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# Membrane order conservation in raft and non-raft regions of hepatocyte plasma membranes from thermally acclimated rainbow trout

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**Abstract**

Homeoviscous adaptation (HVA), the thermal conservation of membrane fluidity/order at different body temperatures, has been observed to varying degrees in different membranes. However, HVA has not been studied in raft and non-raft regions of the plasma membrane (PM) separately. Rafts are ordered PM microdomains implicated in signal transduction, membrane traffic and cholesterol homeostasis. Using infrared spectroscopy, we measured order in raft-enriched PM (raft) and raft-depleted PM (RDPM) isolated from hepatocytes of rainbow trout (*Oncorhynchus mykiss*) acclimated to 5 and 20 °C. We found approximately 130% and 90% order compensation in raft and RDPM, respectively, suggesting their independent regulation. Raft was more ordered than RDPM in the warm-acclimated trout, a difference fully explained by a 58% enrichment of cholesterol, compared to RPDM. Unexpectedly, raft and RDPM from cold-acclimated trout did not differ in cholesterol content or order. Freezing the membrane samples during preparation had no effect on order. Treatment with cyclodextrin depleted cholesterol by 36%, 56%, and 55%, producing significant decreases in order in raft and RDPM from warm-acclimated trout and RDPM from cold-acclimated trout, respectively. However, a 69% depletion of cholesterol from raft from cold-acclimated trout had no significant effect on order. This result, and the lack of a difference in order between raft and RDPM, suggests that raft and non-raft PM in cold-acclimated trout are not spatially segregated by phase separation due to cholesterol.


*Keywords:* Membrane; Hepatocyte; Rainbow trout

## 1. Introduction

Extensive alterations of the plasma membrane (PM) lipid composition are made by poikilotherms in response to changes in environmental temperature [1]. These modifications are thought to offset thermal perturbation of functionally important membrane physical properties. Order, a commonly measured physical property loosely defined as the extent of motions of membrane lipids, is acutely temperature-sensitive. Sinensky [2] observed that thermal acclimation in *Escherichia coli* resulted in a marked reduction of the thermal perturbation of order, leading to the hypothesis that order is actively defended (homeoviscous adaptation; HVA). Since then, HVA has been observed in a wide variety of organisms including bacteria, plants, and animals [3]. The functional significance of HVA may involve the influence of order on the catalytic rate and thermal stability of certain membrane proteins. For example, both the activity and thermal stability of $Na^+/K^+$ ATPase correlate with order [4,5]. Similar correlations between order and thermostability have been observed in rhodopsin and chlorophyll [6,7].

As common as HVA appears to be, the degree of order conservation (the efficacy) varies depending on the membrane examined. This variation may reflect specific physical requirements inherent to the divergent functional roles of different membranes. For example, comparing organellar membranes, mitochondria showed higher efficacies of HVA than microsomes and no HVA was found in sarcoplasmic reticulum from goldfish muscle [8,9]. Additionally, differences in the extent of HVA can exist in different membrane macrodomains as was found between the brush border and basolateral surfaces of the PMs from trout intestinal epithelia [10] and carp enterocytes [11]. These observations led us to speculate that there may be differences in HVA between raft and non-raft regions of the PM.

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It is now generally accepted that the PM contains compositionally and functionally distinct microdomains, called rafts. Rafts are clusters of lipids and proteins estimated to be 300 nm in diameter [12]. Rafts appear to organize some signaling activity, concentrating the interacting proteins of various signal transduction cascades [13]. Compared with the bulk PM, rafts are enriched in cholesterol and lipids with saturated acyl chains [14]. In mammalian cells, rafts form due to interactions between these lipid components leading to the phase separation of liquid-ordered ($L_o$) regions (rafts) from the remaining liquid-disordered ($L_d$) membrane [15,16].

Previous measurements of HVA in the PM reflect a composite of signal from raft and non-raft regions [17–19]. We have found differences in the thermally induced changes in composition and membrane packing between raft and non-raft regions of rainbow trout (*Oncorhynchus mykiss*) hepatocyte PM [20]. Therefore, in this study we have measured order in these separate regions to determine if they differ in HVA. These measurements are also of interest to determine if phase separation between raft and non-raft PM is conserved during thermal acclimation.

In this study we made measurements of order from raft-enriched PM (raft) and raft-depleted PM (RDPM) isolated from the livers of thermally acclimated rainbow trout using Fourier transform infrared spectroscopy (FTIR). We report that, as predicted, rafts were more ordered than RDPM in the warm-acclimated group, and that this is due to a greater cholesterol concentration. In contrast, there was no difference between the order of raft and RDPM in the cold-acclimation group. Furthermore, we found nearly perfect conservation of order in the RDPM and a slight overcompensation of order in the raft membranes. These data suggest that the lipid order of raft and RDPM is regulated independently during thermal acclimation. Furthermore, these results point to an atypical mechanism of segregation of raft from RDPM in membranes of cold-acclimated trout.

