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Rapid Temperature Induced Changes in Physico-chemical Properties of Fish, *Cyprinus carpio* Erythrocyte Subcellular Membranes

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Erythrocytes of the freshwater fish (*Cyprinus carpio*) adapted to 22°C were exposed either to 30°C or 10°C for 2 hr and the temperature dependency of fluorescence polarization of 1,6-diphenyl 1,3,5- hexatriene, embedded into isolated plasma membranes, mitochondrial membranes and microsomal membranes was determined. Fluorescence polarization of phospholipid vesicles prepared from erythrocytes of summer and winter adapted carps was also studied. There was roughly a 20% compensation for temperature when assaying the phospholipid vesicles. Exposure of cells *in vitro* to 10°C resulted in a decrease of fluorescence polarization of the separated membranes alongwith an enhancement of delta 9 and delta 6 desaturation in microsomal membranes. The effect is just reverse when the cells were exposed to 30°C. Thus there is a clear indication of active adaptation in the physical state of the membranes to the prevailing temperature. Possible role of the unsaturated fatty acid in controlling membrane physical properties is discussed.

Key Words: Fluidity, Erythrocyte, Fatty Acids, Temperature, Adaptation, Desaturation

Introduction

Physiological and biochemical reactions in poikilotherms are subject to Vant Hoff's or Arrhenius' rate-temperature laws. Compensation for temperature of vital physiological functions has been reviewed by Precht et al. (1973). A number of physiological process are dependent on membrane structure. The composition as well as the physical state of the membranes profoundly affects these processes. The Singer-Nicholson's membrane model (1972) predicts that membranes are in a liquid crystalline state at the body temperature which in turn may change in the case of poikilothermic animals. A change in the ambient temperature can be regarded as a membrane perturbant which may trigger processes resulting in membrane re-organization favourable for cell to function in the new thermal environment. Adaptation of membrane fatty acid composition and physical properties to temperature by fish liver (Farkas & Roy 1989, Dey et al. 1993b), fish lymphocytes (Abbruzzini et al. 1982), fish

brain (Roy et al. 1992, 1997) have been reported. The fatty acid composition of structural lipids is one of the factors that controls membrane physical properties and an inverse relationship between the content of unsaturated fatty acids and temperature has been reported for several poikilothermic organisms (Farkas et al. 1984, Roy et al. 1991).

All these observations were made on animals exposed to different temperatures for a relatively longer period (i.e. weeks) of time. However, poikilotherms are frequently exposed to rapid thermal fluctuations during day and night or from day to day. It was already shown that the composition of newly formed fatty acids in carp liver depends on the prevailing temperature rather than the thermal history of the fish under both *in vivo* (Farkas & Csengeri 1976) and *in vitro* (Farkas 1984) conditions. Wodtke and Cossins (1991) also reported that fluidity of the carp liver endoplasmic reticulum was readily adjusted to prevailing temperature. Furthermore, it is reported that the plasma membrane of carp erythrocytes reacted to change in temperature, becoming more fluid with decreasing temperature and vice versa (Dey & Farkas 1992, Dey et al. 1993a). We provide here further evidence that some fishes are able to respond immediately to changes in the temperature by modifying fatty acid desaturase activity and membrane microviscosity.

Materials and Methods

Animals : Summer adapted (22°C) and winter adapted (5°C) carps, *Cyprinus carpio*, 1-1.5 kg size, were obtained from a neighboring fish farm (Tisza Fish Farm, Szeged, Hungary). The fishes were sacrificed immediately.

Collection of erythrocytes : Blood samples from 3-4 fishes, were collected from the caudal vein with heparinized syringe. The blood plasma and the buffy coat were removed by centrifugation at 800xg for 10 min. The packed erythrocytes were washed twice with 0.05 M Tris-HCl buffer, *p*H 7.6, containing 0.15 M NaCl at 5°C. A concentrated cell suspension was prepared in the buffer medium. Some cells (about $9x10^9$ cells) were acclimated to 10°C and 30°C for 2 hr along with the cells maintained at room temperature (22°C). During this time of acclimation the cells did not haemolyse.

