmed this but showed a notably higher amount of cholesterol compared with results reported in the literature in both membrane fractions.[5] Conversely, the phospholipid content decreased proportionally in both membrane parts. The results here gave the relative molar content of cholesterol around half for BLM and close to one-third for the BBM when the glycolipids were included. The amount of the glycolipids was high in both membrane fractions, giving a ratio of close to 3:1 to the phospholipids (GL:PL). Thus, the phospholipid content was lower compared with previous reports on fish that could be mainly due to the high proportions of glycolipids in both samples presented here and often ignored in other studies.[23–25] It should be noted that for calculation of the proportional content of various lipids, we assumed only one hexose per lipid molecule. As many glycolipids have more sugar units per molecule, the content of glycolipids is overestimated by this method. The yield of total glycolipids can also be predicted to be higher here than in some older studies due to the use of different methods for extracting the lipids.[25,26] Using the most commonly employed extraction methods,[14,27] we experienced that much of the more polar glycolipids were removed by the aqueous washing step (data not shown). We tested our methodology on rat intestines and obtained similar values for the relative amount from the main lipid classes to those commonly reported (data not shown).

In previous studies, the cholesterol levels in the BLM membrane from fish acclimated to different temperatures remained unchanged but increased in the BBM apical surface.[3,6] This led to the proposal that elevated cholesterol/phospholipid (C/PL) or cholesterol/glycolipid (C/GL) ratios were being used to adjust the viscosity of the membrane.[6] Cholesterol would be the main regulator as the acyl chain composition in the BBM in terms of length, and unsaturation was not significantly altered when fish were either kept at 5 °C or 20 °C. Indeed, the BBM in those studies contained higher cholesterol levels at 5 °C as well as more ordered membranes as measured by diffusion rates of fluorescent probes. Our results, presented here for the BBM, showed full correlation with these cold acclimation studies with higher C/PL and C/GL ratios in the BBM compared with BLM, and we expect cholesterol to play a role in the cold adaptation of the cod intestinal enterocytes. Studies on rainbow trout liver plasma membranes found that cholesterol was enriched in all membrane fractions from warm- compared with cold-acclimated animals.[22,26]

Our current analysis of the phospholipids by TLC tallies with our previous results obtained by ^{31}P-NMR that showed a high amount of sphingomyelin (CerPCho) in the BBM fraction at the level of 58 mol% of the total phospholipids.[7] Other phospholipids were in low amounts except phosphatidylserine (PhtSer) at 6 mol%. On the other hand, the BLM was quite different in this respect to the BBM. There, the level of phosphatidylcholine (PhtCho) was doubled and the level of CerPCho half that observed in the luminal side

of the cells. The content of other phospholipids in the BLM was also low, however, but still significantly higher than in the BBM fraction. These results correspond with previously published results in the literature, where the BLM is believed to have the "normal" cell phospholipid composition. The only caveat here is that the level of CerPCho was high at the expense of phosphatidylethanolamine (PhtEtn) and the BBM showed low amounts of PhtCho while higher amounts of CerPCho maintained the presence of the choline head-group.[2,5] Taking everything into consideration, the high amount of CerPCho in both membrane fractions could be the result of the high cholesterol level as the CerPCho has much higher affinity for cholesterol in the membrane than PhtCho. The latter is usually found in higher amount than our results show for the cod.[28,29]

The homeoviscous adaptation theory (HVA) states that a higher PhtEtn/PhtCho ratio of the membrane is directly related to elevated membrane fluidity necessary to counteract the effects of cold environments. In contrast, elevated CerPCho/PhtCho ratio was correlated with adjustment to a higher temperature, giving less fluidity of the membrane.[3] The basis for the effect of the PhtEtn/PhtCho ratio is believed to be the conical shape of the PhtEtn lipid, often regarded as a destabilizing PL for bilayer formation, and the cylindrical shape of the PhtCho, often taken as a bilayer stabilizing PL.[3] The ability of the CerPCho to both act as a hydrogen bond donor and acceptor while the PhtCho can only act as an acceptor causes a higher CerPCho/PhtCho proportion to give tighter packing of the membrane and, consequently, increase its viscosity.[3] In the present study, the comparison of the relative amounts of these PL ratios proved to be in contrast with the HVA theory, namely, the low content of PhtEtn in the cod membrane fractions, especially in the BBM. In addition, the CerPCho/PhtCho ratio was much higher in the BBM than in BLM. At present, it is not clear how this could be affecting the thermal acclimation of these different compartments of the membrane. The glycerol-based PhtCho and sphingoid-based CerPCho contain the identical choline head-group, and the exchange of the other part may have an effect on the ability of the membrane to perform its physiological function without affecting surface characteristics. However, we still do not know if some of the sphingomyelin may carry an ethanolamine head-group instead of choline,[30] a possible explanation for the lack of PhtEtn in the cod enterocytes' plasma membrane. These questions, we believe, need to be addressed in the future to gain deeper insight into the physiological effect they could have on the ability of the plasma membrane to adapt to different temperatures.