## 2. Materials and methods

### 2.1. Animals

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

### 2.2. Plasma and raft membrane isolations

PMs were isolated from approximately 4 g of liver (pooled from two fish) according to a modification of the procedure of Armstrong and Newman [21] as described previously [18]. The PM was resuspended in working buffer (WB: 0.25 mol $l^{-1}$ sucrose, 20 mmol $l^{-1}$ tricine, pH 7.8, 1 mmol $l^{-1}$ EDTA) and was routinely frozen at −80 °C. PM was separated into raft-depleted PM (RDPM) and raft-enriched PM (raft) using a non-detergent based method [22]. The membrane fractions were separated based on the lighter buoyant density of raft, compared to RDPM. Briefly, the PM was sonicated and then resuspended to 23% OptiPrep before layering a 10–20% OptiPrep gradient on top (for a total of 11 ml in each tube). After centrifugation for 90 min at 72,800 × *g* in a Beckman SW 41-Ti rotor (OP1) the top 5.5 ml (raft) was removed, mixed with 4 ml of 50% OptiPrep in a fresh tube, capped with 250-μl 5% 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 for 20 min at 20,800 × *g* in a refrigerated microcentrifuge (Eppendorf 5417 R). The bottom 5.5 ml from OP1 (RDPM) was diluted with four volumes of buffered saline and pelleted by centrifugation for 1 h at 23,700 × *g* in a Beckman JA 30.50 rotor. The raft and RDPM were routinely frozen at −80 °C for later analysis.

As reported previously [20], we found a large enrichment of the $\beta_2$ adrenergic receptor and adenylyl cyclase and a substantial depletion of the insulin receptor β subunit in the raft-enriched fractions from both warm- and cold-acclimated fish. The degree of enrichment or depletion of these protein markers was roughly equivalent in samples from the two acclimation groups. The raft fractions were also enriched in lipid, at the expense of protein, in both acclimation groups. It is not possible to state with certainty the degree of separation between raft and RDPM regions of the PM since no protein markers are found exclusively in rafts. Even caveolin, the most commonly used marker for caveolae, is found in membranes of other organelles [23]. Nevertheless, this protocol produced two fractions with heterogeneous distribution of proteins and lipids consistent with a reasonable separation of raft and non-raft PM regions.

### 2.3. Cholesterol depletion, cholesterol assay, and protein assay

The role of cholesterol in determining the order of the hydrophobic region of the membrane was determined by depleting raft and RDPM of cholesterol. Samples (raft and RDPM) were split and mixed either with an equal volume of buffered saline (TBS: Tris pH 7.4; 150 mmol $l^{-1}$ NaCl) (control) or 4% methyl-β-cyclodextrin in TBS (treated; 2% final cyclodextrin concentration). Samples were incubated at 25 °C for 20 min and then diluted with TBS to a final

volume of 600 μl. After centrifugation at 20,800 × *g* in a refrigerated microcentrifuge (Eppendorf 5417 R) for 20 min, the supernatant was discarded and the pellet resuspended in 100-μl WB. A coupled cholesterol oxidase fluorometric assay was used to measure cholesterol [24]. Total protein concentration was determined by the bicinchoninic acid method.

### 2.4. Effect of freezing on infrared spectroscopy results

As described in Section 2.2, two freeze/thaw cycles were routinely used during the preparation of raft and RDPM membranes. A modification of the membrane isolation protocol was used to determine the effects of these freeze/thaw cycles on membrane order. PMs isolated by standard methods from approximately 8 g of liver were resuspended in 1 ml of WB. The PM was separated into raft and RDPM by the OP1 centrifugation step, the upper and lower fractions were separated, and the samples were stored at 4 °C overnight. The following morning, raft and RDPM membranes were prepared as described above and then analyzed using FTIR.

### 2.5. Fourier transform infrared spectroscopy

The symmetric stretching vibrations of fatty acid methylene groups are sensitive to motional freedom and thus reflect membrane lipid order [25]. This vibrational mode produces distinct absorbance bands in the infrared spectrum which can be measured using Fourier transform infrared spectroscopy (FTIR). *Increasing* vibrational frequencies are measured as *higher* wave numbers, reflecting *decreasing* order. We measured the methylene symmetric stretching wave numbers of raft-enriched plasma membrane (raft) and raft-depleted plasma membrane (RDPM) of hepatocytes from cold and warm-acclimated trout as a function of temperature. Samples were loaded between two $CaFl_2$ crystals separated by a 50.8-μm Teflon spacer and placed in a thermally controlled sample block (CIC Photonics, Albuquerque, NM, USA). The sample chamber was continuously purged with nitrogen. Using a Perkin Elmer (Wellesley, MA, USA) Spectrum 2000 Fourier transform infrared spectrometer, 75 scans between the wave numbers of 370 and 7800 $cm^{-1}$ were averaged at each temperature. The instrument software package was used to subtract background and identify the peak wave numbers corresponding to the methylene symmetric stretching wave numbers by taking the second derivative of the spectra [26].