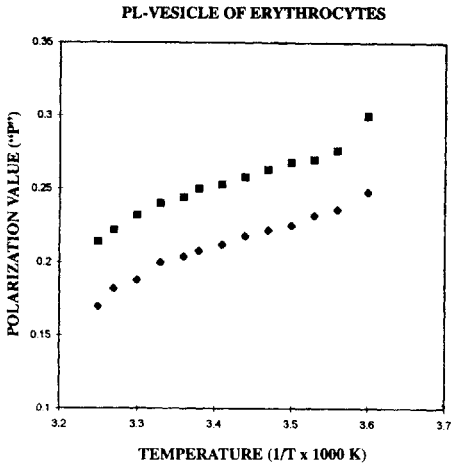
Separation and isolation of membranes: The cells were haemolysed by brief sonication (2x15 sec.) and the total membrane pellets were obtained by centrifugation at 130,000xg for 60 min. The different subcellular membranes were separated by a 3 step sucrose gradient (70%, 42% and 20%) in Tris-Hcl buffer. About 2 ml of total membranes in 2 mM $CaCl_2$ and 5 mM of $MgSO_4$ were layered at the top of a 25 ml of sucrose gradient (5 ml of 70%, 10 ml of 42% and 10 ml of 20%) containing the above salt and centrifuged at 52,000xg (in swinging bucket rotor) for 90 min with acceleration and deceleration of 30 min each. The different membrane fractions (mitochondrial, plasma membrane and microsomal fractions) were isolated and further purified as per the experience of Zevallos

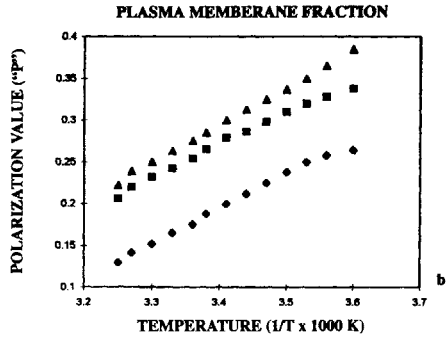
and Farkas (1990). The membrane(s) pellets were suspended in 0.05 M Tris-HCl buffer and stored at (-) 80°C for further analysis the fatty acid composition and measuring the membrane fluidity. The purity of each membrane fractions were tested with suitable marker enzymes, viz. 5'- nucleotidase for plasma membrane (68% of total activity recovered), succinic dehydrogenase for mitochondrial membranes (70% activity recovered) and glucose-6-phosphatase for microsomal membrane fraction (65% activity found).

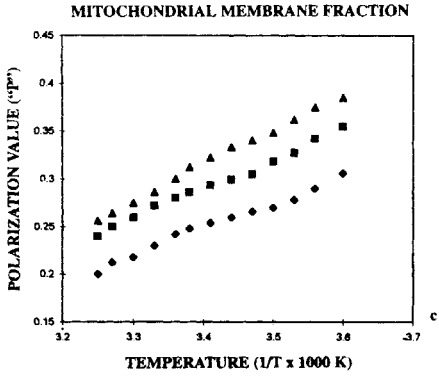
Extraction, separation analysis of lipids: Membrane lipids as well as erythrocytic lipids were extracted according to Roy et al. (1992). The total lipids were further segregated into polar lipids and other lipid subclasses by thin layer chromatography on precoated silica gel G plates (E. Merck, Dramstatdt, Germany) using hexane, diethyl ether, and acetic acid (85:15:1.5, v/v) as solvent system. The spots, after detection with 0.05% ANSA (8-anilino, 1-naphthalene sulphonic acid) in 50% methanol under UV light, were transferred to screw cap vials for transmethylation in the presence of 5% HCl in absolute methanol at 80°C for 2.5 hr. Separation of fatty acid methyl esters was made either by thin layer chromatography (Kates 1986) on silver nitrate impegmented silica gel G plate (after detecting the spots, separated according to the number of double bonds, by spraying with aqueous Rhodamine-B solution) or by gas liquid chromatographic technique using a Hitachi 263-80 gas chromatograph equipped with dual flame ionization detector and Carbowax 20M coated into Supelcoport, 80/100 mesh (Supelco, Pennsylvania) as stationary phase in 2 m x 2.5 mm glass columns (Farkas & Roy 1989).