Fatty Acid Analyses

Fatty acids contribute highly to the viscosity of biological membranes. It is well established that higher unsaturation lowers the transition from liquid-crystalline state to the gel state. Higher unsaturation allows membranes to carry out their bio-physiological function at lower temperatures, and this can be regulated.[3] Studies have also shown that the fatty acid content of dietary fat can have a direct effect on the fatty acid content of lipids in various tissues.[31–33] We, therefore, compared the fatty acid content of the membranes to the diet given to our experimental animals (Supplementary file 1). We could not find any obvious relationship with our membrane fatty acid analysis, with one exception. The fatty acid 22:1n-9 was high in the BLM fraction as well as in the cod diet. For more visual clarity, we have plotted in Figure 4A through C the proportional amount of each fatty acid class from Table 2 for each of the four lipid species, that is, saturated fatty acids (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA).

Saturated fatty aids (SFA) were at a significantly higher level in the inner BLM fraction compared with the corresponding BBM part in all the lipids except the mono-sialylated gangliosides (MG). This was especially noticeable in the PL group. Analysis of the unsaturated fatty acids showed that the main difference was in the MUFA rather than in the PUFA lipids (Figs. 4B and C). We expected the frequently reported high content of PUFA in coldwater fish like cod (see below) to manifest itself, but PUFA proved here to be at similar ratios and in low abundance for all lipid classes with the exception of the NSL fraction, where the level was higher in BBM than in BLM. This was despite the high amounts of eicosahexaenoic acid (EPA, 20:5n-3) (7.6%) and docosahexaenoic acid (DHA, 22:6n-3) (8.7%) in the fatty acids of the diet. Previous studies have shown that the PUFA content might be expected to rise when acclimating animals to lower temperatures.[3] In studies on fish,[24,34,35] this effect was mainly seen in the content of EPA and DHA. The fatty acid content of phospholipids from seven different Atlantic cod tissues

was reported in an earlier study. Compared with our results, higher proportions of PhtEtn and PhtCho and a lower proportion of CerPCho were observed in that study together with a higher content of PUFA.[34] It is interesting to notice that the NSL glycolipid fraction, which is the main lipid species in both membrane parts in a previous study, did not really show the expected link of PUFA with thermal adaptation, as predicted by the HVA theory, in contrast to other lipid species.[4] This might indicate a minor contribution of this lipid species to cold adaptation. Similar to the present study, the content of MUFA in both the BBM and BLM samples was clearly higher in all the other lipid species, thus providing evidence that, for the cod intestinal enterocytes, adapting to the cold is more highly dependent on the MUFA content rather than PUFA. Despite the fact that the theory of HVA predicts higher amounts of PUFA, it is well known that the contributions of the first double bond introduced into a FA towards lowering T_m of the membrane is relatively higher than for each additional double bond. In the BLM fraction, the main overall MUFA contributors were 18:1n-9 and 22:1n-9, with 24:1n-9 in the phospholipids, whereas in the BBM part, a very high amount of 24:1n-9 was seen in the group of phospholipids and both ganglioside groups (Table 2). Another suggestion that a MUFA may be involved in temperature regulation comes from studies on goldfish intestinal mucosa, where 20:1 was twice as abundant in the intestinal mucosa when adaptation temperature was lowered by 20 °C.[36] A rise in 20:4 and 22:5 was also observed in the cold-adapted fish and compensated for by a fall in the amounts of the fatty acids 18:0 and 20:3. The changes in the longer-chain FA were confined to the phospholipids whereas those in C18:0 took place in the neutral lipid fraction.

