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Role of *sn*-1 monounsaturated, *sn*-2 polyunsaturated phospholipid molecular species in membrane adaptation to temperature in poikilotherms

Ph.D. Thesis

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CONTENTS

The present thesis is based on the following publications:

1. "Molecular architecture and biophysical properties of phospholipids during thermal adaptation in fish: An experimental and model study", **E. Fodor**, R. H. Jones, Cs. Buda, K. Kitajka, I. Dey and T. Farkas, *Lipids* 30, 1119-1126 (1995).

   presented in the Results, chapters II, VI: Figs. II.1-3, VI.1-5.

2. "Composition and biophysical Properties of lipids in *Xenorhabdus nematophilus* and *Photorhabdus luminescens*, symbiotic bacteria associated with entomopathogenic nematodes", **E. Fodor**, E. Szállás, Zs. Kiss, A. Fodor, L. I. Horváth, D. J. Chitwood and T. Farkas, *Applied and Environmental Microbiology* 63, 2826-2831 (1997).

   presented in the Results, chapter I: Figs. I.1-3.

3. "Fatty acid composition of the ingested food only slightly affects physicochemical properties of liver total phospholipids and plasma membranes in cold adapted fresh water fish", R. Roy, **E. Fodor**, K. Kitajka and T. Farkas, *Fish Physiology and Biochemistry* 20, 1-11 (1999).

   presented in the Results, chapter IV: Figs. IV.1,2.

4. "Exception to seasonal adaptation: cyanobacterium C*ylindrospermopsis raciborskii*", **E. Fodor**, O. Zsíros, Zs. Várkonyi, Z. Gombos, L. I. Horváth, L. Hiripi, and T. Farkas, (manuscript).

   presented in the Results, chapter I: Figs. I.4-6.

Note: The reprints of articles and complete list of publications are included in the "Appendix to the Ph.D. thesis".

ABBREVIATIONS

ANS	8-anilino-1-naphthalene sulfonic acid
BHT	Butylated hydroxytoluene
CL	cardiolipin
DGDG	diglucosyldiglyceride
DMPC	dimyristoylphosphatidylcholine
DOPC	dioleoylphosphatidylcholine
DOPE	dioleoylphosphatidylethanolamine
DPH	1, 6-diphenyl-1, 3, 5-hexatriene
DPPC	dipalmitoylphosphatidylcholine
ESR	Electron spin resonance
GC	Gas chromatography
H_{II}	hexagonal phase
HEPES	4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid
HPLC	high-performance liquid chromatography
Lα	liquid crystalline phase
LcPUFA	long chain polyunsaturated fatty acid
MLV	multilamellar vesicle
MGDG	monoglycosyldiglyceride
NMR	nuclear magnetic resonance
n-AP	n-(9-anthroyloxy) palmitic acid
n-AS	n-(9-anthroyloxy) stearic acid
n-SASL	n-doxyl-stearic acid (n-(N-oxy-4', 4'-dimethyl-oxazolidin-2-yl) stearic acid)

ODPC	1-oleoyl, 2-docosahexaenoyl-phosphatidylcholine
ODPE	1-oleoyl, 2-docosahexaenoyl-phosphatidylethanolamine
OPPC	1-oleoyl, 2-palmitoyl-phosphatidylcholine
OSPC	1-oleoyl, 2-stearoyl-phosphatidylcholine
PA	phosphatidic acid
PC	phosphatidylcholine
PDPC	1-palmitoyl, 2-docosahexaenoyl-phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
POPC	1-palmitoyl, 2-oleoyl-phosphatidylcholine
POPE	1-palmitoyl, 2-oleoyl-phosphatidylethanolamine
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
$Q_{\|\|}$	cubic phase
R_{ss}	steady state fluorescence anisotropy
SDPC	1-stearoyl, 2-docosahexaenoyl-phosphatidylcholine
SFA	saturated fatty acid
SOPC	1-stearoyl-2-oleoyl-phosphatidylcholine
sn-1	stereospecific numbering

	at the 1st carbon atom in the glycerol backbone
sn-2	stereospecific numbering at the 2nd carbon atom in the glycerol backbone
T_h	temperature of the fluid-to-H_{II} phase transition
TLC	thin layer chromatography
T_m	temperature of the gel-to-fluid phase transition
TPL	total phospholipids
UFA	unsaturated fatty acid

Fatty acids (common name)	***Chain length:unsaturation***
Lauric acid	12:0
Miristic acid	14:0
Palmitic acid	16:0
Palmitoleic acid	16:1 (9-*cis*)
Stearic acid	18:0
Oleic acid	18:1 (9-*cis*)
Linoleic acid	18:2 (9-, 12-*cis*)
γ-Linolenic acid	18:3 (6-, 9-, 12-*cis*)
α-Linolenic acid	18:3 (9-, 12-, 15-*cis*)
Arachidonic acid	20:4 (5-, 8-, 11-, 14-*cis*)
Eicosapentaenoic acid	20:5 (5-, 8-, 11-, 14-*cis*)
Docosahexaenoic acid	22:6 (4-, 7-, 10-, 13-, 16-, 19-*cis*)

INTRODUCTION

Environmental temperature is one of the most important factors affecting the activity, and even the geographical distribution of living organisms.

Animals may avoid adverse temperatures by different ways. Some of them select constant thermal environments to live; these are the so-called cold or warm stenothermic species, while others tolerate large thermal fluctuations (eurythermic species). Among the eurythermic species some animals undergo hibernation in response to unfavourable thermal conditions (like frogs and some fresh water crustaceans) and some migrate to select the most suitable temperature milieu.

Poikilotherms are often exposed to fluctuations in the environmental temperature that might be diurnal, seasonal or even vertical. Membranes of these organisms are the first targets of temperature attack and changing their composition and physical properties according to the prevailing temperature is the common response of many species. It can be hypothesised that animals unable to inherit this kind of control can not survive under adverse thermal conditions.

To understand the temperature-induced responses and the strategies, that the different organisms apply to keep the physico-chemical properties of their membranes within certain limits, remains an intriguing task.

Understanding the process of adaptation of living organisms to varying temperatures at the level of the cell and cell membranes has been the subject of interest since many years. During this time, numerous studies revealed the importance of the physical state, referred as "fluidity", of the membranes in the process of adaptation. Currently, membrane fluidity was invoked as the factor controlling the expression of stress, particularly heat stress, related genes (Vigh *et al.*, 1998). How exactly the adapting

organisms compensate the direct effects of temperature on the level of membranes is still not clear and is under investigation. The basic concepts such as the homeoviscous and homeophasic character of membrane adaptation, used to describe the cell's response to altering temperatures rose from the studies on the bacterium *Escherichia coli.* In these studies a strong correlation between fatty acid unsaturation and growth temperature, as well as between growth temperature and membrane fluidity was found, and the decisive role of unsaturated fatty acids in fluidizing membranes at low temperatures was concluded. However, studies restricted to certain organisms did not give universal explanations for all aspects of adaptation phenomenon even at the level of membrane lipids.

The problem of membrane stability is particularly important for poikilotherms such as fish, whose body temperature varies over the wide range of changes in environmental temperature. Since the phase state and fluidity of membranes are extremely sensitive to temperature variations it is intriguing to study how fish can survive and function over a wide range of temperatures. Fishes had been studied intensively in the last decade and the principle of homeoviscous adaptation was tested in different species and tissues. These investigations were, however, restricted mainly on fatty acid composition analysis of the membrane lipids. The changes occurring at the level of phospholipid molecular species compositions and the need for such a wide range of lipids species remained unknown.

The physicochemical characteristics of the membranes are mostly under dietary or thermal influences therefore diet-induced alterations appearing in the lipid composition of the membranes may affect the temperature tolerance of a given organism. It has been shown that dietary lipids could affect the body temperature of lizards (*Amphybolurus nuchalis* and *Tiliqua rugosa*) in a way that unsaturated fatty acid rich diets reduced and saturated ones increased the body temperature (Geiser *et al.*, 1992, 1994). Studies showed that feeding various fish species with unsaturated fatty acids (UFA) resulted in their

accumulation in liver polar lipids (Olsen *et al.*, 1991; Schwalme, 1992; Bell *et al.*, 1991, 1994) and feeding rats with fish oil resulted an increase of polyunsaturated fatty acids (PUFA) in liver total phospholipids (Farkas *et al.*, 1994). As a response to temperature variation, homeoviscous adaptation (HVA) involves the control of the membrane lipid composition in such a way to ensure an optimal fluidity of the membranes. The possible interference of the dietary fatty acids in HVA is not known.

The body temperature of amphibians, like frogs, follows the changes in the environmental temperature but they undergo hibernation during winter. Whether these organisms regulate their membrane lipid composition and physical properties in the same manner as the winter-active fishes upon their seasonal adaptation or not, is not yet known.

The present thesis attempts to contribute to the answering of the above-mentioned questions, with the specific aim to refine the existing concept on the role of lipids in the process of temperature adaptation and to explain the need for specific molecules of phospholipids in a poikilothermic cell membrane.

AIMS OF THE STUDIES

Depending on their response to temperature change, living beings can be divided into two broad groups. They are either (1) Poikilotherms – their body temperature varies with the ambient temperature or (2) Homeotherms – they always maintain a constant body temperature. Therefore, the change of ambient temperature will pose a particularly serious challenge for the poikilotherms in maintaining cell integrity and cell functions.

Cell membranes are the first major cell components that encounter any change in the ambient temperature. As membranes have to maintain the cell functions at their possible optimum, it is mandatory that they should be able to restructure themselves to adapt to the new environmental temperatures. Till date it is not clear in details, however, how membranes of poikilotherms maintain their structural and functional integrity under temperature stress. One of the major classes of molecules that respond to the temperature change and are also shaping the physical characteristics of the membranes, are the lipid molecules. The last decade has seen a significant amount of study aiming to understand the mechanism and the role of the lipid molecules in the temperature adaptation of the membranes. Although, several discoveries have been made on the organization of the lipids in the membranes, still there is not any 'Global concept' that could describe their role in membrane adaptation to different temperatures.

On the basis of this accumulated knowledge, we aimed to carry on specific studies on the role of lipids in the temperature adaptation of poikilotherms. Experiments were designed using bacteria, fresh-water fish (all-year-round active) species and frogs (winter hibernators) to understand the role of the fatty acid unsaturation and molecular species composition in regulating membrane physical characteristics during temperature adaptation. The experimental approach was to combine the chemical analysis of temperature induced changes in the fatty acid and molecular species composition of the

major lipid classes with the study of the physical characteristics (in terms of membrane order and dynamics assessed by steady-state fluorescence anisotropy of fluorescent probes, and order parameter and rotational correlation time of spin labels) of native and lipid membranes.

In this view the objectives were as follows:

1. To investigate the relation between the fatty acid unsaturation and membrane order and dynamics in bacterial species (symbiotic bacteria of nematodes: *Photorhabdus luminescens*, *Xenorhabdus nematophilus*, and cyanobacteria *Cylindrospermopsis raciborskii*) grown at different temperatures.

2. To investigate the relation between the fatty acid unsaturation and/or phospholipid molecular species composition and membrane order in fish.

a) To assess the changes in the liver fatty acid and lipid molecular species composition of the fresh-water fish *Cyprinus carpio* L. in response to seasonal and short-term temperature adaptation.

b) To compare the physical characteristics of native and phospholipid membranes of livers of the fish *Cyprinus carpio* L. seasonally adapted or short-term acclimated to contrasting temperatures.

c) To correlate the changes occurring in the lipid composition with the physical characteristics of the membranes upon seasonal and short-term temperature adaptation of the fish *Cyprinus carpio* L.

3. To reveal the influence of dietary fatty acids in the process of temperature adaptation of fresh-water fishes.

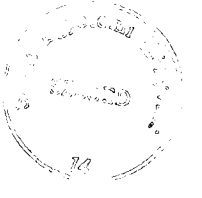
4. To characterize the seasonal adaptation of amphibians to contrasting temperatures at the level of phospholipids and lipid-membrane order.

a) To assess the changes in the liver fatty acid and molecular species composition of frogs upon transition from active to hibernation state.

b) To correlate the changes in the lipid composition with the fluidity of the vesicles made of phospholipid extracts of liver.

5. To confirm the regulatory potential of specific lipids on the fluidity of membranes on model systems of synthetic lipids.

6. With the synthesis of the results obtained from the experiments, to find generalities regarding the role of lipids in temperature adaptation of the membranes.



1. THEORETICAL BACKGROUND

1.1. Biological membranes: structure and dynamics

Biological membranes are delicately composed assemblies of lipids and proteins, and serve multiple functions in living organisms. They separate cells and cell compartments from their environment provide an effective barrier against large variety of compounds, and preserve the particular composition of the cells and compartments. On the other hand, membranes ensure communication with the same environment by passively or actively transporting materials into and out of the cell. Membranes are also the sites of many important cell functions; they act as reaction surfaces and present matrices for enzyme functions, signal and energy transduction. Because membranes perceive and mediate the external signals to the cell machinery, their role is crucial in the adaptation of the cell to the ever-changing external conditions. To accomplish all these functions, a biological membrane has to form a physically solid, yet dynamic entity, establishing dynamic equilibrium between its constituents and between itself and its environment.

Our present view of membrane structure and organisation has emerged from the *fluid mosaic model* of the biological membranes, proposed by Singer and Nicholson in 1972. This model pictures the membrane as a sea of lipids organised in a bilayer and the proteins as floating in the lipid sea. Since its proposal the model went trough a lot of refinements and, by now, numerous studies revealed the complexity and the diversity of the composition and the possible associations among the constituents of the membranes. Emerging aspects of this complex picture of membranes include the followings. Membrane proteins are various and concerning their localisation, can be peripheral, (bound to the membrane surface) or integral, (spanning through the hydrophobic core formed by the lipids). On the other hand, proteins may be present as monomers, or associated in di- or

trimers. The major membrane components, the lipids, represent the greatest diversity in the membranes. Due to their construction, a headgroup and two acyl chains attached to a glycerol backbone, and the possible variety and combination of these elements, (in a fish membrane nearly 10 different headgroups and about 20 different fatty acids) give rise to 100-200 different lipid molecules in certain membranes. It is interesting to note that this great diversity of lipids gives rise to a miscible composition in a membrane.

The above picture of membranes may be even more complicated by the presence of other membrane components, like cholesterol or by the presence of connections with the glycocalix and the cytoskeleton (e.g. in case of plasma membrane), that are also involved in membrane stability and functioning.

There are also other aspects of the membrane complexity, like the structural heterogeneity. This may include not only the transient existence of non-bilayer structures during membrane functioning (De Gier *et al.*, 1985) but also the recently demonstrated presence of lateral lipid domains (Schütz *et al.*, 2000).

Although the fluid-mosaic model of Singer and Nicholson oversimplified the membrane construction, the big merit of the model was to emphasise the fluidity of the structure (Singer, 1975) which should allow a great freedom for the molecules to move laterally, to rotate or to wobble (Cherry, 1979) without disturbing the integrity of the membrane.

1.1.1. Membrane lipids

Phospholipids are the major components of the membranes of animals and they form, together with sterols and glycolipids, the matrix of the membrane. Phospholipid molecules consist of a polar head group esterified to the phosphoric acid of a

phosphoglyceride molecule to which two, non-polar fatty acids (FA) are esterified on the glycerol side. Phospholipids are classified according to the type of the head group that is esterified to the phosphoric acid. Among the phospholipids of the eukaryotic cells the most abundant class is phosphatidylcholine (PC) (about 60%) which is followed by phosphatidylethanolamine (PE) (about 20 %). Besides these two classes, phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG) present in a tissue-specific manner.

In a poikilothermic membrane about 20 different fatty acids can be identified in average. These fatty acids are esterified to lipid molecules in pairs, and give rise to more than 20-25 different molecular species within a phospholipid class like PC or PE. This means that, in poikilotherm's membranes roughly 150 different lipid molecules can be present. At present, the physiological reasons underlying this wide diversity of lipid molecular species are still unclear. The importance of the different phospholipid classes, subclasses and molecular species might be deduced from the existence of specific membrane lipid compositions that are highly preserved under different physiological conditions.