Fluorescence polarization measurements : 1,6 -diphenyl 1,3,5- hexatriene dissolved in tetrahydrofurane was added to the membranes suspended into 0.05 M Tris-HCl buffer, *p*H 7.0 or to phospholipid vesicles, in a ratio of 1:200, and were incubated for 20 min at room temperature. A Perkin Elmer 4A fluorescence spectrophotometer equipped with a temperature regulation unit was used for the measurements. The probe was excited at 375 nm and the emmission was recorded at 420 nm. For correcting the light scattering, readings were made also without added DPH. Fluorescence polarization value ("P") was calculated according to the equation $I_{vv}-I_{vh}.G / I_{vv}+I_{vh}.G$ where I_{vv} and I_{vh} are the fluorescence intensities recorded parallel or perpendicular to the emitted light. G is the polarization correction factor, or Gaussiuss factor, I_{hv} / I_{hh} for vertically and horizontally polarized light.

Desaturase activity : The delta 9 and delta 6 desaturase activities were measured by incubation of erythrocytes (9×10^9) in the presence of either [1-^{14}C] stearic acid or linoleic acid (0.5 uCi). At the end of the reaction the unreacted label was removed by washing the erythrocytes with cold fatty acids in 0.15 M NaCl (Zevallos & Farkas 1990). The lipids were extracted, transmethylated







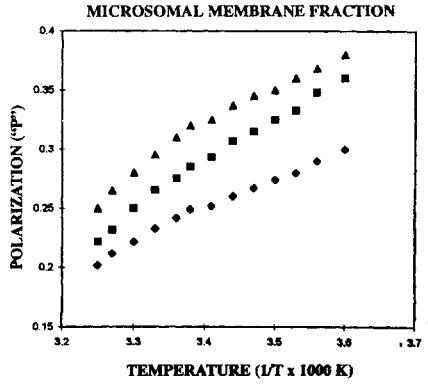


Figure 1. Effect of temperature on DPH fluorescence polarization (Each value is the mean of three determination): a) Phospholipid (PL) vesicles from erythrocytes of summer adapted ([] []) and winter adapted (<> <>) carp, *Cyprinus carpio*. b) Plasma membrane fraction: Erythrocytes from summer adapted, when ambient temperature was 22°C, carps were exposed to either to 10° ± 1°C (<> <>) or 30° ± 1°C (Δ Δ) over the control cells maintained at room temperature ([] []) i.e. at 22° ± 1°C in the laboratory for 2 hr.

c) Mitochondrial membrane fraction : (for rest of the legend refer figure 1b).

d) Microsomal membrane fraction : (for rest of the legend refer figure 1b).

and the fatty acids were separated by TLC mentioned above. Countings were made by a Packard Mod. 4530 liquid scintillation counter. The counts were corrected for quenching and counting efficiency.

Materials: [1-^{14}C] stearic acid (sp.a. 59 μCi/μmol), [1-^{14}C] linoleic acid (sp.a. 60 μCi/μmol) were from New England Nuclear, Boston, Mass. USA), lipid standards, unlabelled fatty acids and DPH from the Sigma Chemical Co. St Louis, Mo, USA.

Results

Effect of short thermal exposure on the physical state of erythrocyte membranes: The fluorescence polarization of diphenyl hexatriene in phospholipid vesicles prepared from erythrocytes of summer and winter adapted fishes is shown in figure la. The polarization value verses temperature curve reveals that the vesicles from the winter adapted fish exhibit roughly 20% higher degree ($P<0.001$) than those of summer adapted fish. To see whether the different membrane types react to rapid changes of the temperature, the intact erythrocytes were exposed to either 10°C or 30°C for only 2 hr and the fluorescence polarization of DPH was determined in the membranes viz. mitochondrial membrane, plasma membrane and microsomal membrane separated. The figures 1b-1d show that exposure of erythrocytes from summer adapted fish to 10°C for 2 hr resulted in 10-20% decrease ($P<0.001$) fluorescence polarization values in all the membranes and conversely, exposure the cells to 30°C for 2 hr resulted 10% increase in fluorescence polarization of mitochondrial membranes ($P<0.10$) and plasma membrane ($P<0.05$). A tendency towards the complete compensation (Precht's type 2) of physical properties membranes was noticed due to exposure of the cells at high temperature for a short period but exposure to low temperature for 2 hr brought near a complete compensation only in plasma membrane and incomplete compensation (Precht's type 3) in the mitochondrial and microsomal membranes.

