

# Thermally induced changes in lipid composition of raft and non-raft regions of hepatocyte plasma membranes of rainbow trout

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## Summary

In poikilotherms, increases in plasma membrane (PM) cholesterol and an increase in the degree of lipid acyl chain saturation commonly accompany an increase in growth temperature. This has typically been interpreted in terms of membrane fluidity/order homeostasis, but these changes would also be expected to stabilize the structure of PM rafts against thermal perturbation. Rafts are microdomains that organize the molecules of many signaling cascades and are formed as a result of interactions between lipids with saturated acyl chains and cholesterol. No study to date has examined the thermally induced compositional changes of raft and non-raft regions of the PM separately. In this study we have measured the phospholipid class composition and fatty acid composition of raft-enriched (raft) and raft-depleted PM (RDPM) of hepatocytes from trout *Oncorhynchus mykiss* acclimated to 5°C and 20°C. In the raft, warm acclimation was associated with a reduction in the

proportion of phosphatidylcholine from 56% to 30% while phosphatidylserine and phosphatidylinositol each increased from 8% to approximately 20% of the total phospholipid. Additionally, there were significantly fewer unsaturated fatty acids in the raft lipids from warm-acclimated (61%) than from the cold-acclimated trout (68%). In contrast, there were no significant changes in phospholipid class or acyl chain unsaturation in the RDPM. These data suggest that changes in raft lipid composition, rather than the PM as a whole, are particularly important during thermal acclimation.

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Key words: raft, trout, *Oncorhynchus mykiss*, lipid composition, cholesterol, fatty acid, poikilothermy.

## Introduction

Poikilothermy requires biochemical flexibility, involving adjustments in many aspects of cellular physiology with changes in growth temperature. Biological membranes are acutely temperature sensitive and thermally induced membrane lipid compositional changes are well documented (Hazel and Williams, 1990). In particular, thermal acclimation has been well studied in the plasma membrane (PM), but compositional analyses have been performed only on whole membranes or separated macrodomains (e.g. apical and basolateral macrodomains of enterocytes; Lee and Cossins, 1990). However, it is probable that the plasma membranes of most cell types contain lipid raft microdomains. Raft microdomains have a distinct composition and act to spatially and functionally segregate the PM, an organization that may be important for signaling and endocytosis (Edidin, 2003).

During acclimation to elevated temperatures, highly conserved lipid compositional changes in the PM include a decrease in the number of double bonds in membrane lipid fatty acids (Hazel and Williams, 1990; Cossins, 1994) and an increase in the mole fraction of PM cholesterol (Robertson and Hazel, 1995). These changes have commonly been interpreted as mechanisms for compensation of membrane fluidity/order,

a physical property that is thought to impact the function of proteins embedded in the membrane (Cossins, 1994).

However, these compositional changes are also consistent with the thermal stabilization of the raft/non-raft phase separation that is thought to give rise to raft structure. In mammals, rafts are enriched in cholesterol, lipids with saturated acyl chains (sphingomyelin in particular), and glycolipids (Fridriksson et al., 1999; Prinetti et al., 2000; Pike et al., 2002). Interactions between cholesterol and lipids with saturated acyl chains are thought to result in the lateral segregation of liquid-ordered ( $L_o$ ) phase raft and liquid-disordered ( $L_d$ ) phase non-raft PM (Ahmed et al., 1997). Sphingomyelin has a particularly strong interaction with cholesterol (Lund-Katz et al., 1988; Ramstedt and Slotte, 1999).

Since lipid phase behavior is temperature sensitive, it is reasonable to hypothesize that compositional changes in the PM act to stabilize rafts in the face of thermal perturbation. The increase in fatty acid saturation and cholesterol content observed in the total PM of animals acclimated to elevated temperature are consistent with the stabilization of rafts against thermal perturbation. Furthermore, we have found that

detergent saturation point, an index of membrane packing strength (and thus raft stability), was conserved in raft but not in raft-depleted plasma membrane (RDPM) of trout liver PM (Zehmer and Hazel, 2003). Curiously, we found raft from warm-acclimated trout to be only slightly more ordered than RDPM but there was no difference in order between raft and RDPM from cold-acclimated trout (Zehmer and Hazel, 2004). Since, in mammals, raft-RPDM segregation is thought to be based on a separation of ordered and disordered phases, it is not clear what maintains the separation in fish membranes.

Therefore, to better understand the regulation of the physical properties of raft and RPDM and the mechanism of the segregation of these domains in poikilotherms acclimated to different temperatures, we have conducted compositional analyses of raft and RDPM from rainbow trout *Oncorhynchus mykiss* Walbaum acclimated to 5°C and 20°C. We measured the composition of the major phospholipid classes and found differences in the rafts from the warm acclimated group compared with all other membrane fractions studied. Furthermore, we measured the fatty acid composition of the total lipid extracts as well as phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) resolved from the membrane fractions. Acclimatory changes in unsaturation were larger in raft than RDPM and the patterns of change differed between PtdCho and PtdEtn.