Bacterial cell membranes differ from the animal membranes in that they contain generally higher amounts of PE in their phospholipids than PC, and that the fatty acid (FA) composition of the lipids is less diverse. The majority of bacterial FA-s is short-chain and is ranging from 12 to only 18 carbon atoms in length.

Differing from the animal membranes, the photosynthetic membranes possess galactolipids as major lipids. In these membranes, about 50% of the lipids are monogalactosyl diacylglycerols (MGDG), followed in abundance by digalactosyl diacylglycerols (DGDG), sulphoquinovosyl diacylglycerols (SDG) and

phosphatidylglycerols (PG). The maximum lengths of the FA-s of these lipids are also found to be of 18 carbon atoms.

Being amphiphilic molecules, lipids, when dispersed in water can spontaneously form bilayer structures, with the hydrocarbon core sandwiched between the two layers of hydrated head groups. Lipid bilayers are very elastic and at the same time very stable structures. The stability of a lipid bilayer is determined by the hydrophobic, Van der Waals attractive forces involved in maintaining the structure of the fatty acid chains, by the electrostatic interactions and by hydrogen bonds between the polar head groups and the water molecules. The study of lipid membranes is closely related to the study of liquid crystals (Seelig, 1976). Liquid crystals are substances exhibiting both liquid and solid properties. They may be as fluid as water but at the same time the constituent molecules are ordered along a local axis, the director. This is also a characteristic of lipid bilayers, which can be regarded as an orientationally anisotropic two-dimensional fluid or smectic liquid crystal.

1.1.2. Phase state of membrane lipids

Lipids are polymorphic and can assume different phase states depending on the temperature, on the pressure, on the degree of hydration and on the chemical composition of the lipid itself. Three kinds of major phases had been identified in phospholipids and in phospholipid mixtures of biological origin: the lamellar, the hexagonal and the cubic phases. Transitions between these phases can be driven by changes in the temperature.

Lamellar phase spans a broad temperature range, however, a variety of lamellar phases can appear depending on the actual temperature. At low temperatures, the lamellar gel phase (L_β) predominates in which the fatty acyl chains are fully extended, adopting

mostly *all-trans* configuration with restricted mobility resulting in a highly ordered chain packing. Gel phases can also exhibit structural diversity: the fatty acyl chains can be tilted with respect to the bilayer normal ($L_{\beta'}$), the lamellae can be rippled with tilted chains ($P_{\beta'}$) or in the case of mixed-chain phospholipids, an interdigitated phase ($L_{\beta\bullet}$) can appear.

At higher temperatures the acyl chains melt and form a fluid lamellar or liquid crystalline phase (L_α). The transition from the gel to liquid crystalline phase is called the main phase transition and occurs at a specific temperature noted as T_m. In the fluid phase there is a pronounced loss of membrane order, a reduction of the bilayer thickness and a net increase in the volume per lipid molecule. This is the consequence of the large increase of the amount of *gauche* conformers at the expense of the formerly dominating *all-trans* conformers upon the phase transition from gel to liquid crystalline state.

Although the lamellar, liquid-crystalline phase state predominates in biological membranes throughout the physiological conditions, nonlamellar structures can be formed transiently during biologically important processes like membrane fusion (Ellens *et al.*, 1989), or at the extremes of the normal physiological conditions (De Gier *et al.*, 1985).

1.1.3. Orientational order and packing in membrane systems

The orientation of the molecules in a membrane bilayer is more probable to be perpendicular to the plane of the membrane than being parallel. Thus it can be assumed that the bilayer normal is an axis of certain symmetry for the molecules and that they are essentially cylindrically symmetric. As a result, the orientational distribution function, f (θ), for a lipid or a lipid-like probe molecule will depend only on the angle θ between the membrane normal and the molecular axis and will give the probability of finding a molecule oriented with its long axis at an angle θ relative to the membrane normal.

Orientational order (and dynamics) in membrane systems are studied mainly with probe techniques (fluorescence depolarisation or electron spin resonance spectroscopy) that monitor the behaviour of extraneous, lipid-like, probe molecules embedded in the membrane structure at the smallest possible concentration compatible with an acceptable signal/noise ratio. It is assumed that each probe molecule experiences a local orienting potential imposed on it by the surrounding lipid molecules and the orientational distribution function will be given by the Boltzmann distribution corresponding to this orienting potential. In the interpretation of fluorescence depolarisation measurements, the form of the potential is generally assumed to be a square-well with infinite walls at an arbitrary angle θ_0 ($0 \leq \theta_0 \leq \pi/2$) (wobble-in-cone model). On the other hand the electron spin resonance spectroscopy uses the Brownian diffusion model were the form of the orienting and potential and orientational distribution function $f(\theta)$, is given as an infinite series of Legendre polynomials of the angle θ. The moments of the expansion are called *order parameters*. The commonly used order parameter, S, is identical to the lowest moment, $\langle P_2 \rangle$ that has the expression:

$$\langle P_2 \rangle = \langle 3\cos^2\theta - 1 \rangle / 2 = S$$

The angled bracket $\langle \ldots \rangle$ denote an average over the distribution. To characterise the general form of $f(\theta)$, the minimum knowledge of the two lowest moments $\langle P_2 \rangle$ and $\langle P_4 \rangle$ ($\langle P_4 \rangle = \langle 35\cos^2\theta - 30\cos^2\theta + 3 \rangle / 8$) are required that are accessible experimentally in a model independent way. It has to be emphasised that the orientational distribution function defines a static equilibrium, a time-averaged orientation of the molecules and does not contain information about their dynamic behaviour. The orientational order is connected to the molecular packing in the membrane, packing being the number of molecules per unit area. If the orientational order is high the lipid chains are extended and tightly packed, if

the order is low, the chains have irregular conformation and thus occupy a larger volume and surface area.

1.1.4. Molecular dynamics in membrane systems

Different kind of molecular motions in the membranes are dynamic processes, which can be described by diffusion coefficients, correlation times or frequencies. The motional properties of the major membrane components such as proteins and lipids are rather complex and the frequencies of intra- and intermolecular motions range over many orders of magnitude in the liquid-crystalline state. Typical time scales for the various motions are indicated in Table 1 (Marsh, 1988).

Table 1. Typical time scales for the motion of membrane components in a fluid-lipid environment

	D_T (cm^2sec^{-1})	ν_j (sec^{-1})	$D_{R\|\|}$ (sec^{-1})	$D_{R\perp}$ (sec^{-1})	τ_j^{-1} (sec^{-1})	τ_f^{-1} (sec^{-1})
Protein	10^{-8}	_	10^5	~0	_	~0
Lipid	10^{-7}	10^8	10^9	10^9	10^{10}	10^{-5}

D_T and ν_j refer to the translational diffusion of the proteins and the lipids, respectively. $D_{R||}$ and $D_{R\perp}$ refer to rotational diffusion. τ_j^{-1} refers to the *trans-gauche* isomerisation in the lipid chains, and τ_f^{-1} refers to the trans-membrane flip-flop of the lipid molecules (Marsh, 1988).

Considering the lipid hydrocarbon chains, the intramolecular -CH_2 bond vibrations are the fastest motions with frequencies above 10^{14} Hz, being followed by the CH_2-CH_2 bond rotations that give rise to the *trans-gauche* isomerization of 10^9-10^{11} Hz depending on the position of the segment in the chain. The lipid molecules can rotate around their long molecular axis and can perform limited rotations around their perpendicular axis, within the plane of the membrane, both motion have diffusion coefficients of 10^9 Hz.

Intermolecular motions, such as the lateral diffusion and trans-bilayer flip-flop jumps of lipids, are slower with exchange frequencies of 10^{7} Hz and 10^{-5} Hz, respectively.

The dynamic processes can only be studied with techniques whose intrinsic time scale is comparable to the time scale of the processes themselves. The wide range of motional modes can not be covered entirely by a single physical technique of study. Every physical measurement has its own inherent time scale and relative to that the rate of a conformational change can be fast or slow. If the motion is slower than the time-window of the technique, the gathered data will represent a distribution of instantaneous events, while if the motion is faster than the time scale of the technique a flash photo of the process is obtained. Therefore, when choosing a technique for the study of certain motion, it is very important to take into consideration the time scale factor.

1.2. Techniques to measure lipid order, dynamics and phase states in biological membranes

The most common techniques to study the orientational order in membranes are nuclear magnetic resonance (NMR), electron spin resonance (ESR), polarised fluorescence, polarised Raman and linear dichroism spectroscopies. The common basis for the use of these techniques is the anisotropic interaction of certain molecules in the bilayer with an externally applied field. Thus, these interactions depend on the molecular orientation relative to the bilayer normal and yield information about the orientational distribution of the molecules.

Vibrational spectroscopic techniques such as Raman and infrared (IR) spectroscopy, are sensitive to rapid (10^{13}-10^{15} Hz) motions, can detect bond vibrations but are rather insensitive to large-scale intra- and intermolecular motions. The frequencies of the -νCH_2 stretching vibrations are sensitive to the conformation of the fatty acids by

responding to changes in the ratio of the *trans/gauche* segments of the acyl chains. Therefore, this technique is suitable to monitor temperature induced conformations of the hydrocarbon chains, and also to quantify the distribution and localization of specific rotational conformers whereas no direct information on motional rates of the whole lipid molecule could be obtained.

Deuterium nuclear magnetic resonance spectroscopy (^{2}H-NMR) is sensitive to relatively slow motions (10^3-10^6 Hz) of the lipid hydrocarbon chains. The technique requires the introduction of exogenous pre-deuterated fatty acids into the membrane lipids. From spectral line shape analysis, the time averaged carbon-deuterium bond orientational order parameter and the hydrocarbon chain segment motion rates can be determined without ambiguity. Since gel and liquid-crystalline lipid domains have different line shapes, this technique can also be used to monitor lipid chain-melting phase transition (Davis, 1983).

Due to their intrinsic time scale, ESR and polarised fluorescence techniques are particularly suitable for studying the dynamics of the lipid molecules. The intrinsic time scale of the polarised fluorescence experiments is determined by the lifetime of the excited state (about 10^{-9} s) and in the magnetic resonance experiments this is determined by the anisotropy of the magnetic interactions (10^{-7} - 10^{-9} s). This time scale covers the range of fatty acid chain rotational motions, no wonder they are the most widely used methods in membrane studies. These two techniques were also used in the present thesis and are discussed below in more details.

Membrane phase-state had been measured by both ESR and fluorescence polarisation techniques, however, differential scanning calorimetry (DSC) is superior to these techniques. This technique does not use any probe as a reference and accurately determines the temperature, enthalpy and entropy accompanying lipid phase transitions.

Nevertheless, because DSC operates on long time scale, it can not provide direct information on the dynamics of the membrane order and rates of the hydrocarbon chain motions.

1.2.1 Fluorescence depolarisation spectroscopy

In the last decades fluorescence polarisation measurements have yielded valuable information about lipid structure and dynamics in membranes. In these techniques the rate of depolarisation of apolar fluorophores embedded in the membranes is measured upon excitation with polarised monochromatic light. In the steady-state measurements the emission of the fluorophore is measured under continuous illumination. The steady-state fluorescence polarisation (P) and anisotropy (R_{ss}) are determined by the emission intensities measured through an analyser, oriented parallel (I_V) and perpendicular (I_H) to the direction of polarisation of the exciting light, as follows (Lakovitz, 1983):

$P= (I_V-I_H)/(I_V+I_H)$;

$R_{ss}=(I_V-I_H)/(I_V+2I_H)$

From the two expressions, it follows that the two parameters are closely related, $R_{ss}=2P/(3-P)$, and both express the degree of polarisation of the light. However, anisotropy is the one used more frequently as it characterises the light emitted by the fluorophore. The extent of the depolarisation of the emission of the fluorophore in the membrane reflects the degree to which a population of photoselected excited fluorophores loses its initial selective orientation and becomes randomised. Until the end of 1970s the steady-state anisotropy measurements had been interpreted in terms of "microviscosity" where the rate of the rotational diffusion of the probe was thought to reflect the viscous drag imposed by its local apolar environment (reviewed in Shinitzky and Barenholz, 1978). The apparent "microviscosity" of the membrane interior was estimated by comparing the measured

fluorescence anisotropy with that of obtained in reference mineral oils by using the classical hydrodynamic expressions of Perrin. Time resolved fluorescence decay measurements revealed, however, that the steady-state fluorescence anisotropy measured in lipid membranes is determined by the cone-angle to which the rotation of the fluorophore is restricted by the molecular packing of the lipids (a static factor), rather than by its rotational rate (a dynamic factor). In the time-resolved measurements a photo-selected group of excited rod-like fluorophores is created at time zero by a flash of polarised light and the decay of the emitted polarised fluorescence is followed in time. According to the measurements performed on lipid membranes, the anisotropy decay is exponentially approaching a limiting value, r_∞. Assuming a single exponential decay for the total fluorescence intensity, the steady-state fluorescence anisotropy can be expressed by two separate components, a fast decaying dynamic part, r_f and an almost constant part, r_∞:

$$r_s = r_f + r_\infty$$

where r_f is related to both fluorescence lifetime τ and rotational relaxation time of the reorientation of the long molecular axis φ of the fluorophore:

$$r_f = (r_0 - r_\infty)/(1+\tau/\varphi)$$

where r_0 is the maximal fluorescence anisotropy corresponding to the all parallel orientation of the emitting dipoles i.e. absence of any rotational motion of the fluorophore, φ is the rotational correlation time for the reorientation of the long molecular axis, r_∞ is the non-zero limiting anisotropy of the fluorophore in the membranes due to the final anisotropic distribution of emitting dipoles. This latter phenomenon reflects the structural order in membranes. Thus the dynamic part, r_f is in turn proportional to the microviscosity while r_∞ is proportional to the square of the lipid-order parameter (Hildenbrand and Nicolau, 1979; Heyn, 1979; Jähnig, 1979). The relative contribution of the two

components to the steady-state anisotropy is determined by r_∞ and the ratio τ/φ. In biological membranes, however, the contribution of r_∞ is predominant. The value of r_s will be in between the values for r_0 and r_∞ and will be positioned by the ratio φ/τ. Van Blittersvijk *et al.* (1981) compiled a set of experimental data for fluorescence anisotropies obtained in a variety of artificial and biological membranes, to establish a relation between r_s and the limiting anisotropy r_∞. They found that the contribution from r_∞ to r_s was zero for very small r_s values and was increasing with increasing values of r_s, approaching 100% for the theoretically maximum value of r_s=0.4. In the region of $0.13<r_s<0.28$ the relation between r_s and r_∞ was linear corresponding to the formula: $r_\infty = (4/3)r_s - 0.1$. Thus, from steady-state measurements the lipid order parameter could be calculated.

1.2.2. Electron spin resonance spectroscopy of membranes

Electron spin resonance (ESR) spectroscopy detects transitions between the Zeeman levels of paramagnetic molecules situated in static magnetic field. Transitions between these levels may occur if the electrons can absorb exactly the amount of energy, which separates the Zeeman levels. These transitions may be achieved by applying an additional oscillating magnetic field. When the resonance conditions are met with this field the transition from the lower to the higher Zeeman level occurs by absorption of the oscillating magnetic field energy. The absorption line shape is determined by the intra- and intermolecular interactions of the magnetic dipole with its local environment.

The natural occurrence of paramagnetism in biological systems is very low, and is limited to the occurrence of transition metal ions or free radicals produced by external radiations. However, the introduction of external paramagnetic molecules can make possible the study of such systems. Spin labelling electron spin resonance spectroscopy is widely used to report the physical state of the biological membranes. This technique

introduces stable, paramagnetic molecules into the membrane and records their resonance absorption characteristics. The spectral parameters, -such as the position and the widths of the hyperfine lines- of the spectrum depend on the rate and the amplitude of the rotational motion of the probe molecule in its particular environment. Consequently, when incorporated into native or artificial membranes, information about the relative conformational freedom of the membrane components can be obtained (Griffits and Jost, 1976).