### 2.6. Chemicals used

Cholesterol oxidase was purchased from Calbiochem (San Diego, CA, USA). The bicinchoninic acid total protein assay kit was from Pierce (Rockford, IL, USA). Other biochemicals were from Sigma (St. Louis, MO, USA) and all other chemicals were of analytical grade.

### 2.7. Statistical evaluation

Comparisons between raft and RDPM from the same PM prep were made with paired *t*-tests. All other comparisons were made with unpaired *t*-tests. All tests were one-tailed unless otherwise noted. In all cases, a $P$ value $\leq 0.05$ was accepted as indicating statistical significance.

## 3. Results

### 3.1. Lipid order of plasma membrane rafts and RDPM

In mammals, raft microdomains are thought to be in the liquid-ordered phase ($L_o$), and thus more ordered than the remainder of the liquid-disordered ($L_d$) plasma membrane (i.e. the raft-depleted plasma membrane; RDPM) [16]. However, the effect of thermal acclimation on this relationship has not been examined. Therefore, we measured the order of raft-enriched PM (raft) and RDPM of hepatocytes from rainbow trout acclimated to 5 and 20 °C (Fig. 1). Surprisingly, the order of raft and RDPM were nearly coincident in the membranes from both the cold- and warm-acclimated trout (Fig. 1A and B). Furthermore, membrane order was not significantly different between raft and RDPM at the acclimation temperatures of either cold-acclimated (Fig. 1A, arrow; $P = 0.437$) or warm-acclimated (Fig. 1B, arrow; $P = 0.269$) fish.

Due to these unexpected results, we chose to collect more membrane and conduct a second set of measurements, focusing on the physiological assay temperatures 5 and 20 °C. As observed previously, the order of raft and RDPM from the cold-acclimated fish did not differ at their acclimation temperature (Fig. 1C; $P = 0.320$). However, unlike the first data set, rafts from the warm-acclimation group were slightly, but significantly, more ordered than RDPM (Fig. 1C; $P = 0.011$). This apparent discrepancy between data sets was resolved through an analysis of the individual sample pairs (i.e. raft and RPDM separated from a single PM preparation) at their respective acclimation temperatures (Fig. 2). In this analysis, the wave number obtained from an individual raft sample was subtracted from that obtained from the paired (from the same preparation) RDPM. Sample pairs with raft more ordered than the RDPM would produce a positive number (Fig. 2 includes data from the freeze/thaw experiments that will be discussed below in addition to the data included in Fig. 1). The data from the cold-acclimation group are distributed roughly equally above and below the zero line, demonstrating no consistent relationship between the lipid order of raft and RDPM (Fig. 2A). In contrast, data from the warm-acclimation group are consistently positive (with one outlier from the first study), indicating that rafts are more ordered than their paired RPDM (Fig. 2B). A reanalysis of the first data set using a paired *t*-test with the outlier excluded