## Materials and methods

### Materials

Authentic lipid standards were obtained from Avanti (Alabaster, AL, USA). Fatty acid methyl ester standards were from Nu Chek Prep (Elysian, MN, USA). Methanolic HCl was from Supelco (Bellefonte, PA, USA). Methanol, ethanol and chloroform were of analytical grade, obtained from Fisher (Fairlawn, NJ, USA). Biochemicals, including OptiPrep density gradient medium, were from Sigma (St Louis, MO, USA) and all other chemicals were of analytical grade. Methanol and ethanol were distilled in the presence of KOH and were used within 1 month. Chloroform was washed with distilled water to remove water-soluble contaminants, dehydrated with anhydrous sodium sulfate, distilled and stabilized with 1% (v/v) methanol and then used within 1 month. Aluminum-backed thin layer chromatography plates (silica gel 60; 20 cm×20 cm with 2.5 cm concentrating zone) were from EM Science (Gibbstown, NJ, USA). Syringe filters (25 mm Acrodisc® filters with 0.45 µm PTFE membranes) were from the Pall Corporation (Ann Arbor, MI, USA).

### Animals

Rainbow trout *Oncorhynchus mykiss* Walbaum were obtained from the Alchey National Fish Hatchery in Whiteriver, Arizona, and were maintained at the Animal Resource Center of Arizona State University. Fish were housed in recirculating freshwater aquaculture systems consisting of circular fiberglass tanks; water temperatures were controlled using flow-through chillers. Animals were acclimated to 5°C

or 20°C for at least 3 weeks before use in experiments. Fish were held under a constant 12 h:12 h L:D cycle and were fed daily with Rangen Inc. (Buhl, ID, USA) trout food to satiation.

### Plasma and raft membrane isolations

PMs were isolated from approximately 8 g of liver (pooled from several fish) according to a modification of the procedure of Armstrong and Newman (1985) as described previously (Hazel et al., 1992). The PM was resuspended in working buffer (WB: 0.25 mol l<sup>-1</sup> sucrose, 20 mmol l<sup>-1</sup> tricine, pH 7.8, 1 mmol l<sup>-1</sup> EDTA disodium salt) and was separated into raft-depleted PM (RDPM) and raft-enriched PM (raft) using a non-detergent based method (Smart et al., 1995). The lower buoyant density of raft, compared to RDPM, was used as a means of separating the membrane fractions. Briefly, the PM was sonicated and then brought to 23% (v/v) OptiPrep before layering a 10–20% (v/v) linear OptiPrep gradient on top (for a total of 11 ml in each tube). After centrifuging for 90 min at 72 800 g in a Beckman SW 41-Ti rotor (OP1) the top 5.5 ml (raft) were removed, mixed with 4 ml of 50% (v/v) OptiPrep in a fresh tube, overlaid with 250 µl 5% (v/v) OptiPrep, and centrifuged for 90 min at 72 800 g (OP2). The raft membrane concentrated at the top of the OP2 tube was collected with a Pasteur pipette, diluted with three volumes of buffered saline and was centrifuged to a pellet at 20 800 g for 20 min in a refrigerated microcentrifuge (Eppendorf 5417 R). The bottom 5.5 ml from OP1 was diluted with four volumes of buffered saline and was centrifuged for 1 h at 23 700 g in a Beckman JA 30.50 rotor to form the RDPM pellet.

### Lipid extractions

Lipids were extracted from membrane samples using the method of Bligh and Dyer (1959). Briefly, 2.4 ml of aqueous membrane suspension was mixed with 9 ml of chloroform-methanol (1:2) and incubated for 10 min. The addition of 3 ml of chloroform and 3 ml of 0.88% aqueous KCl resulted in the formation of two phases. The lower hydrophobic phase (containing the lipids) was recovered and transferred to a 50 ml boiling flask. The solvent was driven off using a rotary evaporator. The sample was dehydrated by adding 5 ml of absolute ethanol followed by rotary evaporation. This procedure was repeated twice. The dried lipids were then transferred to an autosampler vial using a small volume of chloroform-methanol (2:1). Butylated hydroxytoluene (BHT) was added to a final concentration of 50 mg l<sup>-1</sup> to inhibit oxidation and the lipids were stored at -20°C.

### Protein and lipid analyses

Total protein was determined colorimetrically by the method of Bradford (Bradford, 1976). Phospholipid concentration was measured by colorimetric phosphate analysis after complete hydrolysis of total lipid extracts or isolated PtdCho and PtdEtn (Ames, 1966). A coupled cholesterol oxidase fluorometric assay was used to measure cholesterol (Crockett and Hazel, 1995).

### Thin layer chromatography

Lipids were separated into major phospholipid classes using thin layer chromatography (TLC). Lipids in chloroform–methanol (2:1) were spotted onto the preabsorption zone of TLC plates (approximately 1.5 mg lipid per plate). The solvent was driven off under nitrogen. The developing solvent was prepared immediately prior to use: chloroform–methanol–acetic acid–water (50:37.5:3:1.7; Holub and Skeaff, 1987). The developing solvent was poured into the TLC tank, fully wetting the filter paper lining the sides of the tank. The dried TLC plates were immediately placed in the tank, the top sealed, and developed for 1 h and 15 min.

After removal of the plates, residual solvent was dried under a stream of nitrogen. Each TLC plate was positioned between two plates of glass, leaving one edge of the TLC plate exposed. The exposed lipids were stained in an iodine chamber and identified against authentic standards. The separated lipid classes were recovered by scraping the silica gel (from the region of the plate not exposed to iodine) off the aluminum backing into test tubes. The lipid classes were extracted from the silica gel by adding 7 ml of chloroform–methanol (2:1), centrifuging briefly in a clinical centrifuge, and recovering the supernatant into a conical boiling flask. This procedure was repeated twice for a total volume of 21 ml solvent per sample, which was filtered through a 0.45  $\mu\text{m}$  PTFE (polytetrafluoroethylene, commonly known as Teflon<sup>®</sup>) syringe filter. The solvent was driven off using a rotary evaporator and then a small volume of chloroform–methanol (2:1) was used to transfer the lipids to autosampler vials. BHT (50 mg l<sup>-1</sup>) was added and the vials were stored at -20°C.