The spin labelled molecules used in membrane studies are stable nitroxide free radicals that are usually synthetic analogues of fatty acids or natural lipids (Marsh, 1985). The resonance absorption is detected between the Zeeman levels of the unpaired electron of the N-O bond in the spin labelled molecule and the ESR spectrum consists of three lines due to the interaction between the spins of the electron and the nucleus of the ^{14}N atom. The use of lipids or fatty acids that are labelled at different carbon atoms along the hydrocarbon chain, made possible the study of the molecular mobility at various depths of the membrane, with a resolution of 1-1.3Å, (Páli *et al.*, 1992; Bartucci *et al.*, 1993). The time window of the conventional ESR spectroscopy is determined by the spectral anisotropy of the nitroxide group and it ranges between 10^7-10^{11}Hz. When the molecular re-orientational motion is sufficiently slow, the ESR spectrum of randomly oriented molecules consists of a set of overlapping spectral lines corresponding to different orientations. As the molecular tumbling rate increases, the anisotropic line shape transforms to a single, motionally averaged, isotropic spectrum. The transition from the one to the other takes place at a well-defined limiting frequency of $3x10^8$ sec^{-1} (McConnell, 1976). Above and below the limiting frequency, the motional range is divided into so called fast and slow motional regimes being controlled by different physical processes that determine the shape of the spectrum.

The 5-doxilstearic acid spin probe incorporated into lipid vesicles gives a spectrum with well separated outer hyperfine component, $2A_{max}$, of the hyperfine tensor, where A_{max} is defined as one half of the separation of the outer hyperfine extrema (Figure 1).

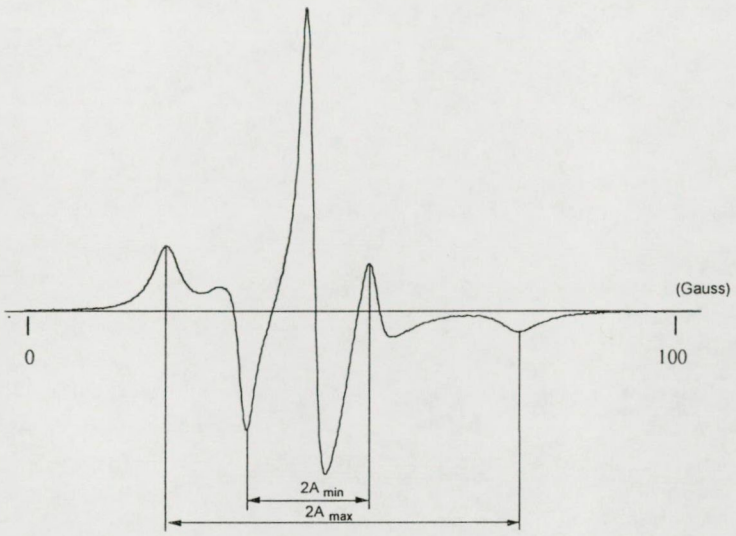


Figure 1. Representative experimental spectrum of 5-SASL-spin probe incorporated into lipid (PDPC) vesicles, recorded at room temperature. The outer and inner hyperfine splitting extrema, $2A_{min}$ and $2A_{max}$, are indicated.

The magnitude of this spectral parameter decreases monotonically from its rigid limit value as the motion of the spin probe becomes more rapid (Freed, 1976) as for example when the temperature is increased (Figure 2).

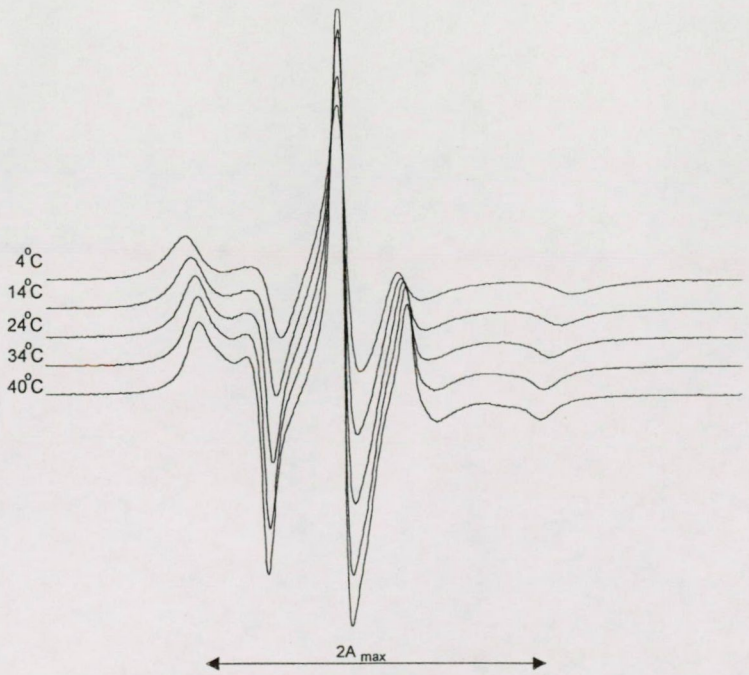


Figure 2. Representative experimental spectra of 5-SASL-spin probe incorporated into lipid (PDPC) vesicles, recorded at different temperatures showing the decrease of the magnitude of the outer hyperfine splitting $2A_{max}$ as the temperature increases.

Therefore, $2A_{max}$ can be used to characterise the motional freedom of the probe molecule in a given environment and consequently will also give information, indirectly, about the so-called "fluidity" of the membranes. When the motion is fast but restricted the angular amplitude of the wobbling motion of the probe molecule along its long axis is directly related to the order parameter which can be calculated according to the relation (Seelig, 1976) :

$$S_{zz}= (A_{max}-A_{min})/(A_{zz}-0.5(A_{xx}+A_{yy}))$$

where A_{xx}, A_{yy} and A_{zz} represent the hyperfine splitting values of the spin label oriented in a single crystal host with its x, y and z molecular axes parallel to the external magnetic field. In the case of spin labelled stearic acids the denominator in the above equation is equal to 27.55 Gauss. The spectral parameters, A_{max} and A_{min} are indicated in Figure1.

In the studies where the membrane order and changes in the phase state are to be monitored, usually the order parameter is plotted against the temperature. In the cases where the inner hyperfine splitting, $2A_{min}$ can not be measured, $2A_{max}$ is used to be plotted without evaluating the order parameter (Hubbell and McConnell, 1971) to give information about the above-mentioned parameters of the membranes.

The rate of rotational motion of spin labels incorporated in a membrane can be characterised by the rotational correlation time, $\tau(r)$. In this case shorter correlation time means faster motion, i.e. a more fluid structures of the membrane and vice versa. In the case of fast motions $\tau(r)$ can be calculated from the spectral parameters according to the relation of Kivelson (1960):

$$\tau(r)=0.65 \times W_0[(h_0/h_{-1})^{1/2}-(h_0/h_{+1})^{1/2}]$$

Where W_0, h_0, h_{-1} and h_{+1} are spectral parameters that are indicated in the Figure 3.

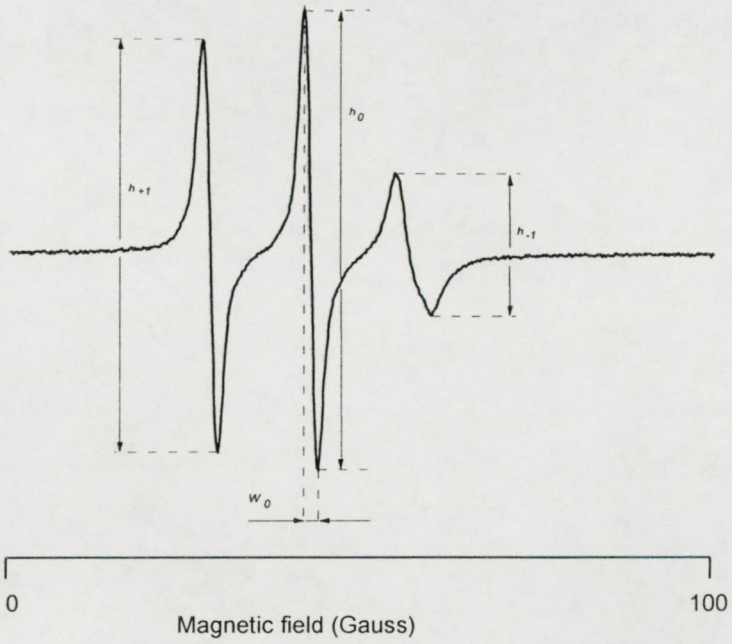


Figure 3. Representative experimental spectra of 16-SASL-spin label incorporated into vesicles made of total phospholipid extracts of fish liver, recorded at room temperature. The spectral parameters used in the calculation of the rotational correlation time, $\tau(r)$ are indicated on the figure.

Membrane phase-transitions are manifested by changes in both the amplitude and the rate of the motion of the membrane molecules, thus the spin label ESR method is well suited to study phase transitions in membranes. The characteristic spectral parameters change abruptly at phase transition temperatures in pure lipid systems (Hubbell and McConnell, 1971). Natural membranes having a more heterogeneous composition do not show sharp phase transitions. However, characteristic points in the temperature dependence of the order parameter or the rotational correlation time can still be used to identify phase transitions (Pesti *et al.*, 1985).

1.2.3. Membrane "fluidity"

"Membrane fluidity" is a widely used concept in membrane research (Shinitzky, 1984) however, it lacks a precise definition. This ambiguity is due to the fact that the membrane differs from isotropic fluids where the motional freedom of the particles can be described with a single parameter, the fluidity, being equal to the reciprocal of viscosity.

The membranes are anisotropic two-dimensional fluids, with heterogeneous composition and the motional mobility of molecules could quantitatively be described by only a set of parameters.

At present it can be said that "membrane fluidity" is an operationally defined term and it is quantified by using one of the several biophysical techniques presented above, that measures the rotational characteristics of probe molecules. Thus each technique will provide information specifically about the type of motion which affects the spectroscopic property in question and thus reflects different aspects of the "fluid" condition i.e. of the molecular motional restriction. Two important notes has to be made here: The first is that the data obtained by one technique may not necessarily agree with those obtained by another technique, and the second is that for a more complete characterization of membrane "fluidity", it is advisable to use two or more techniques in parallel.

In the study of the thermal adaptation, generally, the lipid intramolecular motional rates and ordering, referred as "fluidity", and the phase-state of membranes are found to be regulated in a compensatory manner by the organisms. These physical characteristics were measured in the last decades using different spectroscopic and calorimetric methods. Comparing the data obtained by different techniques on fluidity or phase state during temperature adaptation, some techniques yield qualitatively the same or complementary information, while others gave contradictory results. A substantial amount of experimental data has been accumulated on prokaryotes, and based on these a critical evaluation of the suitability of different biophysical techniques to monitor membrane fluidity and phase states was made by McElhaney in 1994. He concludes that non-perturbing spectroscopic techniques like ^{2}H-NMR, FTIR or calorimetric methods are more reliable in measuring motional rates, order parameter, or phase state than those utilising probe molecules, like fluorescence polarisation or ESR spectroscopy, questioning especially the latter. However,

a similar comparison and evaluation of techniques are not possible on eukaryotes since there are not enough experimental data. The use of extrinsic probe molecules is based on the assumption that they distribute uniformly in the membrane, do not disturb the membrane characteristics at the concentrations used (1:100-200 probe:lipid), and that they reflect accurately membrane properties. It is necessary to recognise that probe techniques generally provide an average of the probe positions thus the membrane is treated as a single hydrophobic phase although microdomains may be present. The common probes used, like 1,6, -diphenyl-1, 3,5-hexatriene (DPH) or the nitroxy labelled fatty acid spin probes are not able to differentiate between microdomains and yield a weighted average of the probe position. With the introduction of localised fluorescence probes like the anionic and cationic derivative of DPH, 3(p (6-phenyl) 1,3,5-hexaqtrienyl) propionic acid (DPH-PA) and trimethylammonium DPH (TMA-DPH) this problem could be partly overcome as they localise in the outer leaflet of the membrane. A vertical differentiation of the membrane properties was made possible by the use of n- (9-anthroyloxy) stearic and palmitic acid fluorescence probes and n-doxylstearic acid spin probes. These are based upon a fatty acid chain and localised within the membrane with their carboxyl end at the membrane water interface and their terminal methyl group being toward the core of the bilayer (Cooper and Meddings, 1991).

1.3. Mechanisms of temperature compensation in membranes

The physical properties of the lipid molecules and of the lipid assemblies are exquisitely sensitive to changes in temperature. In fact, temperature determines the rates of the molecular motions of all membrane constituents and the phase state and order of the membrane lipids. A change in the temperature will have thus a direct and prompt effect on any membrane. Therefore, temperature-induced perturbations of the membrane

organisation pose a serious challenge to the maintenance of its physiological functions. This holds especially for aquatic poikilotherms because, the temperature of their body follows the ambient water temperature, for most gill breathers, within 1°C (Block, 1991). Thus, in these animals, the change of ambient temperature perturbs numerous cellular activities, which are dependent on the membrane structure and function. To offset the direct effects of the temperature on membranes over the wide range of body temperatures, poikilothermic organisms must make compensatory adjustments in the molecular (especially lipid) compositions of their membranes. The most common adaptation method in poikilothermic animals is the introduction of lipids matching the physical properties required by the membranes for normal functioning at the altered temperatures (reviewed by Williams and Hazel, 1994).

1.3.1. Homeoviscous and homeophasic adaptation

In 1974, using ESR spectroscopy and 12-doxylstearic acid spin probe, Sinensky reported that the fluidity of the vesicles made of extracted membrane lipids, of cells of *Escherichia coli* grown at 43°C was lower than that of lipids from the 15°C grown cells. Moreover, when compared at the culturing temperatures, the fluidities were similar. He termed this maintenance of similar membrane fluidity at different growth temperatures as *Homeoviscous adaptation.* The same phenomenon was reported by Cossins and Prosser (1978) and by Cossins *et al.* (1987) in synaptosomal membranes of various fishes and rats. They found that the polarisation values of DPH were inversely related with the cell or body temperature, and when compared at the respective body or cell temperatures these values were nearly equivalent. Since then the compensatory regulation of the membrane fluidity upon temperature variation had been reported in many poikilothermic species both in genotype comparisons and in phenotypic thermal acclimation, although with different

homeoviscous efficacy (homeoviscous efficacy is defined as the observed change in the gel-to-liquid-crystalline lipid phase transition temperature due to altered growth temperature, relative to the environmental temperature change imposed), ranging between 20-100 %.

As the perturbation of membrane structure is more severe during a phase transition, McElhaney (1984) proposed that the homeoviscous response in fact serve the prevention of a phase transition. In studies made on *E. coli,* he found that the growth and the cell functions were not perturbed over a wide range of temperature, as long as at least 50% of lipids remained in liquid state. He proposed that *homeophasic adaptation* is the primary means of normal growth at different temperatures for microorganisms. Numerous examples were further accumulated which confirmed the adaptive alterations of the membrane lipid phase behaviour in microorganisms. For example, in *Anacystis nidulans* the onset of the phase separation in plasma membranes was shifted from 5°C to 16°C in the cells grown at 28 and 38°C, respectively. In the same manner, the region of the phase separation of the thylakoid membranes, found between 0 and 26°C in cells grown at 38°C was shifted to between –5 and 16°C in the cells grown at 28°C (Furtado *et al.*, 1979; Murata, 1989).

In poikilotherms, the phase transition is more difficult to assess. In few cases where phase transitions have been detected acclimatory responses had also been reported (Farkas *et al.*, 1984). What became clear from these studies is that the normal physiological conditions of functioning require fluid state in a membrane and in these conditions the fluid-to-gel phase transition temperature of the membrane lipids should be well below the physiological temperature range. In more recent studies it is becoming evident that not only the main phase transition temperature but also the bilayer-to-hexagonal phase transition temperature is regulated in the same adaptive manner. In this sense, the cells

seem to try to maintain an optimal ratio between the lamellar and non-lamellar structure-forming lipids (Rietveld, 1993; Rilfors, 1993). The regulation of the acyl chain composition would be such to enable microorganisms to grow in a window between the lamellar gel phase and non-lamellar phases (Morein, 1996).