Effects of short term acclimation on the fatty acid composition of the erythrocyte membranes: Major indices of fatty acids in erythrocytes of summer- and winter-adapted carp are given in table 1. In agreement with previous results, the levels of total saturated fatty acids was decreased with lowering the temperature resulting in a decrease in the ratio of total saturated to unsaturated fatty acids ($P<0.01$) and in an increase in the ratio of total n-3 to n-6 polyunsaturated fatty acids ($P<0.005$). The same trend was observed with erythrocytes exposed to two extreme temperatures for a short period of time (table 2). However, the different membrane types reacted differently to the temperature shifts as far as the individual fatty acids are concerned. For instance, changes in the oleic acid (18:1) were significant only in the mitochondrial membrane for both the temperature upshifted and downshifted ($P<0.025$), in the temperature upshifted microsome ($P<0.005$) and in

Table 1. *Fatty acid composition of erythrocyte phospholipids of summer and winter adapted carp,* ***Cyprinus carpio*** L.

Fatty acids	Summer adapted (n=4)	Winter adapted (n=4)
Total saturated (TS)	40.4 ± 1.6	32.5 ± 1.7
Total unsaturated (TUS)	59.6 ± 2.0	67.5 ± 1.8
TS / TUS	0.7	0.5
Oleic acid	18.5 ± 0.4	21.8 ± 0.3
Total n-3 fatty acids	10.6 ± 0.3	15.4 ± 0.4
Toatl n-6 fatty acids	30.5 ± 1.0	29.8 ± 1.3*
n-3 / n-6	0.34	0.54

Note: The sample size "n" means different set of erythrocytes.

* Except this, all values of winter adapted fish are statistically significant ($P < 0.05$) with comparison to that of summer adapted fish.

Table 2. *Effect of up- and down shift of temperature on fatty acid composition of erythrocyte subcellular membranes.*

Fatty acid	Plasma membrane			Mitochondria			Microsome		
Temperature	22/10	22/22	22/30	22/10	22/22	22/30	22/10	22/22	22/30
12:0	-	-	-	1.0	4.2	4.2*	11.3*	12.2	10.2
14:0	2.0	4.4	4.6*	1.6	3.0	4.1	3.0	10.3	10.7*
16:0	34.5	40.8	45.2	25.3	32.6	38.3	20.3*	20.6	24.8
16:1	2.4	0.2	0.4*	0.8*	0.7	0.5*	0.9*	0.6	0.8*
18:0	5.9	13.1	12.1*	7.0*	6.7	8.9*	4.0	8.3	11.8
18:1n-9	11.4	8.7	9.2*	19.2	14.6	10.2	11.3*	10.2	7.6
18:2n-6	6.8	3.2	2.4*	9.4	9.0	7.6	12.0	10.8	8.5
18:3n-3	3.1*	2.5	1.5	7.2	3.4	3.2*	6.6	5.2	3.5
20:1n-9	1.1	2.2	1.3	2.4'	1.9	1.6*	1.2*	1.4	1.1*
20:2n-6	1.6	0.8	0.4	1.8*	2.0	2.2*	7.1	2.9	1.8*
20:4n-6	13.4*	16.3	16.4*	14.1*	16.2	13.2	10.0*	9.5	10.5*
20:5n-3	4.6	2.3	2.1*	1.8	0.6	0.5*	1.1*	1.4	2.2
22:4n-6	3.2	1.2	1.5*	0.6	2.2	2.4*	3.2	1.2	1.4*
22:5n-3	3.8	1.2	1.6*	2.1	0.9	1.2*	1.2*	1.4	2.6
22:6n-3	6.2	3.1	1.2	5.7	1.5	1.9	4.2	3.0	2.5*

Note: the values are the mean values of 4 samples or different set of erythrocytes.
* Except these, all values are statistically significant ($P < 0.05$) in compare to that of cells maintained at 22°C (22/22).

the temperature downshifted plasma membrane ($P<0.01$). On the other hand, docosahexanoic acid (22:6) differed significantly from the control in all the experimental condition in all the membranes ($P<0.05$ - 0.005) except for the temperature upshifted microsome and mitochondrial membranes.