### Preparation of fatty acid methyl esters

Fatty acid methyl esters (FAMES) were prepared from total lipid extracts and TLC-separated lipid classes by the acid catalyzed method described by Christie (2003). Lipids were transferred from their storage vials to 15 ml glass tubes, the solvent was driven off under nitrogen, and 2 ml of 3 M methanolic HCl added. The tubes were placed in a heat block at 65°C, capped with a marble, and allowed to reflux for 2 h. The tubes were removed and 5 ml of 5% (w/v) NaCl was added. The FAMES were extracted with 3.3 ml hexane three times, the hexane was pooled in a fresh tube and then washed with 4 ml of 2% (w/v) KHCO<sub>3</sub>. The hexane was then recovered to a fresh tube and dried with anhydrous sodium sulfate. The hexane was again recovered to a fresh tube, leaving the sodium sulfate behind, and the majority of the solvent driven off under nitrogen. The remaining solvent was transferred to an autosampler vial, 50 mg l<sup>-1</sup> BHT was added, and the vials were stored at -20°C.

### Gas chromatography – Mass spectrometry

FAMES were separated using a Varian (Palo Alto, CA, USA) Star 3400cx gas chromatograph fitted with a Supelco SP 2380 column (30 m × 0.25 mm i.d. × 0.2  $\mu\text{m}$  film) with a helium carrier linear flow rate of 30 cm s<sup>-1</sup>. The injector and detector manifold were at 250°C. The column oven was maintained at 100°C for 2 min postinjection. The oven

temperature was then increased at a rate of 4°C min<sup>-1</sup> for 30 min to a temperature of 220°C. At 30 min the oven temperature was increased at 50°C min<sup>-1</sup> to 250°C and held at this temperature for 5 min to clear the column. The separated FAMES were detected using a Varian Saturn 4D mass spectrometer with a scan rate of 1000 ms with a mass range between 50 and 400 m z<sup>-1</sup>. FAMES were identified by retention time and fragmentation pattern compared against authentic standards and a fragmentation pattern library. Quantification was by peak integration and expressed for individual FAMES as percentages of the total in each run.

### Statistics

The percentages of each phospholipid class (e.g. phosphatidylcholine) from raft and RDPM from both acclimation groups were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. For the fatty acids, the unsaturation index was calculated as the number of double bonds per 100 fatty acids (the percentage abundance of each fatty acid × number of double bonds, summed for all fatty acids). Percentage unsaturation and unsaturation index were analyzed separately using one-way ANOVAs and Tukey's test. In all cases a *P* value of 0.05 or lower was accepted as indicating statistical significance.

## Results

### Acclimation effects on phospholipid classes

We used thin layer chromatography (TLC) and phosphate assays to measure the phospholipid class composition of lipids extracted from raft-enriched plasma membrane (raft) and raft-depleted plasma membrane (RDPM) from trout acclimated to 5°C and 20°C. Exposing the TLC plates to iodine vapor revealed five major bands which were identified as phosphatidylethanolamine (PtdEtn), phosphatidylinositol (PtdIns), phosphatidylserine (PtdSer), phosphatidylcholine (PtdCho) and sphingomyelin (SM; Fig. 1).

Temperature acclimation had no effect on the phospholipid class composition of the RDPM (Fig. 1A). The major phospholipids of the RDPM were PtdCho and PtdEtn, which made up approximately 48% and 31% of the detected lipids, respectively; of the minor phospholipids, PtdIns made up approximately 10%, whereas PtdSer and SM constituted approximately 5% each. By contrast, in the raft, acclimation to increased temperature resulted in a significant decrease in PtdCho (*P*=0.001, from 56% to 30%) with concomitant increases in both PtdIns (*P*=0.040, from 8% to 20%) and PtdSer (*P*=0.002, from 8% to 19%; Fig. 1B). Although PtdCho was clearly the major phospholipid of raft from cold-acclimated fish, followed by PtdEtn and the minor phospholipids PtdSer, PtdIns and SM, in raft from warm-acclimated fish all phospholipids, with the exception of SM, were present in roughly equivalent proportions. Notably, SM did not increase in abundance in the raft fraction in response to warm acclimation