1.3.2. Acyl chain restructuring

The best-documented phenomenon accompanying the temperature acclimation or adaptation of both lower and higher organisms (reviewed by Hazel, 1989; Orr and Raison, 1990; Russel and Fukunaga, 1990) is the alteration of the lipid acyl chain composition *per se*. In these studies the total fatty acid composition of either a single lipid class or, a total lipid extract from an isolated cell membrane, from a tissue, from a whole organ or from an organism were analysed. It has been generally observed that the reduction of the proportion of saturated fatty acids (SFA-s) and a corresponding increase in the proportion of unsaturated fatty acids (UFA-s) was the response to the lowered temperature. Compiling a set of data obtained on a variety of metazoan poikilotherms, Hazel (1988) established the following generalities: (i) SFA levels decline 19 % on average, for a 20°C drop in growth temperature, resulting in a 1.3- to 1.4-fold increase in the unsaturation ratio (UFA/SFA); (ii) the unsaturation ratio of membrane phospholipids is inversely related with cell or acclimation temperature and (iii) the strength of this correlation varies with the type of the membrane, being strongest for the mitochondria then for microsomes and finally for synaptosomes; (iv) UFA-s that accumulate during cold adaptation may be either monoens or polyenes (PUFA), and the adjustments of PUFA levels are predominating in winter-active species.

Cause and effect relationships between temperature-induced changes in acyl chain unsaturation and specific aspects of membrane structure and function have not been rigorously demonstrated.

1.3.3. Phospholipid molecular species restructuring

The majority of the studies conducted to understand the role of lipids in adaptation of the membranes to different temperatures were focused on the analysis of the gross fatty acid composition of the membranes and on correlating the observed changes with the physical and functional adjustments that took place. However, fatty acids can be mostly present in the membranes only as integral parts of lipid molecules. The free fatty acid content of membranes is approximately only 4%. The glycerol backbone in a lipid molecule can accommodate two acyl chains in two different positions, termed *sn-1* and *sn-2* positions. Considering that in average 20 different fatty acids can be identified in a poikilothermic membrane, theoretically 20^2 types of different molecules are possible to occur with a single type of headgroup. The specificity (the position, length and saturation) of the lipid acyl chains and the type of the headgroup will all together define the unique feature (structure and physical properties) of a given phospholipid molecule. By this way, each phospholipid species will have a defined capacity and role in adapting membrane structure and functions to the ambient thermal conditions. Thus, when assessing the role of lipids in regulating membrane physical properties and functions, the lipid as an entire molecule has to be considered.

In aquatic poikilotherms about 20-25 different molecular species can be identified in either PC or PE fractions (Farkas *et al.*, 1994). Among them some are abundant, like 16:0/22:6, 16:0/20:4, 18:0/22:6, 18:0/20:4 or 16:0/18:1, while others are present only in low amounts. The pattern of the molecular species composition is sensitive to the

temperature. Similar changes were found in the molecular species composition of PE-s from livers of marine and fresh water fishes evolutionary adapted or seasonally acclimated to differing temperatures. In both cases the amount of 18:1/22:6 and 18:1/20:5 PE was elevated 3 to 4 fold in the cold (Dey *et al.*, 1993a). It was also shown that the pattern of the molecular species changed similarly in case of short-term acclimation process in fish liver and brain, as well (Dey *et al.*, 1993b; Buda *et al.*, 1994).

An interesting and probably very important feature of the molecular species restructuring is the fast "reshuffling", proceeding with a shift in ambient temperature (Thompson, 1989). This means the appearance of new molecular species of phospholipids without altering the fatty acid composition of the membrane. The reshuffling was first illustrated in thermal adaptation on microsomal membranes of *Tetrahymena,* where a rapid fluidisation was observed by DPH fluorescence depolarisation during adaptation from 39°C to 5°C, even before the gross changes appeared in the level of the acyl chain unsaturation (Ramesha and Thompson, 1983). Similarly, cold acclimation from 30°C to 12°C of the green alga, *Dunailella,* led to a rapid increase in di-unsaturated species of PG and reduced proportions of the mixed species with one saturated and one unsaturated chain (Lynch, 1984). Rapid changes in the molecular species pattern have been observed in a Sonoran desert teleost, *Agosia*, as a response to diurnal warming. The percentage of the di-unsaturated PC species declined from 7.23% to 2.46 % in muscle microsomes, between cool morning and warm afternoon that represented a difference of 13°C in the ambient temperature (Carey, 1989). Similarly, when rainbow trout underwent acclimation from 20°C to 5°C, the amount of the disaturated and monoenic molecular species of PC decreased significantly in renal plasma membranes within 8h and 16 h after the temperature change, respectively (Hazel, 1988).

The restructuring of the molecular species seems to be a tool for rapid adjustments in the membrane composition. Because it does not require input from the lipid biosynthetic pathway, it is metabolically inexpensive and probably the only alternative for the restoration of membrane functions when low temperature effectively stops net phospholipid synthesis (Thompson, 1989).

The potency of special lipids to ensure membrane integrity and the need for such a diversity of molecular species in poikilotherms is still not well understood.

1.3.4. Phospholipid polar headgroup restructuring

Among the lipids of the animal membranes, the polar lipids form the major lipid class. Their major representatives are phosphatidylcholines (50-60%) and phosphatidylethanolamines (20-30%) (Hazel, 1979). In the membranes of animals, apart from PC and PE the other polar lipids are phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), sphingomyelin (SM), lyso-phophatidylcholine (LPC) and cardiolipin (CL). The relative percent of the polar headgroup components varies from membrane to membrane and from organism to organism.

In general, the membranes of cold-adapted poikilotherms have more PE and reduced amounts of PC than their warm-adapted counterparts. The ratio of PC to PE tends to be positively correlated with cell or body temperature in specific membranes (Anderson, 1970; Miller *et al.*, 1976; Wodtke, 1978; Christiansen, 1984; Hazel, 1984; Chang and Roots, 1988; Hazel and Landrey, 1988; Pruitt, 1988; Gulik *et al.*, 1990; Sorensen, 1990) but it is less evident in total tissue phospholipid extracts (Hazel, 1989). Furthermore, for rainbow trout undergoing acclimation from 20°C to 5°C, the PC/PE ratio halved within 8 h after the temperature was decreased in renal plasma membranes, indicating that the adjustments of headgroup composition can occur rapidly (Hazel and Landrey, 1988). It is

also interesting to note that in a comparison of winter-active and winter-quiescent crustaceans, only the winter-active species displayed increased proportions of PE (Pruitt, 1988).

Temperature induced alterations in the polar headgroup composition are also common in microorganisms. Over the entire temperature range of bacterial growth there is a general increase in glycolipid content with the increase of the growth temperature (Russell and Fukunga, 1990). Increased ratios of monoglucosyl- to diglucosyldiglyceride are also common features of alga *Dunailella* (Thompson, 1989) and *Acholeplasma* (Rilfors *et al.*, 1984).

1.3.5. Cholesterol modulation

Cholesterol is a surface-active steroid that is readily accommodated in most membranes at least up to molar ratios of 1:1, relative to the phospholipids. Cholesterol lies parallel to and buried within the acyl domain of the membrane. The steroid molecule adopts a conical shape due to the small area occupied by the 3β-hydroxyl group located near the bilayer surface and to the larger hydrophobic end area occupied by the flexible alkyl tail in the membrane interior (Rilfors *et al.*, 1984). Consequently, cholesterol disturbs the packing of the gel phase lipids and decreases the size of the co-operative unit involved in the gel/fluid transition, causing the phase transition to broaden or even disappear (Yeagle, 1985). Conversely, fluid phase lipids are ordered by cholesterol, particularly in the segments of the acyl chains which are closest to the headgroup (Meddings, 1989; Yeagle, 1985). In addition to ordering and phase transition broadening effect, cholesterol also promotes hexagonal phase formation. As a result of these effects, the cholesterol-doped bilayer is thinned below the gel/fluid transition and thickened at higher

temperatures. It was shown that cholesterol interacts more with saturated than with unsaturated phospholipids (Finean, 1989).

A regulatory role for cholesterol in adaptation to temperature has not been definitively established, although cholesterol can exert profound effect on the physical and functional properties of the membranes. In most cases, the cholesterol content of the membranes is positively correlated with growth temperature (Wodtke, 1978, 1983; Chang and Roots, 1989). In many cases cholesterol levels do not vary with the acclimation history (Cossins, 1977; Buckley, 1985; Sorensen, 1990) indicating that the growth temperature has no consistent effect on the cholesterol/phospholipid ratio of the membranes.

1.3.6. The role of proteins

Structural and catalytic properties of proteins such as optimal temperatures, the temperatures of their thermal denaturation, substrate specificity, catalytic efficiency, and temperature dependence of their kinetic constants (e.g. K_m) depend on the genetically determined primary structure of the protein in question. In terms of thermal stability, organisms that have inhabited in cold environment for generations may have experienced mutations causing some of their proteins to become relatively heat labile; such mutations would not be lethal unless the organism were transferred to a warmer environment. It is therefore not surprising that variations in protein functioning associated with adaptation to a particular thermal environment are correlating with genetically determined differences in amino acid composition and sequence.

One of the well-investigated proteins, which play an important role in cold adaptation of poikilotherms, is the thermal hysteresis protein (THP). This protein produces a thermal hysteresis (i.e. lower the freezing point of water below the melting point). They produce anti-freeze proteins, which lowers the freezing point, but not the melting points

(DeVries, 1972, 1986). These unique, usually repeating sequences of the anti-freeze proteins with their preponderance of hydrophilic side chains (sugars in glycoproteins and hydrophilic amino acids in the other) apparently allow the proteins to hydrogen bond to the surface of a potential seed ice crystal. Anti-freeze proteins are most common in freeze avoiding species where they function to lower the freezing point and/or promote super-cooling of body water. These proteins have been most thoroughly studied in cold water marine teleost (bony) fishes (DeVries *et al.*, 1972, 1986; Feeny *et al.*, 1986; Davies *et al.*, 1988, Davies and Hew, 1990; Cheng *et al.*, 1991). The range of the thermal hysteresis generally is around 1-1.5°C in fishes. In addition to decreasing the freezing point without significantly lowering the vapour pressure, the anti-freeze proteins can promote super-cooling (Zachariassen and Husby, 1982) and inhibit ice nucleators (Duman *et al.*, 1991; Parody-Morreale *et al.*, 1988). Another effect of the anti-freeze proteins, which is a potential advantage for the freeze tolerant fishes (those that can survive freezing of their extracellular water) is the inhibition of the re-crystallisation activity (Knight *et al.*, 1984, 1988) of proteins even at concentrations well below those required to detect thermal hysteresis activity. Fish typically produce multiple sizes of THP-s with similar primary structures (Cheng *et al.*, 1991). The largest known animal (fish) THP is ~ 34 kDa (Cheng *et al.*, 1991).

The heat-shock phenomenon has been reported in cultured cells of rainbow trout (RTG-2) and in bacteria. A distinct class of heat-shock polypeptides has the size of about 70 kDa; these are called hsp 70 proteins. The induction of a heat-shock polypeptide has been shown to be very fast, within 5 minutes after inducing with sodium arsenate (Kothary *et al.*, 1984). This heat-shock protein was also reported in *Salmo gairdnerri* (Kothari and Candido, 1982).

The protein-lipid interaction is another major factor that influences membrane adaptation, some of them may rigidify or others may fluidise the membranes. It has been shown that some proteins when interacting with phospholipids lowered the fluidity of artificial membranes (Papahadjopoulous *et al.*, 1975). It has also been shown by Monte Carlo simulation technique that due to the fluctuating nature of the phase transitions in lipid membranes, the perturbation of the integral proteins may be of significant range and extend up to a large number of lipid layers, depending on the temperature and on the degree of the mismatch (i.e. varying protein length in an artificial lipid membrane) between the lipid and protein hydrophobic thickness (Maria and Ole, 1991). The lateral mobility of protein on the cell surface and the slow rate of the protein diffusion, comparable to that of the lipid molecules in the same membrane can uncouple the cytoskeleton from the lipid bilayer rendering the membrane more fluid or *vice versa* (Berk and Hochmuth, 1992). Therefore, it may be concluded that in poikilotherms and in other organisms such as bacteria, heat-shock proteins and lipid-protein interaction plays an important role in membrane adaptation to temperature.

1.3.7. The regulation of lipid metabolic enzymes in poikilotherms

Lipid metabolism plays important role in thermal adaptation by regulating fatty acid composition in the membranes. The enzymes that regulate the fatty acid composition (Figure 4) of the membranes are compartmentalized either within the endoplasmic reticulum where phospholipid synthesis takes place or within the organelles where the fine adjustment of the phospholipid molecules is done. It is unclear at present to what extent these two sites are responsible for determining the highly individual fatty acid patterns of membranes.

The fatty acyl coenzyme A pool occupies a central position in the biosynthesis of complex lipids. All fatty acids, whether dietary origin or produced *de novo* form acyl Co A-s before being incorporated into membrane lipids. PUFA are produced by sequential microsomal desaturation and elongation of the 18:1 (n-9), oleic, the 18:2 (n-6), linoleic and the 18:3 (n-3), linolenic acids. The elongation and desaturation of the parent fatty acids in the n-9, n-6 and n-3 families are performed by enzymes called desaturases and elongases (Figure 4).

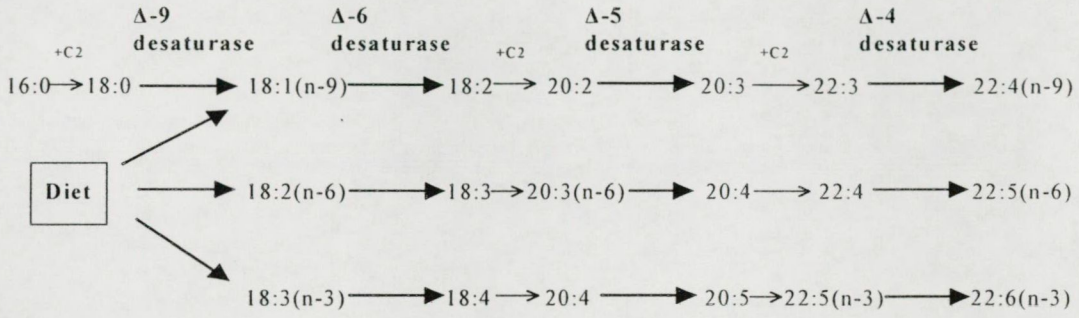


Fig 4. Pathways of elongation and desaturation of the (n-9), (n-6) and (n-3) series of fatty acids. (Bell *et al.*, 1986)

The relative contribution of unsaturated fatty acid production to total lipogenesis in fish has been estimated by determining the pattern of [^{14}C]-acetate incorporation into specific fatty acids. It has been seen that regardless of the growth temperature, an acute drop in the assay temperature stimulates the desaturation and the elongation enzymes (Ninno *et al.*, 1974; DeTorrengo and Brenner, 1976) leading to production of unsaturated fatty acids (Farkas and Csengeri, 1976; Hazel and Prosser, 1979).

Sellner and Hazel (1982a) demonstrated that hepatocytes from trout acclimated at 5°C or 20°C had a substrate preference 18:3(n-3) > 18:2(n-6) > 18:1(n-9) in desaturation and elongation, especially when assayed at 5°C, thus explaining the increase in the (n-3)

series of unsaturated fatty acids from the 5°C acclimated trout in comparison with the 20°C acclimated trout.

Selective incorporation of fatty acids could occur at several points of the *de novo* synthesis of phospholipids, including acyl-transferase reactions and the phospho- and CDP-diglyceride transferase reactions (Figure 5).