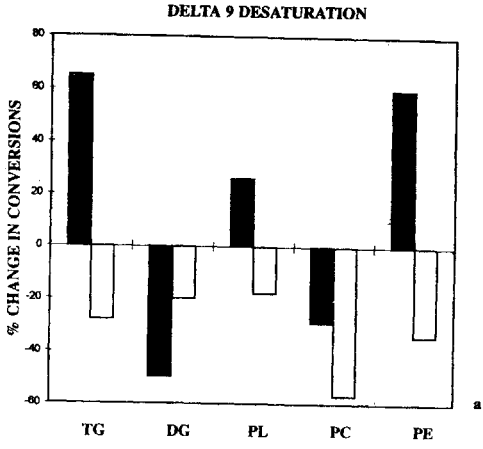
Desaturation of fatty acids in response to temperature shifts

Since the fatty acids are considered as the most powerful compounds to influence membrane physical properties, it was interesting to study whether short term thermal change affect the formation of unsaturated fatty acids or not. Incorporation of labeled stearic (18:0) or linoleic acid (18:2) into total lipid was linear with the time upto 150 min at 30°C and upto 8 hr at 10°C (data not shown). In figures 2a & 2b the delta 9 and delta 6 desaturase activities of the cold (10°C) and warm (30°C) incubated erythrocytes are shown relative to those measured at room temperature (22°C) incubated cells. Exposure to cold resulted in an increase in the activity of both (delta 9 and delta 6) desaturases and the newly formed fatty acids were directed into almost all lipids classes separated. Interestingly, incubation at 10°C had a negative effect on incorporation of oleic acid in diacylglycerols and phosphatidylcholine. Triacylgycerols and phosphatidylethanolamine were the compounds to accumulate maximum (60-65% more) oleic acid at this temperature. There was no such selectivity with y-linolenic acid (18:3) which appeared in all lipid classes in order triacylglycerols, diacylglycerols, phosphatidylethanolamine and phosphatidylcholine. On the other hand, a drastic decrease in delta 9 and delta 6 desaturase activity, relative to the controls, was noticed in the cells incubated at 30°C. The most sensitive responses were seen with diacylglycerols and phosphatidylcholine (50-60% decrease) in the case of delta 9 desaturation and with phiosphatidylethanolamine and phosphatidylcholine (50-60% decrease) in the case delta 6 desaturation.

Discussion

Fish erythrocytes are nucleated and thus they are expected to conduct all metabolic processes characterizing all cells in the fish body. From the data given in figures 2a & 2b, it is evident that they can take up fatty acids from their environment, desaturate them and direct into the different lipid classes which involves the presence of different desaturases, phospholipases, acyltransferases, lipid carrier proteins etc. From the figures 2a & 2b, it may also be inferred that these processes are selectively affected by the thermal environment of the cells.

It has repeatedly been observed that membranes in cold adapted fish are more fluid than in warm adapted ones and this is regarded as a manifestation of homeoviscous adaption first described for *E. coli* (Sinensky 1974). In agreement with this, phospholipid vesicles from cold adapted carp erythrocytes proved to be more fluid than those from warm adapted ones (figure



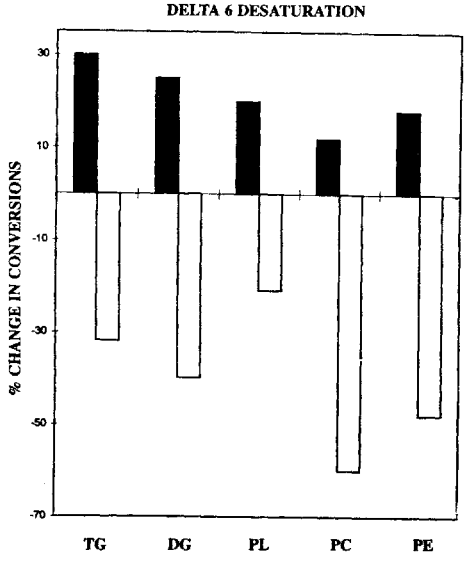


Figure 2. Efffect of short term thermal acclimation on delta 9 and delta 6 desaturase activity of carp erythrocytes. Intact cell were labeled with proper precursors and were exposed to either low or high temperature. The lipids were separated into different classes and then were converted into methyl ester and were further separated on TLC plate to determine the radioactivity (see materials and methods).