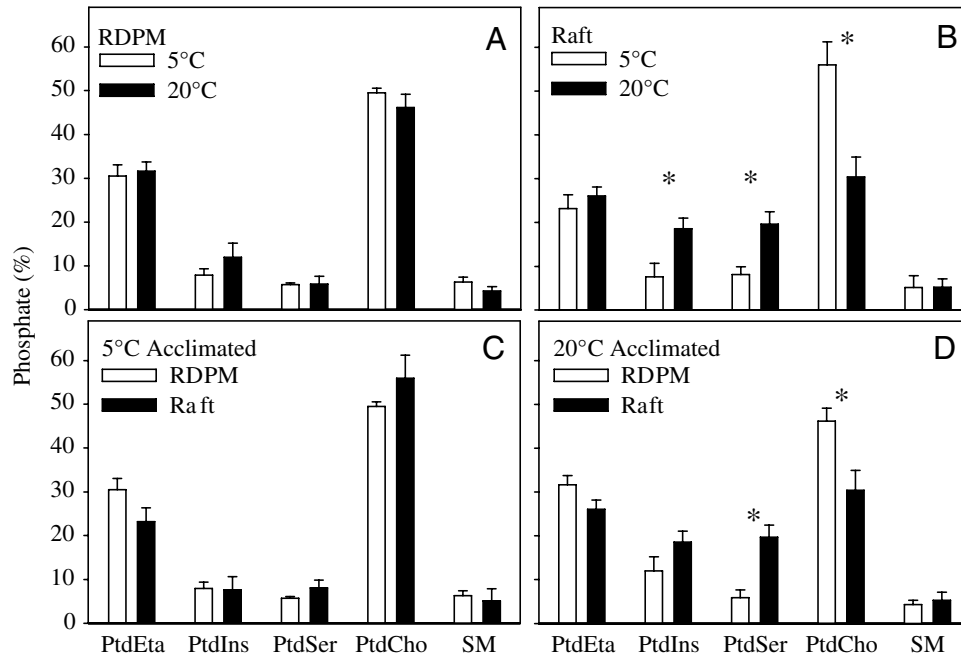


Fig. 1. Raft-enriched plasma membrane (raft) and raft-depleted plasma membrane (RDPM) phospholipid class composition. Total lipids extracted from isolated membranes were separated by TLC and the major phospholipid classes were quantified by phosphate assay. (A) RDPM from 5°C-acclimated (open bars) and 20°C-acclimated (filled bars) trout. (B) Raft from 5°C-acclimated (open bars) and 20°C-acclimated (filled bars) trout. (C) RDPM (open bars) and raft (filled bars) from 5°C-acclimated trout. (D) RDPM (open bars) and raft (filled bars) from 20°C-acclimated trout. PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; SM, sphingomyelin. Values are means + S.E.M. ( $N=4$ ).

#### Comparison of phospholipid classes in raft-enriched plasma membrane and raft-depleted plasma membrane

Raft and RDPM from cold-acclimated trout did not differ in phospholipid class composition (Fig. 1C). In both subdomains, PtdCho and PtdEtn were the major components while PtdSer, PtdIns and SM each made up less than 10% of the total phospholipid. In contrast, raft and RDPM did differ significantly in warm-acclimated fish: the proportion of PtdSer was significantly higher in rafts (20%) than RDPM (6%;  $P<0.001$ ) whereas the proportion of PtdCho was lower in raft than in RDPM (30% vs 46%;  $P=0.028$ ; Fig. 1D).

#### Fatty acid analyses

We next examined the fatty acid content of lipids extracted from raft and RDPM from cold- and warm-acclimated fish. Fatty acid methyl esters (FAMES) were prepared from total lipid extracts and isolated PtdCho and PtdEtn, and were analyzed with a gas chromatograph fitted with a mass spectrometer. Seventeen major FAMES were resolved. The major species resolved from the total lipid extracts were, in order of abundance, 22:6n3 (26.8–29.2%), 16:0 (17.7–23.9%), 18:1n9 (13.1–15.3%), 18:0 (9.6–10.3%) and 18:2n6 (5.9–8.3%) which, collectively, made up approximately 80% of the total FAMES (see complete data in Tables S1–S3 in supplementary material). We were primarily interested in patterns of unsaturation since double bonds influence membrane order and the interaction of phospholipids with cholesterol. We calculated two indexes of fatty acid

unsaturation. The unsaturation index (UI) is the number of double bonds per 100 molecules of fatty acid. With this measure, either a small number of highly unsaturated fatty acids or a large number of monounsaturates can produce the same UI number. Therefore, we also calculated the percentage of unsaturated fatty acids, i.e. the number of fatty acids with one or more double bonds in a pool of 100. The data are presented on two dimensional graphs with percentage unsaturation on the abscissa and the UI on the ordinate. Additionally, because of their differing effects on membrane physical properties, we analyzed the monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) separately, plotting the percentage of fatty acids in each group in bar graphs.

#### The total fatty acid composition of raft and RDPM domains

We first examined the fatty acid composition of total lipid extracts of raft and RDPM (Fig. 2). For reference, in this and subsequent similar figures, isopleths are plotted (dotted lines) representing the region on the graph where an increase in percentage unsaturation is achieved by adding fatty acids with an average specified number of double bonds (Logue et al., 2000). For example, if all unsaturated fatty acids had three double bonds, the lower isopleth in Fig. 2A would mark the UI for any given level of percentage unsaturation. Increases in unsaturation that parallel an isopleth represent an increase in the proportion of unsaturated fatty acids having the same average number of double bonds, while those that cross