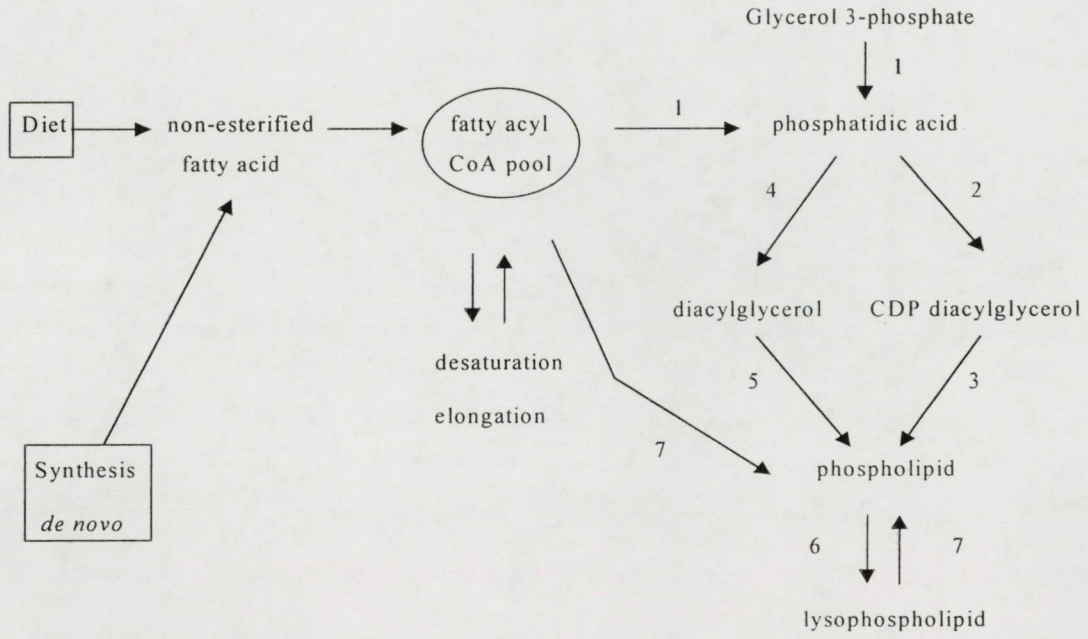


Figure 5. Schematic pathways of phospholipid metabolism showing stages at which specificity for particular fatty acids can be exerted. 1. *Acyltransferase*; 2. *Cytidyltransferase;* 3. *CDP diacylglycerol phosphatidyl transferase*; 4. *phosphatidate phosphatase*; 5. *phophotransferase*; 6. *phospholipase A2/A1*; 7. *acyltransferase.* (Bell *et al.*, 1986)

The preferential incorporation of specific fatty acids into phospholipids during temperature acclimation has been shown in isolated trout hepatocytes (Sellener and Hazel, 1982b). Preferential incorporation of unsaturated fatty acids into PC during low temperature acclimation has been shown by Leslie and Buckley (1976) and Zwingelstein *et al.* (1978). During both acute and chronic cold exposure the phospholipids of trout microsomes incorporated specifically C20 PUFA rather than C18 unsaturated fatty acids (Hazel, 1979;

Sellener and Hazel, 1982c). Cold acclimation in these microsomes exhibited preferential incorporation of the (n-3) and (n-6) fatty acids (Hazel *et al.*, 1983).

Cold is a major stress factor for all living organisms. Poikilothermic animals respond adaptively to chronic cold by a suite of cellular responses that compensate to varying extent for the rate-depressing effects of cooling. The most widespread response is to increase the level of unsaturation of the membrane phospholipids to offset the cold-induced stress. The enzymes involved have not been clearly identified, and the mechanism that controls their activity is also unknown.

Cooling of carp liver causes a large increase in the activity of the Δ-9 desaturase (Schunke and Wodtke, 1983), the enzyme that incorporates the first double bond into the saturated fatty acids (Macartney *et al.*, 1994). It was shown that in carp liver cold can induce a 8-10-fold increase in the specific activity of the microsomal stearoyl coenzyme A desaturase (Δ-9 desaturase) (Tiku *et al.*, 1996).

Tiku *et al.* (1996) has also shown that in cold adaptation the monoenes were increased at the expense of saturates. The predominant effect was the replacement of saturates by monounsaturates in the *sn*-1 position of phosphatidylethanolamine, a reaction that is mediated by the Δ-9 desaturase (Enoch *et al.*, 1976). Tiku *et al.* (1996) has suggested that cold-induced Δ-9 desaturase gene expression arise from two sources. The first is that pre-existing latent desaturase becomes activated within 24-48 hours after initiation of the cooling. This might include the release of the sequestered enzyme or its activation by posttranslational modification. Carp desaturase is notable in that it has many consensus sites for phosphorylation, myristoylation and N-glycosylation. The second is the cold-induced gene transcription, thus, the increased amount of the desaturase transcript resulting in an increased amount of the desaturase protein after 3-5 days of cooling. The

time-course of both events correlates with the changes in lipid saturation and in the membrane lipid order upon cooling.

1.3.8. The time-course of adaptive mechanisms

Species, like fish, experiencing rapid changes in body temperature must be able to modulate rapid, reversible (or arrestable) changes in physical properties of their membranes according to the actual external temperature. The first illustration of these rapid changes in membrane fluidity was given in microsomal membranes of *Tetrahymena* by Ramesha and Thompson in 1983. Wodtke and Cossins (1991) found that endoplasmic reticular membranes of carp liver showed a 61-92 % compensation in fluidity within 24 h after transferring from 30°C to 23°C. Similarly, *in vitro* acclimation of trout hepatocytes at 5°C isolated from 20°C acclimated fish fluidised their plasma membranes within 6 hours. This fluidisation was offsetting 45-50 % of the cold-induced increase in the membrane order, which is approximately one half of the fluidity compensations of a fully acclimated trout *in vivo* (Williams and Hazel, 1995). The fact that the effects of temperature are immediate and the organisms possess means for rapid membrane modifications was well illustrated in earlier study (Dey and Farkas, 1992) made on carp erythrocytes. When these erythrocytes were subjected to heating or cooling cycles *in vitro*, erythrocytes showed a nearly complete compensation of the membrane fluidity within 40-60 min. Fluidity compensations occurred in response to both warming (to 25°C) of the erythrocytes of cold acclimated (5°C) fish and cooling (to 5°C) those of warm acclimated (25°C) fishes.

The mechanisms responsible for these initial, rapid alterations in biophysical properties as a response to altered temperatures, termed 'emergency homeoviscous adaptation' (Hazel, 1995) of the fish membranes have not yet been thoroughly studied, but presumably reflect metabolic adjustments capable of rapidly altering the lipid composition

of a membrane in an adaptive manner. Both the molecular species composition and the phospholipid class composition of a membrane have been shown to respond rapidly to the thermal challenge. The nature of these metabolic adjustments varies with the type of restructuring involved.

2. MATERIALS AND METHODS

2.1. Experimental Organisms

The fishes, frogs and symbiotic/photosynthetic bacteria species investigated in the studies are listed in Table 2.1 where the adaptation/growth temperature and the feeding habit of fishes, are also indicated.

Table 2.1. List of the species investigated

Species		**Water/growth temperature**
Fresh water fishes	**Feeding habit**	
Cyprinus carpio L.	omnivorous	5°C, 25°C
Hypopthalmichthys molitrix	phytoplankton	5°C
Aristichthys nobilis	zooplankton	5°C
Ctenopharyngodon idella	aquatic weeds	5°C
Amphibians		
Rana esculenta		5°C, 25°C
Bacterial species		
Photorhabdus luminescens (symbiont of *Heterorhabditis* sp.)		18°C, 28°C
Xenorhabdus nematophilus (symbiont of *Steinernema* sp.)		18°C, 28°C
***Cylindrospermopsis raciborskii* (blue-green alga)**		25°C, 35°C

Fishes were obtained from the local fish farm, Tisza Halászati Szövetkezet, Szeged. All fishes were fresh water species and were collected at the end of summer when the water temperature was between 23-26°C for more than two months (considered "summer-adapted" or "25°C-adapted" fish) or at the end of winter (considered "winter-adapted" or

"5°C -adapted" fish) when the temperature of the water was 4-5°C for more than two months. Livers were removed on the spot and were placed immediately into liquid nitrogen or into chloroform:methanol 2:1 (v/v) containing BHT as an antioxidant. Samples were transported on dry ice and processed immediately or stored at -80°C until processing.

Frogs (*Rana esculenta*) were collected at their active state at the end of summer, in a neighboring fishpond, when the temperature was above 25°C for more than two months. Livers of 5 animals were removed and total phospholipids were extracted for further analysis. Part of the animals were placed into a low temperature environment and acclimated to 4-5°C for 3 months. After this acclimation/hibernation period their livers were removed and the same analyses were carried out as for summer adapted animals.

Bacteria were cultured in the laboratory of Dr. András Fodor at the Institute of Genetics, Eötvös Loránd University, Budapest. Primary- and secondary-phase variants of *P. luminescens* Hm and *X. nematophilus* N2-4 were grown at different temperatures as follows: phase variants were grown on indicator plates of NBTA (5g of Bacto Peptone, 3g of beef extract, 15g of Bacto agar (Difco, Detroit, MI), 25 mg of bromthymol blue, 40mg of 2,3,5-triphenyltetrazolium chloride, in 1L distilled water, pH 6.8) (Poinar and Thomas, 1984). Both phases can take up triphenyltetrazolium chloride and convert it to formazan and form red colonies. Only the primary forms can take up bromthymol blue and thereby produced dark blue colonies. Single colonies of each strain were removed from indicator plates and transferred into 500-ml Erlenmeyer flasks containing 200 ml of rich liquid medium (20 g of soybean peptone (Sigma, St. Louis, MO), 5 g of yeast extract (Difco, Detroit, MI), in 1L distilled water, pH 6.8). The cultures were shaken in a horizontal shaker for maximum aeration at different temperatures (18 and 28°C for 24, 48, 72, and 96 h). At 28°C, liquid cultures reached the stationary phase within 24 to 28 h at which the bioluminescence and antibiotics production of *P. luminescens* primary cultures were near

maximal; at 18°C maximal levels were reached within 40 to 48 h. Secondary-phase variants usually appeared between 48 to 96 h. At low temperature (18°C), the bacterial growth rate was about half of that of 28°C, with the 48 h cultures having passed the logarithmic phase and entered into the stationary phase.

Cyanobacteria were cultured and thylakoid membranes were isolated in the laboratory and by the group of Dr. Zoltán Gombos, Institute of Plant Biology, BRC, Szeged, as follows: *Cylindrospermopsis raciborskii* was cultivated in modified BG-11 medium containing 10% of the original microelements. A glucose tolerant strain of *Synechocystis* PCC 6803 was grown photoautothrophically in BG-11 medium supplemented with 20 mM HEPES-NaOH (pH 7.5) as described by Wada and Murata (1989). The strains were illuminated with incandescent lamps of 40 $\mu Em^{-2}s^{-1}$ and aerated by sterile air containing 1% CO_2. *Synechocystis* PCC 6803 cells were cultivated in BG-11 medium according to Wada and Murata (1989). The cytoplasmic and thylakoid membranes of both bacteria were isolated by the method of Murata and Omata (1988): the cells after a pre-treatment with lysozyme, were disrupted by passages trough a French pressure cell. The membranes were fractionated by flotation centrifugation on a discontinuous sucrose density gradient.

2.2 Short-term acclimation of fish

Carps (*Cyprinus carpio* L.) adapted to summer or winter temperatures were collected at the end of summer or winter when the temperature of the water was 25°C or 5°C for more than a month, and were kept in tanks with regulated water temperature. These fishes were subjected to temperature shift opposite to their adaptation temperatures by lowering or increasing the temperature of the water in the tanks by 0.5°C/hour. After the temperature reached the desired acclimation value (25°C for the originally winter-adapted, and 5°C for the

originally summer-adapted fishes) fishes were held at this temperature for one more day to allow an equilibration of the organism, after which they were sampled.

2.3. Chemicals and Materials

All reagents and chemicals including spin labels (5- and 16- doxylstearic acid) were purchased from Sigma Chemical Co (St. Louis, MO, USA) except those listed below.

Synthetic lipids, 1-palmitoyl-2-docosahexanoyl-phosphatidylcholine (PDPC), 1-stearoyl-2-docosahexanoyl-phosphatidylcholine (SDPC), 1-oleyl-2-palmitoleyl-phosphatidyl-choline (POPC), 1-oleyl-2-docosahexanoyl-phosphatidylcholine (ODPC), 1-palmitoyl-2-oleyl- phosphatidylethanolamine (POPE), 1-oleyl-2-docosahexanoyl phosphatidylethanolamine (ODPE) were from Avanti Polar Lipids, Inc. (Birmingham, AL., USA).

Fluorescent labels, 2-, 12-(N-9-anthroyloxy) stearic acid, 16-(N-9-anthroyloxy) palmitic acid, 1,6-diphenyl-1,3,5-hexatriene (DPH) were from Molecular Probes, Inc. (Eugen, OR, USA).

Solvents, Acetonitrile and 2-propanol (HPLC grade) were purchased from Carlo Erba (Milan, Italy).

Silicagel G plates (TLC, 20x20cm) and the high performance Silicagel plates (HPTLC, 10x10cm) used in thin layer chromatography of lipids were from Merck, Darmstadt, Germany.

2.4 Analytical techniques

Extraction of total lipids

Lipids of liver tissues and of related membranes were extracted by chloroform:methanol, 2:1 (v/v) according to Folch *et al.* (1974). The extracted lipids were

dissolved in benzene containing butylated hydroxytoluene (0.1%) and stored at - 20°C until assayed.

Separation of lipid classes

Phospholipids were separated from total lipids by silicic acid column chromatography using chloroform to remove the neutral lipids and methanol to elute the polar lipids. Polar lipids were further separated into phospholipid subclasses by thin layer chromatography on Silicagel G plates using the solvent system of Fine and Sprecher (1978). Phosphatidylcholine and phosphatidylethanolamine were removed, after visualisation with 0.5 % 1-anilino,8-naphthalene sulphonic acid in methanol under 254nm UV light, and extracted with chloroform:methanol:water, 50:50:0.5 (v/v/v).

Galactolipids were separated on Silicagel G plates using the solvent system, benzene:acetone:water, 60:30:4 (v/v/v) and visualised under UV light after spraying with 8-naphtalene-1-sulphonic acid (0.5%) in methanol.

Fatty acid analysis

Lipids were transmethylated with methanol containing 5% HCl at 80°C under inert atmosphere for 3 hours. Fatty acid methyl esters were separated on a 30 m long FFAP column (0.25mm i.d., Supelco, Bellefonte, PA) using a Hewlett Packard gas chromatograph, Model 5890. Peaks were identified by using authentic standards from Sigma (St. Louis, MO).

Separation of molecular species of phosphatidylcholines and ethanolamines

The molecular species composition of isolated diacyl phosphatidylcholines and phosphatidylethanolamines was determined according to Takamura *et al.* (1986). The diradylglycerides obtained by digestion with phospholipase C from *Bacillus cereus* (Sigma)

were converted to their anthroyl derivatives according to Takamura *et al.* (1991). The derivatives were separated into diacyl, alkylacyl and alkenylacyl forms on HPTLC plates (10cm x10cm) using hexane:diethylether:ammonia, 80:20:1 (v/v/v) as solvent and were segregated into individual molecular species on a Supelcosil LC-18 column (particle size 5 mm, 25 x 4.6, Supelco, Bellefonte, PA) as well as on a LC-8 column using acetonitrile:propane-2-ol, 7:3 (v/v) and methanol:water:acetonitrile, 93:5:2 (v/v/v), respectively, as solvents with a flow rate of 1ml/min on a Merck-Hitachi L-6220 pump equipped with a fluorescent detector (Model F 1050). The excitation and emission wavelengths were 360 and 460 nm, respectively. Peaks were identified using authentic diacylglycerol standards (16:0/16:0, 22:6/22:6, 20:4/20:4, 16:0/22:6, 18:0/22:6, 18:0/20:4, 18:0/22:6, 16:0/18:2, 16:0/18:1, 18:0/18:1) from Avanti Polar Lipids Inc. Birmingham, AL. and by comparison of their relative retention times with published data (Bell and Dick, 1991). Known amount of derivatised 12:0/12:0 diacylglycerol was added to the separated spots of diacyl, alkylacyl, and alkenylacyl subclasses before extracting them with water saturated diethylether containing 0.1% BHT, as an internal standard.