Despite a thorough search of the literature, we were not able to find the fatty acid 24:1n-9 (nervonic acid) in the high amounts observed in cod BBM membranes from any other vertebrate. In catfish intestines, 24:1n-9 was detected in fish fed 18:0 at 22 °C to the level of 1.5% but not in fish fed a diet supplemented with fish oils.[37] Thus, diet may affect the amount of 24:1 in some cases. Nervonic acid (*cis*-tetracosa-15-enoic acid; 24:1n-9) is classified as a very-long chain fatty acid and exists in nature as an elongation product of oleic acid (18:1n-9). It is rare in most tissues, but found enriched in nervous tissue.[38] Nervonic acid is particularly abundant in sphingolipids, such as sphingomyelin in the myelin sheath of nerve fibres, but also in the white matter of animal brains and fish tissues.[39] The nervonic acid moiety in human lipids is derived from fish oils.[40] More specifically for fish, nervonic acid has been found in the heart tissue from reared stocks of Atlantic cod[41] and in the scales of Atlantic salmon.[42] Dietary supplementation with nervonic acid might be beneficial for neurological development and function.[38] But generally, in vertebrates, nervonic acid is found in very low amounts at 0.5% to 1.2%.[37,43] The low amounts detected in many previous studies could be related to the fact that most reports terminated their GC-analysis at 22:6n-3 (DHA). Also, due caution must be observed in the analysis of long acyl chain FA such as 24:1n-9 and 22:6n-3 that can have the same retention time on certain GC columns.[43] DHA has been reported to be one of the major fatty acids in fish.[34,44] Therefore, as this high proportion of 24:1n-9 was initially surprising, we performed GC/MS analysis of purified DHA to determine the retention time for that fatty acid on our column (data not shown). This confirmed our results that the peaks were not mixed and both peaks gave the correct mass spectrometry pattern for each FA. Atlantic cod contains elongases that are relatively nonspecific for chain lengths and work both on PUFA and monounsaturated fatty acids.[45] Furthermore, Atlantic cod is not found to have desaturases with the same efficiency as salmonids and some other fish species.[46,47] In fact, the expression of the Δ6 desaturase gene did not appear to be under significant nutritional regulation, with levels in liver and intestine being barely altered in fish fed a vegetable oil blend by comparison with levels in fish fed fish oil.[47] Therefore, we expect that the 24:1n-9 may be the result of elongation of the 22:1n-9 present in the diet, but 20:1 and 18:1 were also present in relatively large amounts and may also serve as precursors. Elongation leading to the production of 24:5n-3 has been verified as attainable by elongases from various fish species transformed into yeast. These enzymes have been shown in other animals to work with n-9 substrates but less efficiently than n-3 or n-6. Elongase from cod had higher activity on n-6 substrates than n-3.[45]

Sphingolipids containing very-long acyl chains are a minor component of plasma membranes in most mammalian cells as noted above. The effect that very long chain sphingolipids have on phase transition in model membranes has been studied using differential

scanning calorimetry. These include studies on the 24:0 and 24:1n-9 fatty acids N-linked to CerPCho.[48–50] From these studies it is evident that introducing 24:1n-9-CerPCho instead of 24:0-CerPCho and introducing CerGal instead of the choline head-group dramatically decreased the membrane transition temperature. This was most likely due to the kink in the extended 24:1 acyl chain preventing it from packing tightly into organized membranes, whereas the 24:0-sphingolipids formed highly packed and ordered domains as shown by fluorescence quenching measurements.[48] The present study indicates that both the longer chain MUFA (24:1n-9) and the exchange of the diacylglycerol with the sphingoid-base attached to the choline head-group might well contribute to the thermal acclimation in the cod. Therefore, we propose that this may be a newly discovered method to maintain the membrane fluidity in the cold through the unfavorable packing order of the long chain MUFA.

Calculated indices are often used to evaluate the ability of the membrane to adapt to different temperatures. In accordance with HVA theory for cold adaptation,[3,4] a higher unsaturation index and higher PUFA over SFA ratio would be prime indications for lowering of the viscosity of the membrane, causing it not to pack as tightly. On the other hand, it is generally assumed that a higher average chain length would point in the direction of warm acclimation due to the ability of longer acyl chains to pack more tightly and reduce the viscosity of the membranes.[3,35] Unlike the BLM, the BBM side of the cell can be expected to be more atypical in comparison with non-epithelial cells. It has been documented earlier that BBM shows the inverse compensation behavior compared with BLM when either rainbow trout (*Oncorhynchus mykiss*) or common carp (*Cyprinus carpio*) were acclimated to the cold, that is, the BBM was shown to become more viscous[4,6] when adapting to cold. A number of observations have been made to suggest that fluctuations in the microenvironments of the two membrane sides of the endothelial cells could lead to differences in the physiological properties such as the detergent efficiencies of bile salts on the BBM. In contrast with BLM, the unsaturation indices of different lipid species in the BBM were much higher than in BLM. This correlates fully with the HVA theory of cold adaptation.