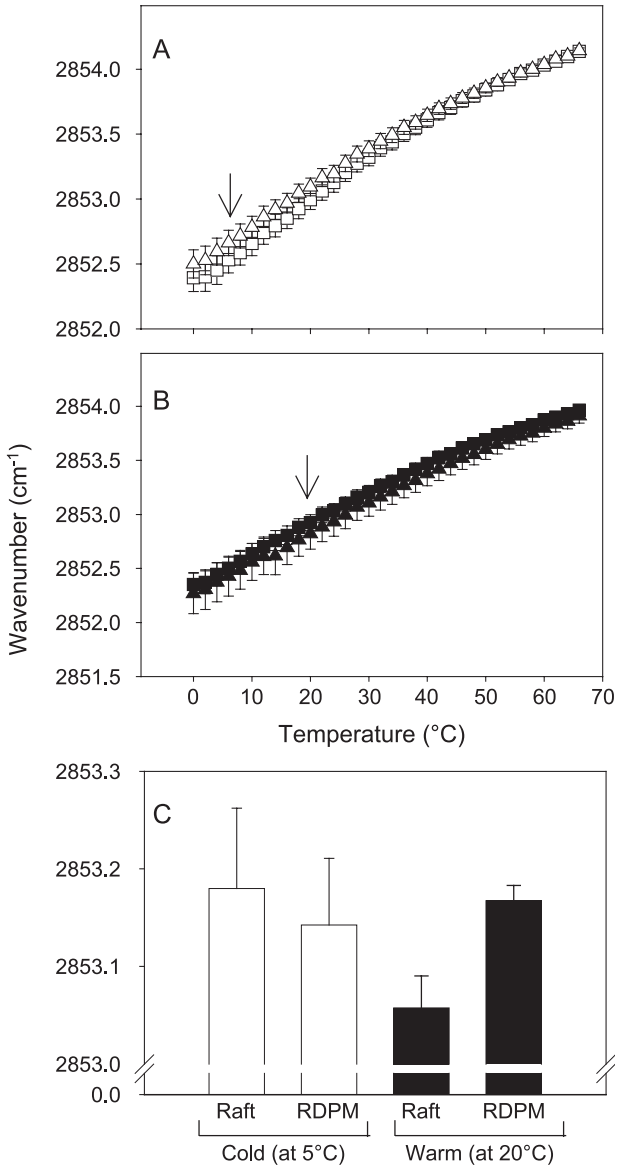


Fig. 1. Comparison of order in raft and RDPM from cold-acclimated and warm-acclimated trout. Two independent data sets are presented. (A) Order of raft (open triangles) and RDPM (open squares) from cold-acclimated trout data set 1 assayed from 0 to 66 °C. Arrow denotes assay at physiological temperature. (B) Order of raft (filled triangles) and RDPM (filled squares) from warm-acclimated trout data set 1 assayed from 0 to 66 °C. Arrow denotes assay at physiological temperature. (C) Raft and RPDM from cold-acclimated (open bars) and warm-acclimated (filled bars) trout data set 2 assayed at physiological temperatures. Values are means and S.E. from (A and B) six independent experiments or (C) four independent experiments.

gave a near significant difference ($P=0.051$), with raft more ordered than RDPM.

### 3.2. Thermal acclimation of lipid order in rafts and RDPM

We next examined the two data sets to determine if order was conserved in raft and RDPM during thermal acclimation. Acclimatory changes offsetting the disordering effect of increased temperature can be detected by comparing the order of two membranes at a common temperature. A greater order of the membrane acclimated to a higher temperature indicates order compensation. Furthermore, if the order of the two membranes are the same when measured at their respective acclimation temperatures, perfect order conservation is present.

Order compensation was evident in RDPM from the first study only at supraphysiological temperatures (above about 30 °C) (Fig. 3A). In the physiological temperature range, the order of the RDPM from the cold-acclimated and warm-acclimated fish were nearly coincident, indicating a lack of order conservation. A *t*-test comparing the order of RDPM samples measured at their respective physiological temperatures (Fig. 3A, arrows) detected a significant difference ($P=0.023$), indicating a significant thermal perturbation of this physical property. However, a pattern of order compensation was evident in RDPM from the second data set.

In this data set, lipid order in the RDPM fraction did not differ significantly between acclimation groups when measured at the respective physiological temperatures ($P=0.367$; Fig. 3B, compare left- and right-most bars), and the efficacy of conservation was 93%.

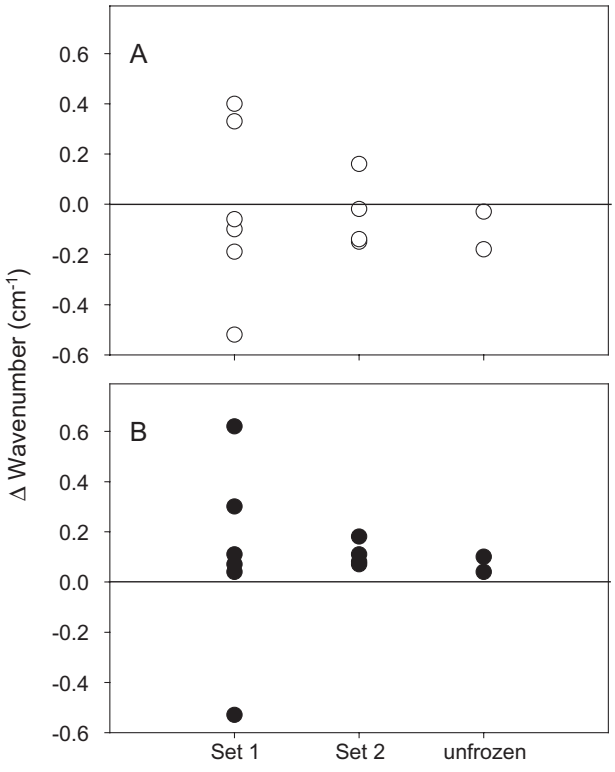


Fig. 2. Comparison of order of individual pairs of raft and RDPM from cold-acclimated and warm-acclimated trout. Values were generated by subtracting the wave number of raft from RDPM for each data pair. Positive values reflect raft with greater order than RDPM. Three data sets are presented: sets 1 and 2 (see Fig. 1) and a third set from an additional experiment with unfrozen membrane (see text). (A) Data pairs from cold-acclimated trout. (B) Data pairs from warm-acclimated trout.