Preparation of subcellular membranes (mitochondria and plasma membrane) of fish liver

Livers of 3 fish were cut into small pieces and were homogenised in ice cold 0.25M sucrose, 2mM $CaCl_2$, 5mM $MgSO_4$, 10mM Hepes-KOH buffer, pH 7.5 in a Dounce homogeniser. Isolation of plasma- and subcellular membranes were done according to Van Amelswoort *et al.* (1978) by a combination of differential centrifugation and discontinuous sucrose density gradient centrifugation at 4-6°C. The crude homogenate was centrifuged for 10min at 600g. The supernatant was collected and centrifuged for 20minutes at 4000g to produce a pellet containing mitochondria and the supernatant containing the plasma and microsomal membranes. The mitochondrial pellet was collected and washed two more times

in the same buffer and at same speed, collected in 10mM Hepes buffer, pH 7.5, in small volume, and stored at -80°C. The supernatant containing both plasma membranes and endoplasmic reticulum (ER) was layered on top of 1.45M sucrose, 10mM Hepes, pH 7.5 solution to form a two-step sucrose density gradient of equal volumes in the centrifuge tubes. The gradient was then centrifuged at 40000g for 45minutes in a swing-out rotor (SW-27) to separate the plasma membranes from the ER membranes. Plasma membranes were collected as the interface formed between the two phases and were further purified according to Williams and Hazel (1994) as follows. The collected plasma membranes were diluted in 0.25M sucrose, 10mM Hepes, pH 7.5 buffer and centrifuged at 15000g for 45min in an angular rotor (SS-34). Pellets were collected by resuspending in 1-2ml of 0.25M sucrose, 10mM Hepes, pH 7.5 buffer and were layered on top of Percol solution (18% Percol solution made in 1.45M sucrose, containing 10mM Hepes, pH 7.5 buffer) and centrifuged at 40000g for 45min in a swing-out rotor (SW-27). The interface layer was collected and diluted in 0.25 mM sucrose buffer and then centrifuged at 180000g for 2hr (angular rotor, Titanium 60). The resuspended pellets were stored in a small volume of 10mM Hepes buffer, pH 7.5 at -80°C.

The purity of the membrane fractions were tested by assaying marker enzymes: Na^+-K^+ ATP-ase for plasma membrane (Jorgenson, 1974), succinate dehydrogenase for mitochondrial membranes (Bonner, 1955) and glucose-6-phosphatase for endoplasmic reticulum (Nordlie and Aron, 1966).

2.5 Preparation of multilamellar vesicles

Phospholipids (250 μg) dissolved in chloroform were uniformly dried on the wall of a round bottom flask under high vacuum for at least 30 minutes to remove the organic solvent completely, using a rotary evaporator. 4 ml of 0.2 M Tris-HCl buffer, pH 7.4 was added and

the system was incubated for 15 minutes at about 10°C above the main transition temperature of the lipid species or at 40°C in case of extracted total phospholipids. Multilamellar vesicles were prepared by vigorous vortexing for 2 minutes followed by 5 minutes sonication in a bath type sonicator. The dispersion was allowed to equilibrate at room temperature for at least 30 minutes before using.

2.6 Spectroscopic techniques

Electron spin resonance spectroscopy

Electron spin resonance studies were carried out on a Bruker (Karlsrue, Germany) ECS-106 Series 9 GHz X-band spectrometer using 100 kHz modulation frequency. Spectra were recorded with medium microwave power (10 mW) and at low magnetic field modulation (0.1 mT).

Spin labelling was done as follows: 5-or 16-doxylstearic acid was incorporated into samples in typically 1:1000 mol/mol spin-label/sample-phosphorus ratio where the phosphorus content of the samples was estimated according to the procedure of Rouser *et al.* (1970). In the case of lipid samples, multilamellar vesicles (MLV-s) were prepared by co-dissolving the phospholipid (1mg dissolved in chloroform) and the spin probe (dissolved in ethanol) and dried simultaneously. MLV-s were prepared in 0.2 mM Tris-HCl buffer, pH 7.4, as described earlier. Membranes were labelled by injecting the ethanol solution containing the spin label (5μl of 0.2 mg/ml solution) into the 1 ml aliquots of aqueous membrane suspension, of approximately 1 mg/ml phospholipid concentration while vortexing thoroughly. Samples were transferred into 1 mm internal diameter glass capillaries (Kimax, USA) and pelleted by low-speed centrifugation before placing into the resonator cavity. The spectrometer was equipped with a temperature controller unit (using compressed air flow) so that the temperature of the sample could be regulated with ±0.1°C accuracy. Spectra were recorded in

a heating cycle in 2°C steps from -30°C to +30°C and from 0°C to 40°C, respectively. The temperature was incremented by 0.5°C/min, and at each temperature 3 scans were added to give the final spectrum.

For 5-doxylstearic-acid spin label, the outer hyperfine splittings ($2A_{max}$) of the spectra (Figure 1) were measured and used to characterise the membrane ordering state at a given temperature (Freed, 1976). In case of 16-doxylstearic-acid the rotational correlation time of the probe was calculated according to Kivelson (1960) from the spectral parameters indicated on Figure 3.

Fluorescence spectroscopy

Steady-state fluorescence anisotropy measurements were carried out using an L-format Hitachi MPF-2A (Tokyo, Japan) spectrophotofluorimeter or in certain cases a T-format SLM-8000 fluorimeter (Champaign, IL, USA). Both instruments were computer controlled and possessed temperature regulation facility of the cuvette chamber. A Xenon arc lamp was used as light source. The excitation and the emission wavelengths were set using monochromators in both cases. The excitation wavelength was 360 nm for the DPH fluorescent label and 364 nm for the anthroyl derivative probes, the emissions were recorded at 430 and 446 nm, respectively.

In case of the L-type fluorimeter the steady-state fluorescence anisotropy, R_{ss}, was calculated as:

$R_{ss} = (I_{VV} - ZI_{VH}) / (I_{VV} + 2ZI_{VH})$,

where I_{VV} and I_{VH} are the emission intensities recorded with polarizers parallel and perpendicular to the direction of the vertically polarised excitation light; Z, is an instrumental correction factor accounting for the different sensitivities of the detection system for vertically and horizontally polarised light. Z can be calculated from the ratio of horizontally and

vertically emitted light intensities (I_{HV} and I_{HH}) at horizontal excitation: $Z = I_{HV}/I_{HH}$. In case of the T-type fluorimeter the steady state anisotropy was calculated as:

$R_{ss} = (R_V/R_H-1)/(R_V/R_H+2)$,

where R_V and R_H are the ratios of the intensities detected in the two emissions channels with the excitation polariser in the vertical and horizontal position, respectively.

The temperature of the sample was controlled by a circulating water bath and measured directly in the cuvettes with a platinum electrode inserted into the cuvette just above the light beam. Measurements were done between 5 and 40°C using a heating rate of 0.4°C/min. Samples were prepared as follows: lipid and probe stock solutions in chloroform or THF were mixed at a ratio of 1000:1 (mol/mol) and MLV-s were prepared as described earlier. The final concentrations in the cuvettes were 250μg lipid in 3.5 ml buffer. Membrane suspensions were diluted in 10 mM Hepes-KOH buffer, pH 7.4, to give a final protein concentration of 1mg/ml. Labeling with DPH was accomplished by drying 6μl probe of 2mM THF solution to the wall of a round bottom flask and adding 4ml membrane suspension followed by thorough mixing.

2.7 Determination of change point temperatures

The temperature profiles of the outer hyperfine splitting parameters of 5-doxylstearic spin label incorporated into phospholipid vesicles showed changes in the slope at characteristic temperatures. To find the exact temperature value(s) of a change-point (a change-point is a point where a line suddenly changes slope, but the line is continuous) the procedure of Jones and Dey (1995) was applied.

3. RESULTS

I. ABSENCE OF DIRECT RELATION BETWEEN FATTY ACID UNSATURATION, MEMBRANE FLUIDITY AND GROWTH TEMPERATURE IN BACTERIAL SPECIES

Thermal adaptation had been studied widely on bacteria during the last decades. The basic concepts of thermal adaptation of membranes, such as homeoviscousity (Sinensky, 1974) and homeophasicity (McElhaney, 1984) rose from widely studied species like *E. coli*. A general outcome of the studies was, that the ratio of the saturated to unsaturated fatty acids in the lipids correlates with the growth temperature and membrane biophysical properties. A positive correlation between fatty acid unsaturation and membrane 'fluidity' was found and the decisive role of the unsaturated fatty acids in fluidizing membranes at low growth temperatures was accepted as a general concept. .

Three different examples of bacteria are shown below where no correlation was found between the growth temperature, the fatty acid composition, and the biophysical properties of the membranes.

I.1. *Photorhabdus luminescens* and *Xenorhabdus nematophilus* symbiotic bacteria associated with entomopathogenic nematodes

The effect of temperature on the lipid fatty acid composition and structural organization was studied on two species of symbiotic bacteria *Photorhabdus luminescens* and *Xenorhabdus nematophilus*. Primary and secondary cultures of the bacteria were cultured at low (18°C) and high (28°C) temperatures to evaluate their response of adaptation to temperature. The analysis of the fatty acid composition and physical properties of their lipids at different temperatures was aimed to reveal whether their lipid membranes reflect a homeoviscous response to temperature changes or not. The results

obtained on primary cultures are presented below. Secondary cultures gave qualitatively the same results.

I.1.1. The fatty acid composition of total lipids and the structural order of vesicles made of total lipid extracts

In general, 25-26 different fatty acids eluting between 10:0 through 18:1 were identified in the total lipid extracts of both species. Table I.1 compares the major fatty acids (>1%) of primary cultures of *P. luminescens* and *X. nematophilus*, at different culturing temperatures. On the basis of population densities, cultures grown at 18°C for 96 hours were comparable to cultures grown at 28°C for 72 hours.

Table I. 1. Fatty acid composition of the total lipid extracts of the primary cultures of *Photorhabdus luminescens* and *Xenorhabdus nematophilus* grown at two different temperatures. Values represent weight percentages.

	P. luminescens		*X. nematophilus*	
Fatty acids	**28°C** (72h)	**18°C** (96h)	**28°C** (72h)	**18°C** (96h)
<14:0	nd	11.30	22.86	24.94
14:0	19.57	23.26	21.12	17.26
14:1	5.97	4.77	4.18	4.13
15:0	1.43	1.87	0.04	0.06
16:0	22.05	19.31	24.95	22.33
16:1	13.15	24.28	9.27	11.63
17:0	10.19	5.22	0.14	nd
cp17:0	16.95	1.96	4.83	8.47
18:0	2.09	nd	nd	0.71
18:1	8.13	8.03	12.61	10.47
S/U	**2.00**	**1.64**	**2.65**	**2.48**

S/U: total saturated to unsaturated fatty acid ratio; cp: cyclopropane; nd: not detected. Pooled lipid samples from two separate cultures were used for the determination of the fatty acid composition.

Changes in the fatty acid composition as a response to lowered growth temperature are shown in Fig.I.1. The values of 28°C cultures were taken as reference (100%) and the effect of low growth temperature is shown as the percentage change of their levels.

In the lipids of primary cultures of *P. luminescens* grown at 18°C there was an increase in short-chain saturated (<14:0), saturated (14:0) and monounsaturated (16:1) fatty acids, in parallel with the decrease of 14:1 monounsaturated, 17:0 saturated, and cyclopropane 17:0 fatty acids when compared to those of 28°C cultures. These changes resulted in a decrease of total saturated-to-unsaturated (S/U) ratio at low growth temperature (Fig.I.1).

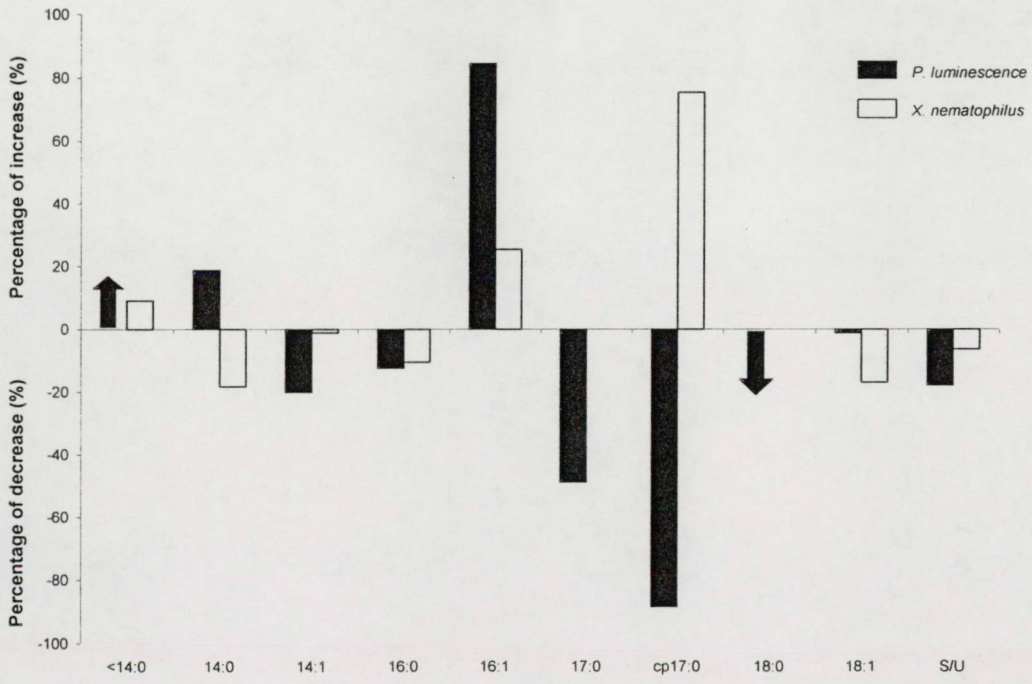


Figure I.1. Cold-induced changes in fatty acid composition of *P. luminescens* and *X. nematophilus*. The values represent the percentage of increase/decrease of fatty acids as compared to values at 28°C (values at 28°C were assumed to be 100%). Arrows represent the appeared (upward arrow) and disappeared species (downward arrow).

In the lipids of *X. nematophilus* grown at 18°C there was a significant increase of short-chain and cp17:0 fatty acids, and a decrease of the saturated (14:0, 16:0) and monounsaturated (18:1) fatty acids. The total S/U ratio of the fatty acids decreased by 6.4% as compared to 28°C-grown cells.

In the adaptation of the two bacteria the common observation was the higher amounts of the short-chain, monounsaturated 16:1, and the lower amounts of palmitic acid (16:0) at low growth temperatures. The other fatty acids were regulated in a species-specific manner, like 14:0 and cp17:0. Therefore these fatty acids may not be universal factors under regulation during temperature adaptation in the symbiotic bacteria studied.

One of the major differences between the primary cultures of *P. luminescens* and *X. nematophilus* is a higher percentage of short chain fatty acids (<14:0) in the latter at both growth temperatures. There were some characteristic differences with respect to the amounts of the monounsaturated fatty acids. The lipids from *P. luminescens* contained higher amount of 14:1 and 16:1 than *X. nematophilus* at both growth temperatures, while *X. nematophilus* contained more 18:1 at both temperatures. The levels of 16:0 increased in both cultures at higher temperature.

The structural orders of membranes made from the lipids of the bacterial species were determined by electron spin resonance spectroscopy. 5-doxylstearic acid spin label was incorporated into the vesicles and the outer hyperfine splitting parameters ($2A_{max}$) were compared (Figure I.2.A, and B) at the two growth temperatures.