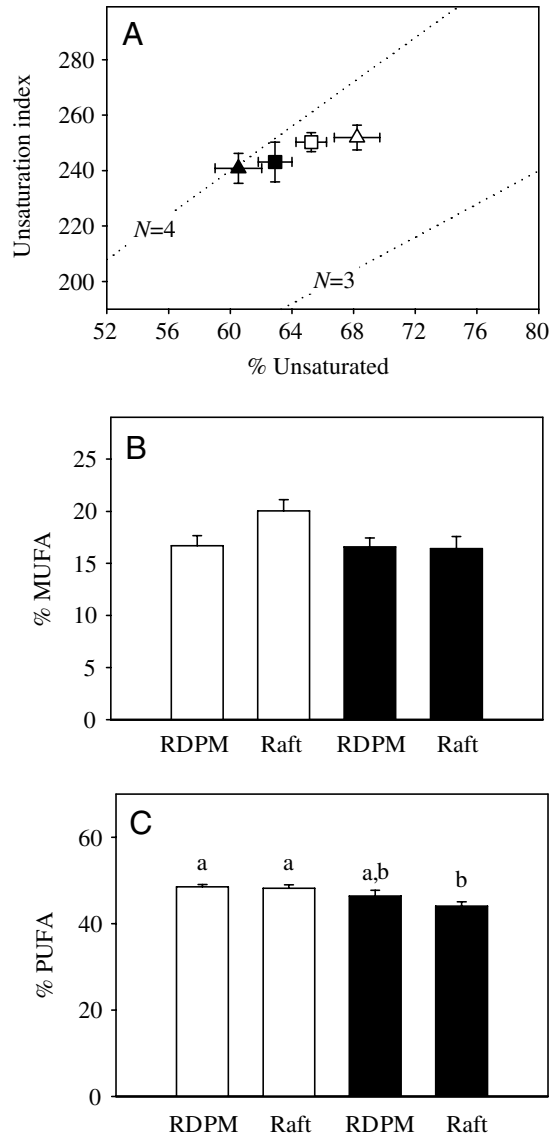


Fig. 2. Indexes of total fatty acid unsaturation of membrane fractions. (A) Percentage of unsaturated fatty acids and unsaturation index of raft (triangles) and RDPM (squares) from 5°C-acclimated (open symbols) and 20°C-acclimated (filled symbols) fish. The dotted lines are isopleths representing the relationship between percentage unsaturation and unsaturation index if each unsaturated fatty acid had three ( $N=3$ ) or four ( $N=4$ ) double bonds. (B) Percentage of monounsaturated fatty acids (MUFA) and (C) percentage of polyunsaturated fatty acids (PUFA) in raft and RDPM from 5°C-acclimated (open bars) and 20°C-acclimated (filled bars) fish. Bars with a common lower case letter do not differ significantly. All values are means  $\pm$  S.E.M.,  $N=4$ . Raft, raft-enriched plasma membrane; RDPM, raft-depleted plasma membrane.

isopleths represent a change in the average number of double bonds per fatty acid.

Comparing total fatty acid compositions, there were no significant differences in UI among the samples (ANOVA  $P=0.411$ ). However, there were differences in the percentage of unsaturated fatty acids (ANOVA  $P=0.008$ ) with increased

unsaturation in the samples from the cold-acclimated fish (Fig. 2A). This trend was small and not significant between the RDPMs ( $P=0.617$ ), but the rafts did differ significantly ( $P=0.006$ ), reflecting a greater acclimatory response. Raft and RDPM did not differ in percentage unsaturation in either cold- ( $P=0.399$ ) or warm- ( $P=0.562$ ) acclimated fish.

There were no significant differences in percentage MUFA among the samples (ANOVA  $P=0.082$ ) (Fig. 2B). PUFA levels were slightly, but significantly lower (ANOVA  $P=0.024$ ) in raft from warm-acclimated trout (44.1%) than raft (48.2%;  $P=0.045$ ) or RDPM (48.6%;  $P=0.029$ ) from cold-acclimated trout (Fig. 2C).

#### Phosphatidylcholine

We next examined changes in fatty acid composition of PtdCho during thermal acclimation. There was a significant ANOVA ( $P=0.050$ ) for percentage unsaturated fatty acids, but there were no significant pairwise comparisons among the samples (Fig. 3A). The ANOVA of the UI data was significant ( $P=0.017$ ) and the general trend of the data reflected decreases in UI (across the isopleths;  $N=4$ ) with warm acclimation rather than changes in percentage unsaturation. This pattern indicates that in PtdCho, acclimatory changes in unsaturation occur primarily in the form of an increased number of double bonds per unsaturated fatty acid, without change in the proportion of unsaturated fatty acids. However, multiple comparisons demonstrated no significant differences between the rafts ( $P=0.083$ ) or the RDPMs ( $P=0.141$ ) and raft and RDPM had nearly identical UI values within each acclimation group.

Examination of the patterns in the MUFA (Fig. 3B) and PUFA (Fig. 3C) data also shows a reduction in the number of double bonds per fatty acid with warm acclimation. With cold-acclimation there were significant decreases in MUFA (ANOVA  $P<0.001$ ) in both raft ( $P=0.010$ ) and RDPM ( $P=0.017$ ). The ANOVA for PUFA was nearly significant ( $P=0.051$ ), although the only significant pair-wise comparison was between raft from cold-acclimated and RDPM from warm-acclimated trout ( $P=0.036$ ). Thus in PtdCho, warm acclimation increases the content of MUFA at the expense of PUFA (i.e. fewer double bonds per unsaturated fatty acid). There were no significant differences in MUFA or PUFA between raft and RDPM within an acclimation group. However, in the warm-acclimated group there was a near-significant increase ( $P=0.052$ ) in MUFA in raft, compared to RDPM.