Relatively little is known about the effects of temperature on membrane lipids in fish. Studies on carp[4] and rainbow trout[6] intestines showed an increased proportion of cholesterol in cold environments, but neither the PhtEtn/PhtCho nor the overall amount of PUFA rose as would be expected according to the homeoviscous theory. Our results agree with this. It should be noted that in carp grown at 10 °C compared with 30 °C MUFA were reduced at 10 °C by half in PhtCho whereas the reverse was observed for MUFA in PhtEtn.[4] PUFA followed the opposite trend to MUFA. Thus, different types of lipid changed differently on exposure to the low temperature. The fatty acid composition of scraped intestinal mucosa was studied in two Antarctic fish kept without feeding for two weeks and compared with warm-blooded fish.[24] A higher amount of PhtEtn and a lower amount of CerPCho was found, which is in disagreement with the present results. However, the percentage of unsaturated FA was clearly higher in the Antarctic fish, particularly with regard to MUFA. This is in line with our observations with PUFA levels being no different from common values, but MUFA content being increased. A high content of PUFA has been reported in some coldwater fish, but that was not evident in our study. PUFA content in flesh from coldwater fish compared with fish from temperate regions was high, but it was not related to the geographic location of the species examined.[51] In a mixed brain-cell population from carp (*Cyprinus carpio*) that was acclimated to either summer (23 °C–25 °C) or winter (5 °C) temperature, fluidity, as measured by electron spin resonance, showed a high degree of compensation (80%) for temperature, whereas phospholipids separated from both types of animals exhibited only around 10% compensation. The fatty acid composition of the brain total phospholipids did not vary with adaptation to temperature, and it was concluded that the total amount of DHA (22:6), a prominent PUFA in membranes, and the phospholipids played only a minor role in adjusting the membrane physical properties to temperature.[52] We can agree with that result from our present results. In synaptosomes from goldfish acclimatized to 5 °C or 25 °C there was a trend toward an increase in unsaturation at low temperatures, particularly in PhtCho.[35] Another study on goldfish intestines found that 20:1 was twice as abundant in the intestinal mucosa when adaptation temperature was lowered by 20 °C. Rises in 20:4 and 22:5 were also observed in the cold-adapted fish, and a fall in the amount of 18:0

and 20:3 occurred.[36] In the present study, the amount of PUFA in the intestinal border was not particularly greater than in warm blooded animals like the rat[23,25] and similar to other cold-adapted teleost.[24] Thus, adaptation strategies may also be tissue specific.

The lipid composition reported here appeared not to be a reflection of the diet lipids. The influence of diet on heart fatty acid of two stocks of reared Atlantic cod was studied previously under identical conditions for over 3 years. It was demonstrated that neither environmental factors nor diet caused the observed difference in fatty acid composition.[41] This suggested that the observed difference between stocks of cod was purely genetic.

Conclusion

Our results show clearly that both the outside BBM and the internal membrane fractions of the intestinal enterocytes from Atlantic cod have different content of the main lipid classes and distinct patterns of lipid species and fatty acids. We propose that this selection helps in maintaining their physiological function in cold environments. However, the detailed mechanism is still unknown. Therefore, it would be of great interest to conduct further research on such lipid profiles from other species of fish grown at different temperatures especially in cells isolated from intestinal enterocytes. A further extension would be to chart the lipid composition of the lipid rafts to compare with the main bilayer and to determine the proteome in both niches. Fish make up the largest group of all vertebrates, and many are exploited for commercial purposes, including the Atlantic cod. Interest in fish farming, where feeding and the well-being of the animals depends largely on the health of the digestive tract, will clearly benefit from further studies on the polar cells that are at work in the intestines.

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Competing Interests

Author(s) disclose no potential conflicts of interest.

Author Contributions

Conceived and designed the experiments: GAG, EK, and BA. Analyzed the data: GAG. Wrote the first draft of the manuscript: GAG. Contributed to the writing of the manuscript: BA and EK. Agree with the manuscript's results and conclusions: GAG, BA, and EK. Jointly developed the structure and arguments for the paper: GAG and BA. Made critical revisions and approved final version: BA. All authors reviewed and approved of the final manuscript.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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