The mean of data from 4 different set of of erythrocytes are represented in the figure. The solid bar represents the cells exposed to 10°C and the open bar represents the cell exposed to 30°C.

a) The percentage of delta 9 desaturation at 22°C was as follows: triacylglcerol (TG) = 27.3, diacylglycerol (DG) = 30.0, phospholipid (PL) = 22.3, phosphatidylethanolamine (PE) = 24.3, and phosphatidylcholine (PC) = 19.0.
b) The percentage of delta 6 desaturation at 22°C was as follows: TG = 12.1, DG = 12.0, PL = 18.7, PE = 24.3 and PC = 19.2

la). Exposure of erythrocytes into two extreme temperatures (10°C, 30°C) brought about changes in their fluidities in harmony with the concept of homeoviscous adaptation. However, only the plasma membrane showed a tendency of perfect compensation in response to downshift or upshift of the temperature, while all other erythrocyte membranes displayed near partial compensation or incomplete compensation according to Precht's classification of 1973 (figures 1b - 1d).

The erythrocytes of fish are more sensitive to the fluctuation of environmental temperature and thus the erythrocytic membrane brought such intelligent changes towards homeoviscous adaptation within a very very short period i.e., 2 hr. A perfect compensation has been reported for lymphocytes of 27°C and 17°C acclimated pinyfish (Abbruzzini et al. 1982). Our present data, alongwith the previous one (Dey et al. 1993a) permit the speculation that the plasma membrane of erythrocytes, and probably of all other cells, gives the most sensitive response to thermal changes. This would explain why no or incomplete compensation was observed with the different brain membranes of *Channa punctatus* (Roy et al. 1992) and *Clarias batrachus* (Roy et al. 1997) kept at two extreme temperatures. The present results give support to earlier hypothesis (Zevellos Farkas 1990) proposing that desaturation of fatty acid is regulated by the physical state of the membranes. In our experiments the activity of both delta 9 and delta 6 desaturases varied with the measured fluidity of the microsomal membrane in a way that the more rigid membranes converted more stearic to oleic, and linoleic to linolenic acid and vice versa. However, the question remains open as to the unsaturated fatty acids accumulated during the temperature downshift would be sufficient volume to modulate the fluidity of the membranes according to the demand.

The slight and significant accumulation of polyunsaturated fatty acids found in this study during cold exposure (table 2) seems not to be sufficient to induce substantial alteration in membrane fluidity. The speculation is based on observation that some physical parameters like, surface areas, thermotropic phase transition temperature of phospholipids containing polyunsaturated fatty acids are rather close (Coolebar et al. 1983). Similar or greater changes in the fatty acid composition of the different brain membranes of *Channa* (Roy et al. 1992) or *Clarias* (Roy et al. 1997) resulted only in a partial compensation of membrane fluidity. Moreover, no changes were found in erythrocyte fatty acids composition of the temperature downshifted carp, although the fluidity of the plasma membrane sensitively followed the change of the temperature as assessed by DPH-PA (Dey et al. 1993a). It is probable that alteration of gross fatty acid composition is only a reflection of changes taking places at structural level of membranes and restructuring of the existing phospholipid is more important in this respect. The

data of figures 2a & 2b suggest that some reorganization of molecular architecture and molecular species composition of phospholipids might have occured in cold exposed erythrocytes since phosphatidylethanolmaine incorporated an appreciable amount of newly synthesized oleic acid. In a current study we found that in cold exposed carp liver slices (Farkas & Roy 1989) oleic acid was directed into the position of *Sn-1* of this phospholipid thus giving rise of formation of 1-monounsaturated, 2 polyunsaturated molecular species. In fact, level of similar phosphatidylethanolmaine is elevated in livers of cold adapted freshwater and marine fish (Dey et al. 1993 b) and shrimps (Farkas et al. 1994) evolutionary adapted to low temperatures. Incorporation of monounsatured fatty acids in position *Sn-1* of phosphatidylethanolmaine render these molecules more conic to stabilize the bilayer in cold. Moreover, appearence of a cis double bond in the position *Sn-1* of phospholipids renders that the membranes are less packed in the upper half of the bilayer as demonstrated in another connection (Farkas et al. 1994). Such structural alterations in some of the constituent phospholipids of erthrocyte membranes might have important in maintaining their structural and functional integrity in response to thermal changes. However, further investigations are needed to prove this for the fish erythrocytes.

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