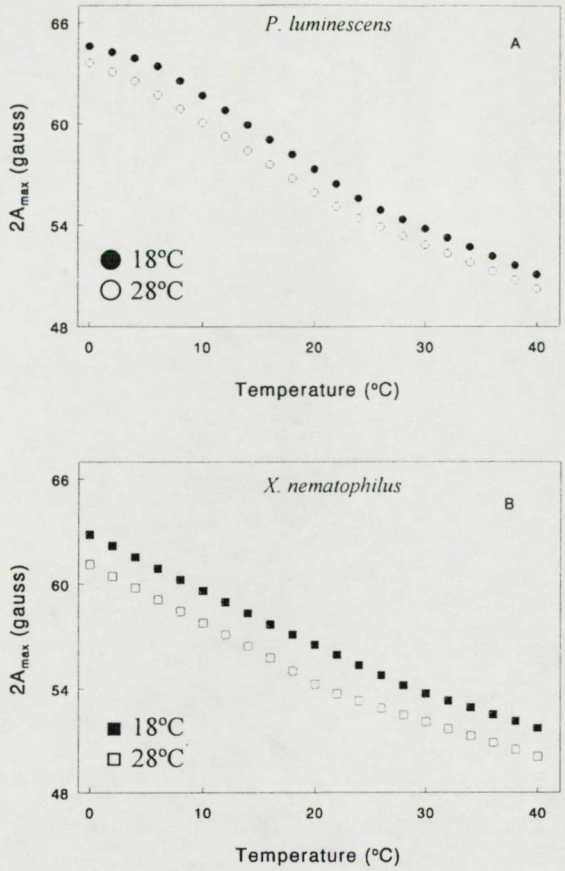


Figure I.2. Temperature dependence of the hyperfine splitting parameter, $2A_{max}$, of 5-doxylstearic-acid spin label embedded in vesicles made of total lipid extracts of primary cultures of *P. luminescens* (A) and *X. nematophilus* (B) grown at 18°C (filled symbols) or at 28°C (open symbols).

Although, the saturated-to-unsaturated fatty acid ratio (S/U) in cultures of *P. luminescens* grown at 18°C was 18% lower as compared to the cultures at 28°C, the $2A_{max}$ parameters measured in the former were higher (Figure I.2. A), indicating a more ordered structure. Similar situation was found for *X. nematophilus* (Figure I.2.B) where the S/U ratio was lower by 6.4 % in the lipids of 18°C cultures than in those of the 28°C.

According to the $2A_{max}$ values the lipids extracted from *P. luminescens* showed more rigid structures than *X. nematophilus* at identical growth temperatures and growth times (Figures I.3.A and B).

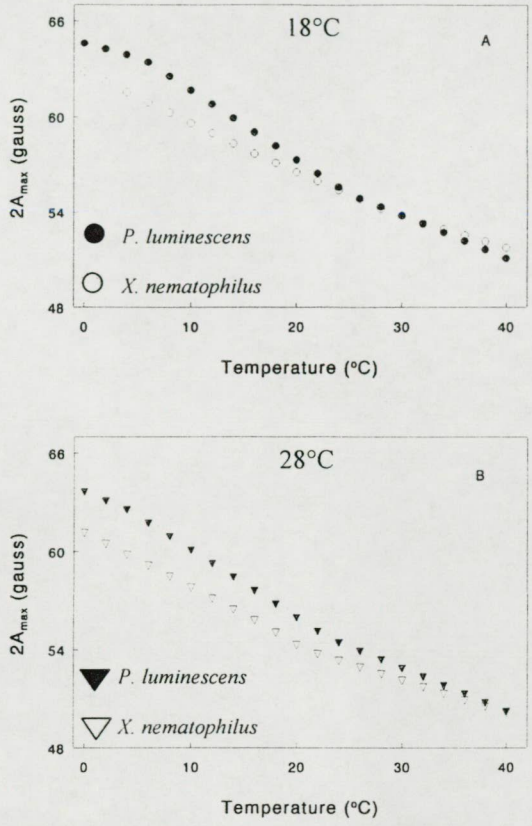


Figure I. 3. Comparison of the temperature dependence of the hyperfine splitting parameter, $2A_{max}$, of the 5-doxylstearic acid incorporated into vesicles made from total lipid extracts of primary cultures of *P. luminescens* (filled circles) and *X. nematophilus* (open circles) grown at 18°C for 96 h (A); and of *P. luminescens* (filled triangles) and *X. nematophilus* (open triangles) grown at 28°C for 72 h (B).

Despite the fact that the saturated-to-unsaturated fatty acid ratio in the lipids of *P. luminescens* was lower than in *X. nematophilus* (Table I.1) in both, 18°C and 28°C cultures (1.64 vs. 2.48 and 2.11 vs. 2.65, respectively), the former showed a more ordered structure than the latter, especially at low measuring temperatures.

I.2. Thermal adaptation of cyanobacterium *Cylindrospermopsis raciborskii*

Cylindrospermopsis raciborskii is of subtropic origin and appeared in temperate regions only in the last 20 years. Since it has been evolutionarily adapted to high water

temperatures, it was extremely interesting to study the rearrangements taking place in the membrane lipids while adapting to low temperatures. Therefore the fatty acid composition and the physical characteristics of the thylakoid membranes of bacteria grown at 25°C and 35°C were studied.

I.2.1. The fatty acid composition and the physical properties of the thylakoid membranes of *C. raciborskii*

Four major polar lipid classes were identified in the total lipid extract of thylakoid membranes (Table I.2).

Table I.2. Composition of thylakoid lipids of *C. raciborskii* grown at two extreme temperatures

	Weight percentage of lipids (%)	
Lipids	**35^{0}C**	**25^{0}C**
MGDG	56.2	57.9
DGDG	14.9	15.1
SL	19.0	18.1
PG	5.5	4.2
UI	4.2	4.7

Cells were grown at the respective temperature for two weeks. MGDG: monogalactosyldiglyceride, DGDG: digalactosyldiglyceride, SL: sulpholipid, PG: phosphatidylglycerol, UI: unidentified.

Monogalactosyldiacylglycerol (MGDG) was the major component followed, in abundance, by sulpholipid (SL), digalactosyldiacylglycerol (DGDG), and phosphatidylglycerol (PG). This distribution was similar to other cyanobacteria (Murata *et al.*, 1992) or thylakoids of higher plants (Webb and Green, 1991). The growth temperature had no effect on the distribution of lipid classes.

The fatty acid composition of the various lipid classes is summarized in Table I.3.

Table I.3. Weight percentage of fatty acid composition of thylakoid lipids of *C. raciborskii* at two extreme temperatures.

	TL		MGDG		DGDG		SL		PG	
Fatty acid	35°C	25°C	35°C	25°C	35°C	25°C	35°C	25°C	35°C	25°C
<12:0	**7.6** ↘	**4.4**	**5.4** ↘	**1.6**	**16.8** ↘	**13.2**	**9.5** ↘	**6.9**	**5.4** ↗	**7.7**
12:0	1.7	1.0	1.2	0.6	2.1	0.7	3.2	2.7	1.9	1.3
14:0	1.3	0.8	1.1	0.5	1.8	0.4	1.1	1.9	2.9	1.2
i16:0	0.5	0.9	nd	0.8	nd	1.3	2.2	nd	2.1	4.2
16:0	**34.5**	**34.5**	**38.0** ↘	**35.2**	**31.6** ↘	**27.5**	**38.6** ↗	**48.1**	**20.0** ↗	**28.9**
16:1	1.9	4.7	1.4	5.1	2.1	5.3	1.6	3.6	8.7	3.8
16:2	1.1	1.2	1.2	0.8	nd	1.9	1.8	2.0	2.0	nd
18:0	**4.1**	**2.9**	**2.2**	**2.0**	**9.1** ↘	**3.2**	**6.4** ↘	**4.7**	**6.1**	**5.9**
18:1	**6.2** ↘	**1.9**	**4.1** ↘	**1.6**	**4.4** ↘	**1.2**	**6.7** ↘	**4.1**	**35.4** ↘	**2.9**
18:2	**17.7** ↘	**1.4**	**22.2** ↘	**1.5**	**12.8** ↘	**1.0**	**15.6** ↘	**1.7**	**6.1** ↘	**1.1**
18:3ω-6	**2.5** ↘	**1.3**	**3.3** ↘	**1.4**	**3.5** ↘	**2.0**	0.6	0.7	**0.4** ↗	**1.2**
18:3ω-3	**7.8** ↗	**21**	**10.0** ↗	**20.5**	**6.9** ↗	**17.9**	**5.9** ↗	**31.3**	**2.0** ↗	**20.7**
18:4	**2.2** ↗	**16.9**	**2.2** ↗	**23.5**	**2.5** ↗	**17.9**	**2.7** ↘	**1.1**	**2.1** ↗	**8.9**
S/U	**1.26**	**0.92**	**1.08**	**0.75**	**1.90**	**0.98**	**1.74**	**1.44**	**0.67**	**1.27**

TL: total lipid extract; i: iso; nd: not detected. For convenience, major fatty acid species were indicated as bold and arrows represent the trend of their change (↗ : increase, ↘ : decrease).

A significant amount of short-chain (<12:0) fatty acids were detected in each lipid class, particularly in DGDG. The dominating fatty acid species were in the order of palmitic, 16:0; linoleic, 18:2(9, 12); α-linolenic, 18:3(9, 12, 15) or 18:3ω-3; oleic, 18:1(9); γ-linolenic, 18:3(6, 9, 12) or 18:3ω-6; stearic, 18:0, and octadecatetraenoic, 18:4(6, 9, 12, 15) acids. As it can be seen in Table I.3 the level of the stearic, oleic and linoleic acids decreased in all four lipid classes, while the levels of α-linolenic and octadecatetraenoic acid increased when the cells were grown at 25°C instead of 35°C. In sulpholipids, the level of 18:3ω-3 increased, but 18:4 fatty acids were not accumulated at 25°C. There was a 27% decrease in the saturated-to-unsaturated (S/U) fatty acid ratio of total polar lipids and 31% and 48% decrease in DGDG and MGDG major lipid classes, upon lowering the temperature.

To follow the effect of low temperatures on the formation of fatty acids, cells grown at 35°C were transferred to 25°C and the lipid composition of the thylakoids was

checked at regular time intervals. As shown in Fig. I.4. A, for MGDG, the level of 18:2 decreased starting from the 3rd hour of incubation at 25°C. α-linolenic and octadecatetraenoic acids started to accumulate at 6th hour. However, the rate of increase of 18:4 was lower than that of 18:3. It has to be mentioned that the level of oleic acid (18:1) also showed a decreasing tendency after 6 hours (data not shown).

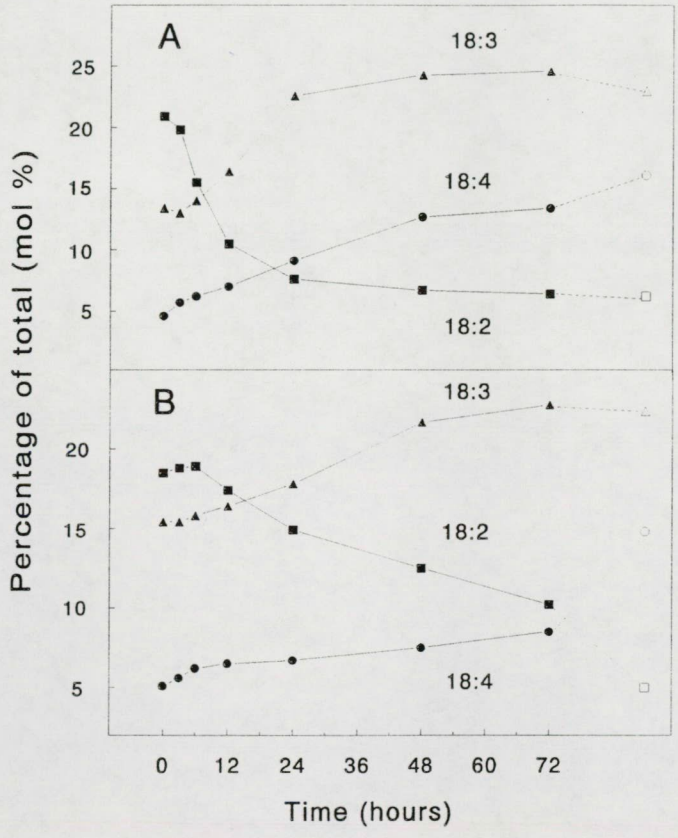


Figure I.4: Time course of fatty acid unsaturation in the thylakoids of *C. raciborskii* for galactolipids after transferring cell cultures from 35°C (0 h) to 25°C. The open symbols represent values from cells grown at 25°C. The decrease of linoleic acid (18:2, ■) is accompanied by the increase of α-linolenic (18:3ω-3, ▲) and octadecatetraenoic acids (18:4, ●) are discussed in the text. (A) MGDG and (B) DGDG.

Qualitatively similar but quantitatively less pronounced trends were observed in case of DGDG (Fig. I.4. B).

The mobility of lipids of the thylakoid membranes were followed by measuring the rotational correlation time of the 16-doxylstearic acid spin probe (16-SASL) and the outer hyperfine splitting of the spin probe 5-SASL incorporated into the membranes. In the entire temperature range studied (0°-60°C) the spectra of 16-SASL could be assigned to

isotropic tumbling in the apolar core of the lipid bilayers. As shown in Fig. I.5.A,for C. *raciborskii*, the temperature dependence of the mobility exhibits a brake around 25°C. Up to this temperature the mobility steeply increases and above it displays a lower rate of increase. For the bacteria grown at 25°C, this steep, pre-brake-point mobility increase was not as significant as for the ones grown at 35°C but the upper sections (>25°C) were similar for the two. This response of the *C. raciborskii* was different from that of *Synechocystis* PCC 6803 (Fig. I.5.B).

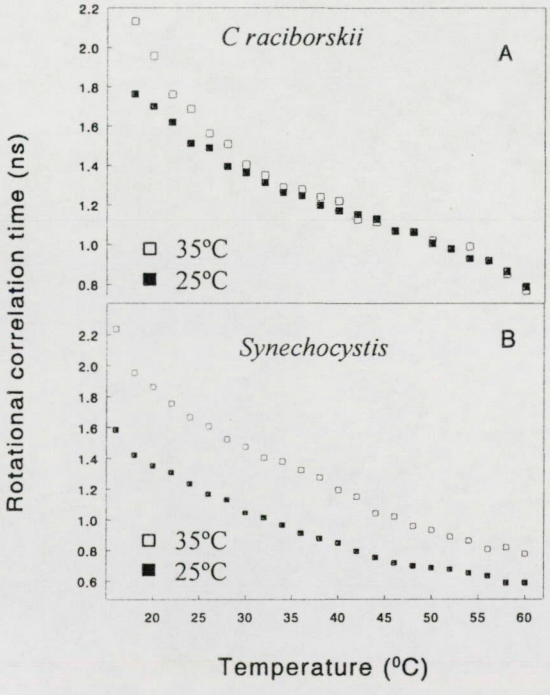


Figure I. 5: Rotational correlation time of 16-doxylstearic acid (16-SASL) incorporated into thylakoid membranes of bacteria grown at different temperatures. (A) Thylakoids of *C. raciborskii* grown at 35°C (open squares) and at 25°C (filled squares); (B) thylakoids of *Synechocystis* PCC 6803 grown at 35°C (open squares) and at 25°C (filled squares). Decreasing rotational correlation time means increasing mobility of the labeled compound.

In the thylakoids of *Synechocystis* PCC 6803 the mobility showed a large change between 0°C and 60°C (Fig. I.5.B). The motional rates of 16-SASL in the thylakoid membranes of *C. raciborskii* grown either at 25°C or 35°C were almost the same as those of for the thylakoids of the *Synechocystis* PCC 6803 grown at 35°C. The spectrum of 5-SASL, where the nitroxyl carrying doxyl group is attached to the C-5 carbon atom of the acyl chain, is anisotropic indicating that the motion close to the polar/apolar interface is confined to a conical angle much smaller than 360° (Griffith and Jost, 1976). In

addition, the motional rate can be determined from the linewidths by using spectral simulations (Seelig, 1976). The spectral anisotropy, i.e. the outer hyperfine splitting ($2A_{max}$), depends on the orientational order parameter and, in case of slow motions, on the motional rate (Freed, 1976). As shown in Fig. I.6.A, in *C. raciborskii* both the absolute value and the temperature dependence of the anisotropy were same in the thylakoids prepared from cells grown either at 25 or 35°C.