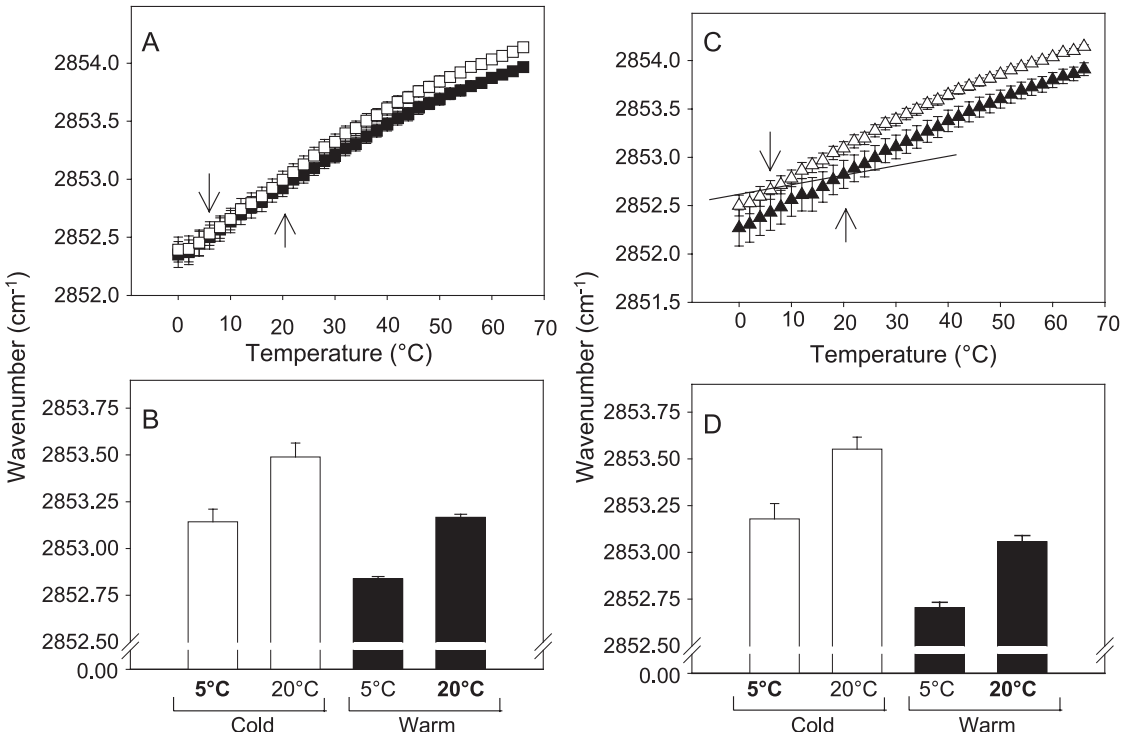


Fig. 3. Effect of thermal acclimation on order in raft and RDPM. Two independent data sets are presented. (A) RDPM from cold-acclimated (open squares) and warm-acclimated (filled squares) trout, data set 1, assayed from 0 to 66 °C. Arrows denote assays at physiological temperatures. (B) RDPM from cold-acclimated (open bars) and warm-acclimated (filled bars) trout, data set 2, assayed at 5 and 20 °C. (C) Raft from cold-acclimated (open triangles) and warm-acclimated (filled triangles) trout, data set 1, assayed from 0 to 66 °C. Arrows denote assays at physiological temperatures. Line indicates conservation of order. (D) Raft from cold-acclimated (open bars) and warm-acclimated (filled bars) trout, data set 2, assayed at 5 and 20 °C. Values are means and S.E. from (A and C) six or (B and D) four independent experiments.

There was also evidence of order conservation in the raft. In both data sets, the raft samples from warm-acclimated fish were more ordered (lower wave numbers) than those from cold-acclimated fish, when measured at a common temperature (Fig. 3C and D). A *t*-test comparing the wave numbers of the raft samples at their physiological temperatures found no significant difference ($P=0.116$, data set 1, Fig. 3C; $P=0.108$, data set 2, Fig. 3D), indicating conservation of order. In the first data set, the conservation efficacy was 54%, meaning that just over half of the thermal perturbation of order was counteracted through warm-acclimation. In the case of data set two, the efficacy was 133%, indicating overcompensation of order.