#### Phosphatidylethanolamine

In PtdEtn, the trend with cold acclimation was for both UI and percentage unsaturation to increase in parallel with the isopleths (Fig. 4A), corresponding to an increase in the proportion of unsaturated fatty acids, without a change in the average number of double bonds. Although percentage unsaturation tended to increase with cold acclimation in both raft and RDPM (ANOVA  $P=0.014$ ), this difference was only significant in rafts ( $P=0.036$ ), not RDPMs ( $P=0.161$ ).

Similarly, increases in UI with cold acclimation (ANOVA  $P=0.037$ ) were nearly significant in raft ( $P=0.061$ ), but not RDPM ( $P=0.274$ ). There were no significant differences in the content of MUFA (ANOVA  $P=0.896$ ; Fig. 4B). The ANOVA for the PUFA was significant ( $P=0.039$ ), although there were no significant pair-wise comparisons (Fig. 4C). However, the most probable cause of the significant ANOVA was the 54.8-44.1% and 50.9-44.4% decreases in PUFA with warm

acclimation in raft and RDPM, respectively. This is consistent with the modulation of the proportion of fatty acids carrying between three and four double bonds, without altering the average number of double bonds on the unsaturated fatty acids (Fig. 4A).

## Discussion

The most consistent pattern demonstrated in these and our previously published data is that acclimatory changes were

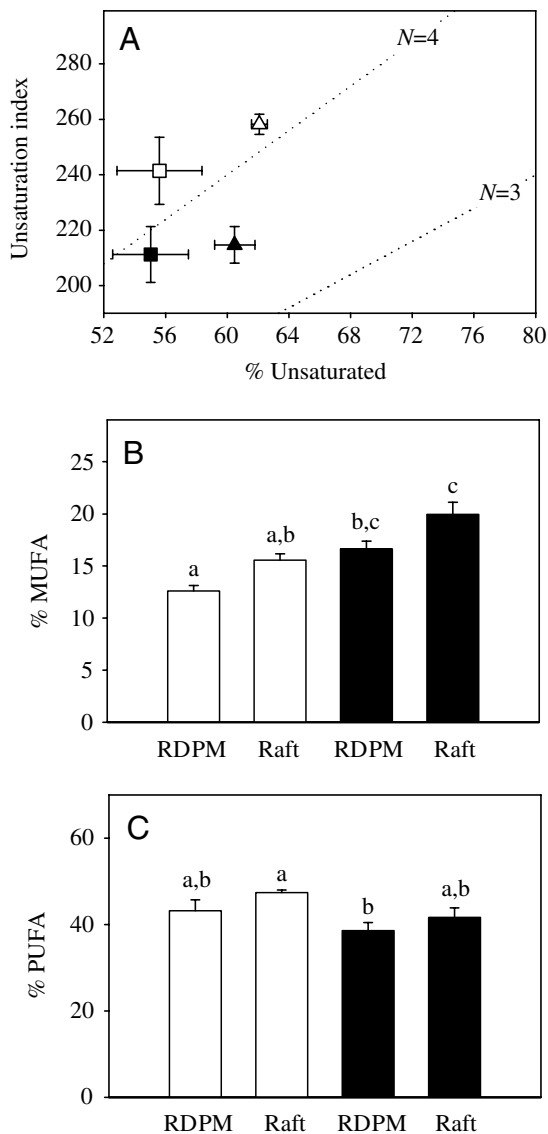


Fig. 3. Indexes of fatty acid unsaturation of phosphatidylcholine in membrane fractions. (A) Percentage of unsaturated fatty acids and unsaturation index of phosphatidylcholine of raft (triangles) and RDPM (squares) from 5°C-acclimated (open symbols) and 20°C-acclimated (filled symbols) fish. The dotted lines are isopleths, as described in Fig. 2. (B) Percentage of monounsaturated fatty acids (MUFA) and (C) percentage of polyunsaturated fatty acids (PUFA) in raft and RDPM from 5°C-acclimated (open bars) and 20°C-acclimated (filled bars) fish. Bars with a common lower case letter do not differ significantly. All values are means  $\pm$  S.E.M.,  $N=4$ . Raft, raft-enriched plasma membrane; RDPM, raft-depleted plasma membrane.