### *3.3. Effect of cholesterol depletion on the lipid order of plasma membrane rafts and RDPM*

Cholesterol is thought to be critical to raft formation in mammals [16] and we have previously found cholesterol to be enriched in rafts from cold- and warm-acclimated trout [20]. Therefore, we used methyl-β-cyclodextrin (MβC) to deplete cholesterol from raft and RDPM membranes to determine its influence on lipid order (Fig. 4). Contrary to our findings in Ref. [20], untreated raft and RPDM from the cold-acclimated group did not differ in cholesterol content (Fig. 4A; $P=0.393$). A 55% reduction in cholesterol significantly decreased lipid order (increase in wave number) in RDPM of cold-acclimated trout, when assayed at 5 °C (Fig. 4A; $P=0.031$). In contrast, there was no significant effect of a 69% reduction in cholesterol on lipid order in rafts from cold-acclimated trout assayed at 5 °C ($P=0.216$).

In the samples from warm-acclimated trout, raft contained 58% more cholesterol than RDPM ($P=0.001$). A 55% reduction in cholesterol significantly ($P<0.001$) decreased lipid order in the RDPM, assayed at 20 °C (Fig. 4B). In raft, MβC treatment reduced cholesterol by 36% to a cholesterol/protein ratio equal to that of untreated RDPM ($P=0.910$, 2-tail). This cholesterol depletion resulted in a significant decrease in order ($P=0.028$) to a value equal to that of untreated RDPM ($P=0.410$, 2-tail).

### *3.4. Effect of freeze/thaw cycles on lipid order in plasma membrane rafts and RDPM*

A recent study has reported effects of freezing and thawing on membrane physical properties [27], prompting us to determine whether freezing impacted the order of raft and RDPM in the present study. We routinely froze samples twice, once after completion of the PM isolation and once following completion of the raft/RDPM separation. To determine if these two freeze/thaw cycles had an effect on membrane order, we prepared membranes with no freeze/

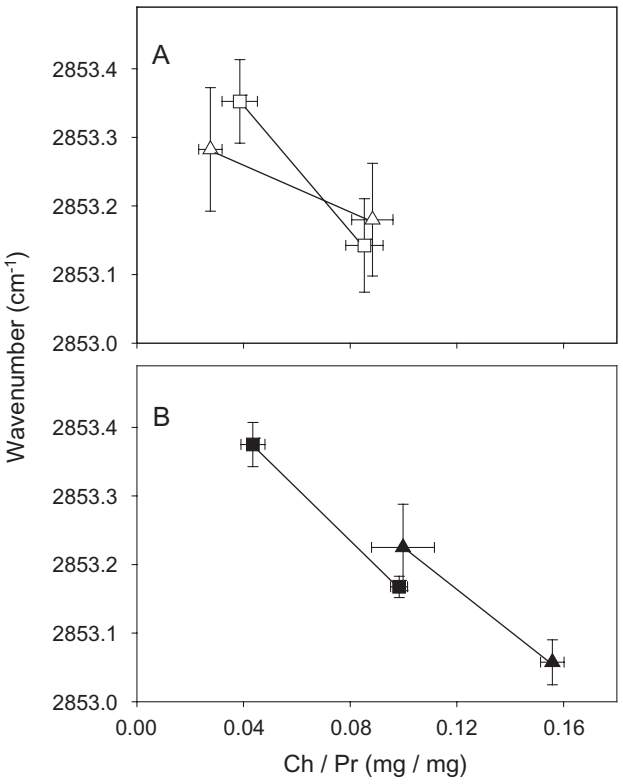


Fig. 4. Effect of cholesterol depletion on order. Isolated membranes treated with methyl-β-cyclodextrin to deplete cholesterol. (A) Raft (open triangles) and RDPM (open squares) from cold-acclimated trout. (B) Raft (filled triangles) and RDPM (filled squares) from warm-acclimated trout. Control samples are right-most and treated samples are left-most symbol of each joined pair. Values are means and S.E. from four independent experiments.

thaw cycles between sacrifice of the animals and FTIR analysis.

A comparison of raft and RPDM of the unfrozen samples assayed at the acclimation groups' respective physiological temperatures demonstrated good correspondence with the data from the second study (Figs. 2 and 5A). In the warm-acclimation group the raft was more ordered than the RDPM, as previously seen. In the cold-acclimation group the unfrozen RDPM were more ordered than the raft, in good agreement with previous observations (Fig. 2).

Examination of the effects of thermal acclimation on lipid order in raft and RDPM revealed an even closer match with the data from the second study (Fig. 5B and C). In both raft and RDPM the absolute values for lipid order were close to those found in the second study and the patterns of order conservation were similar as well. RDPM showed 71% order conservation while the raft membranes showed a 124% overcompensation of order.

## 4. Discussion

In mammals, rafts are thought to be stabilized in the PM by their unusual lipid composition. High concentrations of cholesterol interact with lipids with saturated acyl chains, especially sphingolipids, driving a phase separation of liquid-ordered ($L_o$) membrane (raft) from the remaining liquid-disordered ($L_d$) membrane [16]. The existence of these domains permits the segregation and organization of specific proteins, which is thought to be an important function of rafts. Raft-associated proteins are thought to be targeted to rafts by modification with saturated lipid anchors that are more soluble in $L_o$ than $L_d$ phase membrane [28]. For example, modification with a saturated GPI anchor

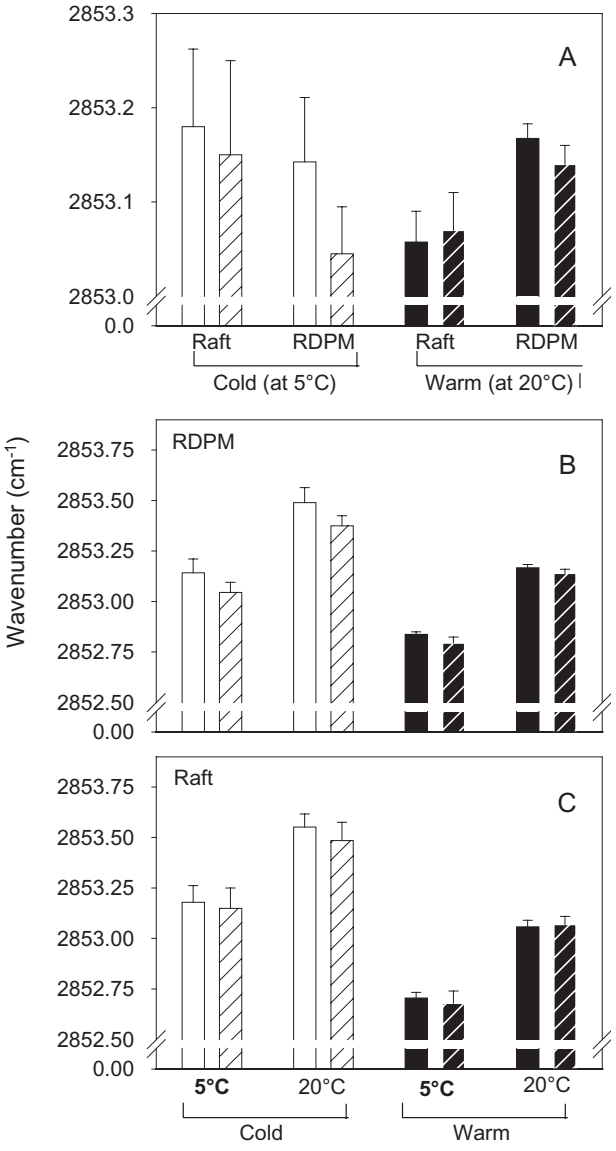


Fig. 5. Effect of freezing membranes on order. Cold-acclimated (open bars) and warm-acclimated (filled bars) samples prepared by standard methods with two freeze/thaw cycles (no hash marks) or no freeze/thaw cycles (hash marks). (A) Comparison of order in raft and RPDM from cold-acclimated and warm-acclimated trout, assayed at their respective physiological temperatures (as in Fig. 1.). (B) Acclimation in RDPM (as in Fig. 3B). (C) Acclimation in raft (as in Fig. 3D). Values are means and S.E. from four independent experiments (no hash marks) or means and ranges from two independent experiments (hash marks).

[29,30], a myristate and palmitate [31,32], or dual palmitate moieties [33] confers raft localization to proteins. Furthermore, the inclusion of unsaturated lipids into rafts causes a displacement of proteins normally localized in there [34].

In this study we found raft-enriched PM (raft) from warm-acclimated trout to be slightly more ordered than raft-depleted PM (RDPM), as predicted (Figs. 1B,C and 2). However, we found evidence that in cold-acclimated trout, the order of raft and RDPM did not differ (Figs. 1A,C and 2). We also found no enrichment of cholesterol in raft, compared to RDPM, from the cold acclimation group (Fig. 4). These results bring into question how cold-acclimated rafts are stabilized, if not by phase separation, and how proteins are targeted to rafts in cold-acclimated trout. Furthermore, the 0.1 wave number difference in order between raft and RDPM from the warm-acclimated trout was small and may not reflect an $L_o/L_d$ phase separation.

Nevertheless, it is important to point out that spatial segregation is apparently maintained in the membranes from both groups of fish. In a previous study we found similar enrichment of the $\beta_2$ adrenergic receptor and adenylyl cyclase and similar depletion of the insulin receptor in rafts, compared to their respective RDPMs, of both warm- and cold-acclimated trout [20]. Furthermore, raft from both acclimation groups were similarly enriched in lipid, at the expense of protein.