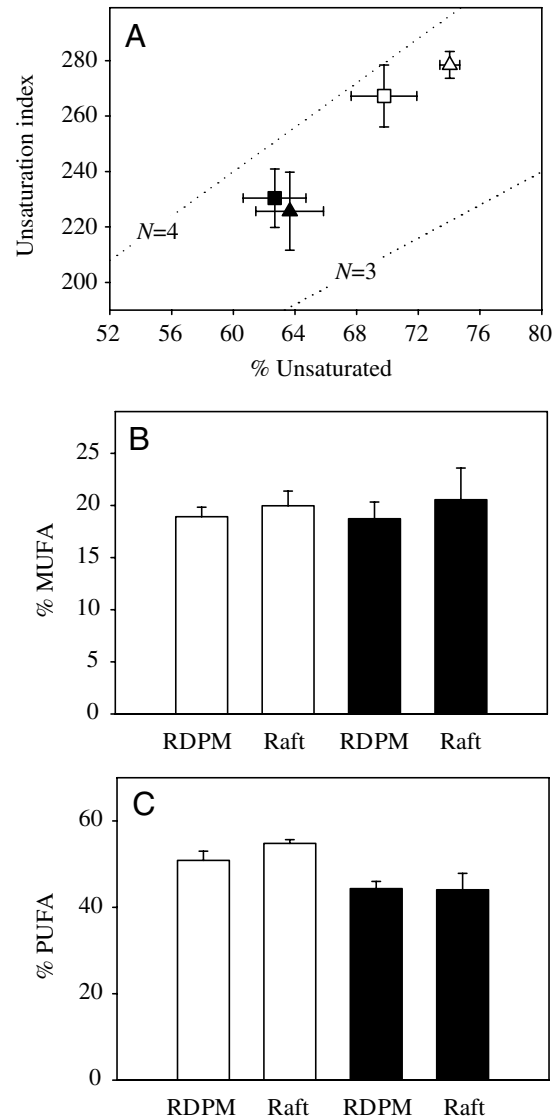


Fig. 4. Indexes of fatty acid unsaturation of phosphatidylethanolamine in membrane fractions. (A) Percentage of unsaturated fatty acids and unsaturation index of phosphatidylethanolamine of raft (triangles) and RDPM (squares) from 5°C-acclimated (open symbols) and 20°C-acclimated (filled symbols) fish. The dotted lines are isopleths, as described in Fig. 2. (B) Percentage of monounsaturated fatty acids and (C) percentage of polyunsaturated fatty acids in raft and RDPM from 5°C-acclimated (open bars) and 20°C-acclimated (filled bars) fish. All values are means  $\pm$  S.E.M.,  $N=4$ . Raft, raft-enriched plasma membrane; RDPM, raft-depleted plasma membrane.

greater in the raft than the RDPM. Large phospholipid class compositional changes were seen in the raft, while there were no changes in the RDPM (Fig. 1A). Likewise, the raft fractions differed significantly in the total percentage unsaturated fatty acids, whereas the RDPM were not significantly different (Fig. 2A). Previously, we found a larger increase in cholesterol concentration in raft than in RDPM with acclimation from 5°C to 20°C (Zehmer and Hazel, 2003). In the same study we found that detergent saturation point, an index of membrane lateral packing, was conserved in raft but not RDPM. Additionally, we found a greater change in order in raft than in RDPM (Zehmer and Hazel, 2004). Collectively, these data suggest that raft properties are particularly important during thermal acclimation.

However, the differences in fatty acid composition found in this study (with thermal acclimation and between raft and RDPM) were surprisingly small. This is difficult to reconcile with the conservation of physical properties described in our previous studies and the theory that rafts are phase separated from RDPM (Ahmed et al., 1997). It is possible that there were larger changes in the fatty acid content of sphingomyelin, which was not measured separately. It is also important to point out that highly polar glycosphingolipids, which are enriched in rafts (Fridriksson et al., 1999; Prinetti et al., 2000; Pike et al., 2002), would not have been efficiently recovered in our preparation. Therefore, the contribution of this important class of lipids to the raft fatty acid composition is unknown.

The second consistent pattern observed in this and in previous work was greater differences between raft and RDPM from warm-acclimated than from cold-acclimated trout. In the present study, this was clearly demonstrated by the class compositional data. While there were no differences in the cold-acclimated group, in the warm-acclimated group PtdSer was enriched in raft compared to RDPM at the expense of PtdCho (Fig. 1C,D). Cholesterol was also more enriched in rafts of warm-acclimated than cold-acclimated fish (Zehmer and Hazel, 2003). Finally, in warm-acclimated trout, raft was more ordered than RDPM while in the cold group, there was no difference in raft and RDPM order (Zehmer and Hazel, 2004).

A predominant model of lateral segregation of raft and RDPM is based on liquid-ordered ( $L_o$ )/liquid-disordered ( $L_d$ ) phase separation (Ahmed et al., 1997). This model predicts substantial differences in fatty acid composition and order that were not observed. Therefore, it is puzzling how raft and RDPM of cold-acclimated fish are segregated. However, it is important to point out that the protein composition and membrane packing strength of raft and RDPM are clearly distinct in both acclimation groups, as shown previously (Zehmer and Hazel, 2003). Therefore, raft and RDPM are distinct in properties and some aspects of their composition, but it is not clear what forms the basis for raft/RDPM segregation in PM of cold-acclimated trout.

A final pattern of interest in the current data involves the markedly different patterns of change in acyl chain unsaturation between the major phospholipids, PtdCho and PtdEtn. With cold acclimation, PtdEtn accumulated a higher percentage of

unsaturated fatty acids without a change in the average number of double bonds (Fig. 4) whereas in PtdCho more double bonds were introduced into the already unsaturated fatty acids (Fig. 3). These results recapitulate those of Logue et al. (2000), who undertook a comparative study of brain synaptosomes isolated from animals adapted to temperatures between -1°C and 41°C. Although the observation of this phenomenon in brain and liver and in both thermal acclimation of a single species (in both raft and RDPM) and adaptation across species, suggests it may be of fundamental importance, the significance of this pattern is not clear.

In summary, acclimatory compositional differences were greater in raft than in RDPM. These included changes in phospholipid class composition and patterns of fatty acid unsaturation. Additionally, there were differences in phospholipid composition between raft and RDPM only in the warm acclimation group. Surprisingly, there were no significant differences in fatty acid unsaturation between raft and RDPM in either acclimation group. The patterns of acclimatory changes in acyl chain unsaturation differed between PtdCho and PtdEtn. Collectively these and previously reported data suggest that regulation of raft properties may be especially important during thermal acclimation.