Perhaps various mechanisms of spatial segregation dominate in different thermal contexts, with $L_o/L_d$ phase separation playing a lesser role at lower temperatures. There is some evidence that lipid microdomains may be formed, at least partially, due to membrane proteins immobilized by connection with the cytoskeleton. Experiments using fluorescence recovery after photobleaching found restrictions to diffusion in membranes but not in protein-free bilayers [35,36]. Single particle tracking experiments found labeled molecules in putative microdomains to move anomalously, as if through a dense field of proteins [37,38]. However, it is not clear how a protein-mediated microdomain would lead to the tighter packing of raft from the cold acclimation group, as determined by its detergent solubility profile [20]. It would be of interest to determine how this is achieved and if this phenomenon is specific to trout or if it is a general feature of membranes adapted or acclimated to low temperatures. Indeed, a survey of raft and RDPM from species adapted to a range of temperatures could determine if there is a relationship between temperature and phase separation in these microdomains.

Differences in the structure of rafts between the two acclimation groups were reinforced by the cholesterol depletion experiments (Fig. 4). In the warm acclimation group, the greater order in raft compared with RDPM was fully explained by differences in cholesterol content (Fig. 4B). In contrast, in cold-acclimated animals, the raft and RDPM did not differ in cholesterol content (Fig. 4A). Furthermore, unlike in the RDPM, cholesterol depletion did not have a significant effect on lipid order in rafts, suggesting a minimal role for cholesterol in raft stability in cold-acclimated trout.

Another aspect of the data deserving consideration is the effect of thermal acclimation on lipid order of raft and RPDM domains. The conservation of membrane order with thermal adaptation or acclimation is a common observation in many organisms and membrane types [3], but this is the first study to consider the raft and RDPM regions of the PM separately. Membrane order is thought to impact the function of membrane proteins by providing an environment conducive to function. For example, the activity of the rat pancreas chloride transporter is positively correlated with lipid order [39], while the function of the PCP-NMDA receptor in rat brain membranes is negatively correlated [40]. The finding of 70–90% order conservation in RDPM (Figs. 3C and 5B) and 120–130% overcompensation in raft (Figs. 3D and 5C) suggests that processes in both regions are sensitive to perturbation in order. Nevertheless, since the degree of compensation differs between these regions, it appears that raft and RDPM are regulated independently, possibly reflecting the different requirements of specific proteins differentially located in each region.

However, another possible interpretation of the overcompensation seen in raft involves the potentially different mechanisms of raft stabilization and protein targeting in cold- and warm-acclimated animals, as discussed above. The greater ordering of raft, compared to RDPM, with warm-acclimation may not reflect differential requirements for order conservation of these two regions. Instead, this may reflect a requirement to establish their phase separation at elevated temperatures. Perhaps at lower temperatures another mechanism for preserving raft/RDPM spatial heterogeneity suffices and only with the introduction of increased thermal energy is phase separation required.

It is important to recognize the differences in results between the first and subsequent studies. The absolute degree of order conservation seen in the raft and RDPM membranes was lower in the first compared to latter studies (Fig. 3). Nevertheless, in the first study, raft showed a higher efficacy of lipid order conservation than the RDPM, similar to the results of subsequent studies (Fig. 3). Furthermore, upon exclusion of an outlying data point from the warm acclimation group, the raft/RDPM comparison of the first study agreed well with that of the later studies (Fig. 2).

The difference in the absolute degree of conservation probably reflects real differences in the physiology of the fish used. These differences may have been due to batches of fish being exposed to different conditions at the hatchery, the age or sexual maturity of the fish (although we routinely excluded fish with developed gonads) or some inconsistency in their care (e.g. spikes in temperatures due to power failure). We have been unable to identify the causative factor. An earlier study of whole, unfractionated trout hepatocyte PM found near perfect order conservation [18]. This is consistent with the findings of the later experiments in this study, suggesting they best reflect the ‘‘normal’’

physiology of these membranes. Regardless, the main patterns in the data presented here were consistent across experiments, as discussed.

Finally, despite a recent report of effects of freezing on membrane physical properties [27], we found the results from frozen and unfrozen material to agree well (Fig. 5). Nevertheless, it is prudent and probably should be common practice to consider the potential effects of freezing and thawing on membrane physical properties, since freezing may well have dissimilar effects on the physical properties of different organisms and membranes.

In summary, the order of raft and RDPM did not differ in samples from cold-acclimated trout. In contrast, raft from warm-acclimated trout was more ordered than its respective RDPM. This difference was fully explained by the greater cholesterol concentration of raft compared to RDPM. Depletion of cholesterol in raft from cold-acclimated trout did not have a significant effect on lipid order, but did significantly decrease order in warm-acclimated trout. We found that the efficacy of order conservation with thermal acclimation was consistently greater in raft than in RDPM. Finally, membranes prepared with no freeze/thaw cycles produced results that agreed well with the results from membranes frozen twice during their preparation. The finding that raft and RDPM from cold-acclimated trout did not differ in order suggests that these regions are not segregated by phase separation, but by some other mechanism.

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