#### List of abbreviations

BHT	Butylated hydroxytoluene
FAME	fatty acid methyl ester
$L_d$	liquid-disordered
$L_o$	liquid-ordered
MUFA	monounsaturated fatty acids
PM	plasma membrane
PtdCho	phosphatidylcholine
PtdEtn	phosphatidylethanolamine
PtdIns	phosphatidylinositol
PtdSer	phosphatidylserine
PUFA	polyunsaturated fatty acids
raft	raft-enriched plasma membrane
RDPM	raft-depleted plasma membrane
SM	sphingomyelin
TLC	thin layer chromatography
UI	unsaturation index

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Table S1. *Percentage fatty acid composition of total lipid extracts*

Fatty acid	Raft				RDPM			
	20°C acclimated		5°C acclimated		20°C acclimated		5°C acclimated	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
16:0	23.9	0.4	17.7	1.1	23.8	0.7	20.1	0.8
18:0	10.3	0.8	9.6	0.2	10.1	0.8	10.1	0.4
20:0			0.1	0.1	0.1	0.1	0.1	0.0
22:0	0.4	0.2	1.2	0.1	1.3	0.0	1.1	0.0
24:0				0.1	0.1			
16:1	0.7	0.7	2.6	0.3	1.5	0.5	1.8	0.2
18:1c	14.5	0.6	15.3	0.6	13.1	0.5	13.9	0.8
18:1t	5.0	1.0	3.3	0.5	1.9	0.4	3.3	0.4
20:1	1.0	0.6	1.5	0.2	1.5	0.2	1.0	0.1
22:1			0.1	0.1	0.1	0.1	0.1	0.1
24:1	0.2	0.2	0.6	0.3	0.5	0.5		
18:2	5.9	0.6	8.3	0.2	6.7	0.1	8.0	0.4
18:3			0.2	0.1			0.1	0.1
20:2	1.3	0.8	1.6	0.1	1.5	0.1	1.6	0.1
20:4	2.5	0.8	4.5	0.1	5.0	0.1	5.2	0.3
20:5	5.2	0.6	5.9	0.1	6.4	0.5	6.2	0.1
22:6	29.2	1.2	27.7	0.7	26.8	1.1	27.5	0.5

Raft, raft-enriched plasma membrane; RDPM, raft-depleted plasma membrane.

Table S2. *Percentage fatty acid composition of phosphatidylcholine*

Fatty acid	Raft				RDPM			
	20°C acclimated		5°C acclimated		20°C acclimated		5°C acclimated	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
16:0	30.5	1.1	29.1	0.6	33.8	1.3	34.5	2.3
18:0	5.7	0.0	6.2	0.1	6.5	0.3	6.1	0.2
20:0	0.7	0.3						
22:0	1.0	0.1	0.9	0.3	1.0	0.1	0.8	0.0
24:0					0.1	0.1		
16:1	2.3	0.1	2.0	0.1	1.0	0.6	1.0	0.3
18:1c	14.4	0.4	11.4	0.3	14.0	0.2	9.9	0.3
18:1t	1.7	0.1	1.8	0.1	3.7	1.0	3.0	0.5
20:1	1.5	0.5	0.9	0.1	0.8	0.3	0.3	0.2
22:1	0.7	0.7			0.7	0.4	1.2	0.4
24:1			0.5	0.3				
18:2	6.0	0.3	5.5	0.2	5.8	0.3	4.6	0.3
18:3	0.1	0.1	0.1	0.1			0.1	0.1
20:2	1.5	0.1	1.2	0.1	1.2	0.4	0.6	0.2
20:4	3.0	0.3	2.4	0.1	1.0	0.6	0.6	0.6
20:5	7.4	0.9	7.9	0.2	6.7	0.3	7.7	0.3
22:6	23.7	1.0	30.4	0.5	24.0	0.7	29.7	1.5

Raft, raft-enriched plasma membrane; RDPM, raft-depleted plasma membrane.

Table S3. *Percentage fatty acid composition of phosphatidylethanolamine*

Fatty acid	Raft				RDPM			
	20°C acclimated		5°C acclimated		20°C acclimated		5°C acclimated	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
16:0	19.2	2.0	10.3	0.7	18.7	0.4	10.6	1.0
18:0	12.5	1.6	8.9	0.2	12.8	1.6	9.9	0.6
20:0	0.1	0.1						
22:0	0.4	0.2	1.1	0.1	0.8	0.1	0.6	0.2
24:0								
16:1	0.7	0.3	0.8	0.3	0.9	0.3	0.8	0.6
18:1c	13.7	1.3	16.4	0.3	14.7	0.6	16.6	0.3
18:1t	4.3	1.2	5.8	1.0	5.0	0.9	9.2	1.1
20:1	2.8	0.9	2.1	0.2	2.2	0.5	1.5	0.1
22:1	2.5	1.4			0.6	0.6		
24:1								
18:2	7.5	0.9	11.4	0.3	8.1	0.3	10.5	0.5
18:3			0.1	0.1				
20:2	0.7	0.4	2.3	0.1	1.5	0.1	2.1	0.1
20:4	8.7	5.2	5.3	1.3	4.7	0.7	3.4	0.2
20:5	3.8	0.6	4.8	0.1	3.9	0.3	4.7	0.3
22:6	23.4	2.2	30.8	1.4	26.2	2.1	30.2	1.7

Raft, raft-enriched plasma membrane; RDPM, raft-depleted plasma membrane.