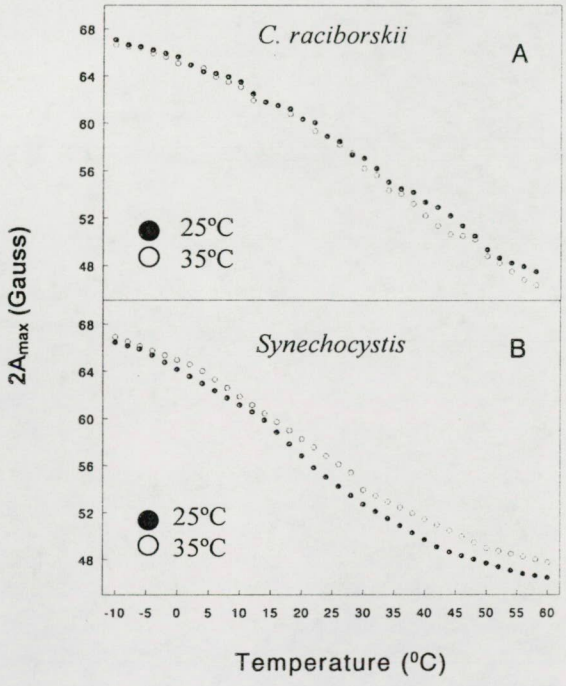


Figure I.6: The temperature dependence of the spectral anisotropy of 5-doxylstearic acid (5-SASL) incorporated into thylakoid membranes. (A) Thylakoids of *C. raciborskii* grown at 25°C (filled circles) or at 35°C (open circles); (B) thylakoids of *Synechocystis* PCC 6803 grown at 25°C (filled open circles) or at 35°C (open circles).

In case of *Synechocystis* PCC 6803, the thermotropic response of the anisotropy was significantly different at the two growth temperatures (Fig. I.6.B). The anisotropy *vs.* temperature curves of *C. raciborskii* were closer to that of *Synechocystis* PCC 6803 grown at 35°C.

II. CHARACTERISTICS OF SEASONAL ADAPTATION TO CONTRASTING TEMPERATURES REFLECTED IN TOTAL PHOSPHOLIPIDS OF LIVER OF THE FRESH WATER FISH *Cyprinus carpio* L.

The fatty acid and the molecular species composition of the liver phospholipids of summer- and winter-adapted (SA and WA, respectively) carps were compared and the possible role of certain lipids in regulating membrane order during the adaptation was investigated.

II.1. Fatty acid composition of liver phospholipids in summer- and winter-adapted carps

The fatty acid composition of the total phospholipid content (TPL) of the liver and the choline (PC) and ethanolamine (PE) phosphoglyceride major subclasses of winter- and summer-adapted carps are shown in Table II.1.

Table II.1. Fatty acid composition of liver total phospholipids, phosphatidylcholines and phosphatidylethanolamines of carps adapted to summer (25°C) and winter (5°C) temperatures.

	Summer-adapted			Winter-adapted		
Fatty acid	TPL	PC	PE	TPL	PC	PE
14:0	0.1	10.6	11.6	0.8	7.0	3.4
16:0	25.5	32.1	21.9	14.4	23.0	15.0
16:1	3.9	5.5	0.4	6.6	11.5	1.1
18:0	17.7	9.2	15.0	8.8	5.1	9.1
18:1	14.2	17.1	10.2	16.0	15.5	15.1
18:2n-6	3.5	3.9	1.6	2.3	2.4	1.1
18:3n-3	1.7	0.3	0.6	2.7	1.5	4.3
20:4n-6	12.0	5.4	13.8	21.6	11.4	24.2
20:5n-3	1.2	1.6	1.0	1.1	0.4	1.2
22:4n-6	1.2	0.8	3.1	2.2	0.6	2.1
22:4n-3	2.7	0.3	3.5	3.4	1.5	3.0
22:5n-3	1.7	1.2	2.5	2.7	2.6	2.8
22:6n-3	14.5	12.0	14.7	17.4	16.9	17.4
Σ LcPUFA	**33.3**	**21.3**	**38.6**	**48.4**	**46.9**	**50.7**
S/U	**0.76**	**1.07**	**0.94**	**0.31**	**0.54**	**0.37**

TPL: total phospholipids, PC: phosphatidylcholine, PE: phosphatidylethanolamine, S/U: ratio of saturated-to-unsaturated fatty acids. LcPUFA: long-chain polyunsaturated fatty acid. Figures represent weight percentages.

As it can be seen in the Table II.1 a major characteristics of the fatty acid composition is the presence of high amounts of long-chain polyunsaturated fatty acids, especially at cold temperature. Characteristic changes upon winter-adaptation were the decrease of the saturated, 16:0 and 18:0, fatty acids and the accumulation of the polyunsaturated, 20:4 and 22:6, fatty acids in all three, TPL, PC and PE lipid extracts analysed. Among the polyunsaturated fatty acids, 20:4n-6 was the most responsive showing approximately 100% increase in all, TPL, PC and PE lipid fractions, whereas 22:6n-6 was less responsive. The level of monounsaturated (16:1 and 18:1) fatty acids increased in all, three, lipid extracts during winter. In the PE 16:1 was almost absent. Oleic acid (18:1) increased by 50% in PE upon winter adaptation, but not in PC. Such cold-induced accumulation of the oleic acid in ethanolamine phosphoglycerides of fish liver had been reported in other cases, too (Dey *et al.*, 1993b). Over all, the changes resulted a 59,2% decrease of the saturated-to-unsaturated fatty acid ratio of the total phospholipids at winter as compared to the summer-adapted state.

II.2. Molecular species composition of diacyl PC and PE lipid classes

In diacyl PC twenty different and in the diacyl PE nineteen different molecular species were identified (Table II.2). The major PC species of the SA fish were 16:0/22:6, 18:0/22:6 and 16:0/18:1, which made up 58.5 % of the total molecular species. In PE, the 16:0/22:6, 16:0/20:4, 18:0/22:6 and 18:0/20:4 species were the major components representing 73.6 % of the total.

Adaptation to winter temperatures resulted in the reorganization of lipid molecular species both in PC and PE:

a) The total amount of saturated/saturated (Sat/Sat) species decreased during winter adaptation regardless of being PC (decreased by 82%) or PE (decreased by 27%).

b) Upon winter adaptation the amount of total saturated/unsaturated (Sat/Unsat) molecular species decreased (by 16% in PC and by 35% in PE), while the amount of total monounsaturated/polyunsaturated (Mono/Poly) species displayed a significant increase: doubled in PCs and tripled in PE. As a result, the ratio (Σ(Sat/Unsat))/(Σ(Mono/Poly)) decreased both in PC (by 61%) and PE (by 77%).

As we can see in Table II.2 the changes were more pronounced among the molecular species of ethanolamine phosphoglicerides than choline phosphoglicerides.

Table II.2. Molecular species composition of liver diacyl phosphatidylcholines and diacyl phosphatidylethanolamines of carp adapted to summer and winter temperatures represented as weight percentages (%).

	PC		PE	
Species[a]	Summer	Winter	Summer	Winter
14:0/16:0	0.4	tr.	tr.	1.3
16:0/16:0	3.7	0.8	1.4	0.3
16:0/18:0	0.3	tr.	0.8	tr.
Σ (Sat/Sat)	**4.4**	**0.8**	**2.2**	**1.6**
16:0/22:6	22.9	23.5	23.1	15.8
16:0/22:5	0.3	1.1	tr.	1.8
16:0/20:4	7.7	11.4	10.0	15.9
16:0/18:2	6.6	9.1	2.7	3.4
18:0/22:6	12.4	6.7	17.0	6.2
18:0/20:5	1.5	tr.	3.0	4.3
18:0/20:4	2.3	1.9	23.5	5.7
16:0/18:1	23.2	11.8	3.4	1.1
18:0/18:1	3.1	1.7	1.2	tr.
Σ (Sat/Unsat)	**80**	**67.2**	**83.9**	**54.2**
18:1/22:6	3.9	6.9	5.4	10.2
18:1/20:5	0.7	4.7	tr.	0.9
18:1/20:4	4.3	8.2	4.8	17.4
18:1/18:2	0.6	0.6	nd.	nd
Σ (Mono/Poly)	**9.5**	**20.4**	**10.2**	**28.5**
16:1/16:1	tr.	tr.	tr.	0.5
18:1/18:1	2.8	3.0	tr.	0.6
18:1/20:1	0.2	tr.	tr.	tr.
Σ (Sat/Unsat) / **Σ (Mono/Poly)**	**8.4**	**3.3**	**8.2**	**1.9**

[a]:The first number denotes the carbon chain length and the second the number of double bonds in the aliphatic chain, the fatty acid before the slash represents the one esterified in the position *sn*-1, while the fatty acid after the slash refers to the one present in the position *sn*-2 of the glycerol moiety. tr.: trace amount. nd: not detected. Sat: saturated fatty acid. Unsat: unsaturated fatty acid. Mono: monounsaturated fatty acid. Poly: polyunsaturated fatty acid. Σ represents the sum.

II. 3. The fluidity of the vesicles made from the liver phospholipid extracts

ESR spectroscopy (using 5-doxylstearic acid spin label, 5-SASL) and steady-state fluorescence spectroscopy (using 2-, 12- and 16-anthroyloxy fatty acid probes, 2-, 12-AS and 16-AP) were used to assess the physical characteristics of phospholipid vesicles.

The outer hyperfine splitting parameter of 5-SASL incorporated into the vesicles made from total phospholipid extracts of SA and WA fish livers were compared over a large temperature range, between -30 and 40°C (Figure II.1).

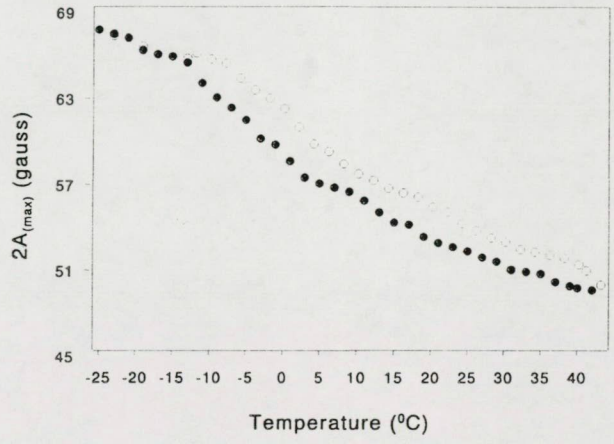


Figure II.1. Temperature dependency of the outer hyperfine splitting, ($2A_{max}$) of 5-SASL in hydrated phospholipids extracted from livers of carp adapted to summer (○) and winter (●) temperatures.

As it can be seen, the values obtained for the SA fish are generally higher through most of the temperature range, indicating a more rigid structure of the vesicles as compared to that of the WA liver extracts. At low temperatures the values are similar in the two samples but in the WA sample a change in the slope occurs at -13°C, which is 5°C lower than that of the corresponding curve of the SA sample (-8°C). This suggests that there might be a 5°C difference between the melting temperature of the gel-phase of SA and WA lipid samples.

The steady-state fluorescence anisotropy of different anthroyloxy fatty acids (2-AS, 12-AS and 16-AP) incorporated into the phospholipid (TPL) vesicles are shown in the Figure II.2.

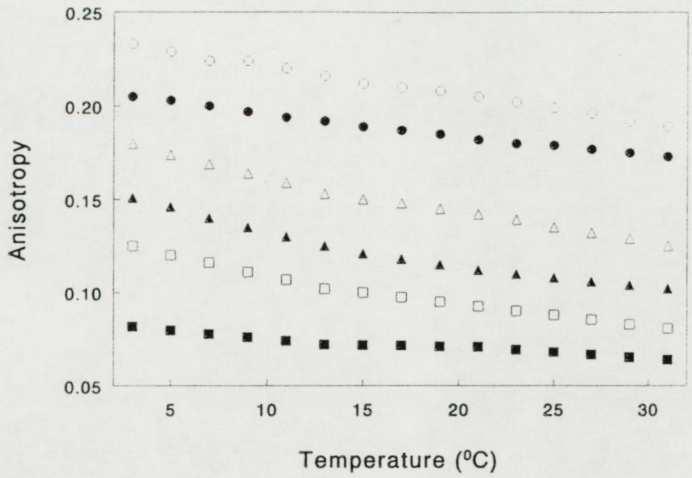


Figure II.2. Temperature dependency of the steady-state fluorescence anisotropy of 2-AS, 12-AS and 16-AP embedded in vesicles made from liver **total phospholipids** of carp adapted to summer (○ 2-AS, Δ 12-AS and □ 16-AP) and winter (● 2-AS, ▲ 12-AS and ■ 16-AP) temperatures.

Each probe incorporated into the bilayer provided information from the level where the fluorophore is attached to its fatty acid chain. Thus 2-AS in the bilayer reports for the upper region close to C-2 carbon atom of the bilayer while 12-AS and 16-AP from the C-12 and C-16 regions, respectively. Lipids of WA fish showed lower anisotropy values at all depths, than those from the SA fish. Similar anisotropy values were found in the C-2 segment at 5°C for the vesicles from the WA fish and at around 24°C for those of the SA fish (see Fig. II.1).

In the following figure (Fig. II.3), the steady-state anisotropy of anthroyloxy probes measured in vesicles made from isolated phosphatidylcholines were compared.

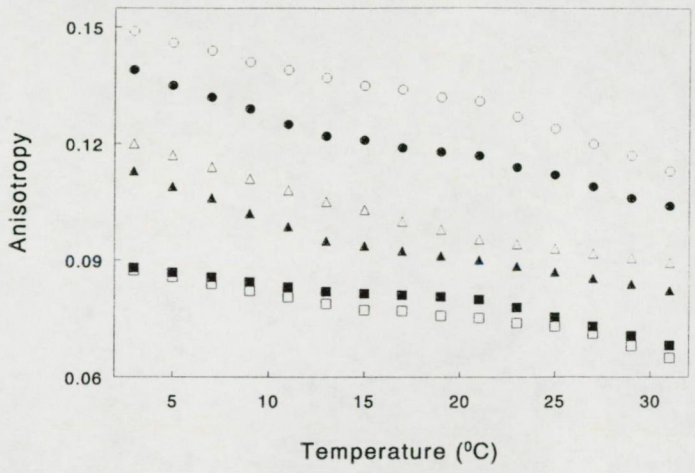


Figure II.3. Temperature dependency of the steady-state fluorescence anisotropy of 2-AS, 12-AS and 16-AP embedded in **phosphatidylcholine** vesicles made from livers of carp adapted to summer (○ 2-AS, △ 12-AS and □ 16-AP) and winter (● 2-AS, ▲ 12-AS and ■ 16-AP) temperatures.

Phosphatidylcholine vesicles isolated from WA carp were less ordered than those of SA carp and they were less ordered at almost each segments than the corresponding total phospholipids (Fig. II.2).

III. SHORT-TERM TEMPERATURE-SHIFT-INDUCED ADAPTIVE CHANGES IN THE LIPID COMPOSITION AND IN THE PHYSICAL PROPERTIES OF MEMBRANES OF CARP LIVER

The following experiments were designed to compare the changes in the lipid molecular species composition and membrane fluidity of the liver membranes during seasonal adaptation and short-term temperature acclimation of fishes.

Fishes (*Cyprinus carpio* L.) being seasonally adapted to winter (5°C) and summer (25°C) temperatures were collected. Part of the winter-adapted fishes was gradually

acclimated ("up-shifted") to 25°C as mentioned in the Materials and Methods. Similarly, summer-adapted fish were gradually acclimated ("down-shifted") to 5°C. Molecular species compositions of the major (PC and PE) lipids and the fluidity of the liver plasma membranes and mitochondria were determined and compared at seasonal adaptation and short-term temperature acclimation temperatures. The physical properties of the membranes were assayed by measuring the steady-state fluorescence anisotropy of intercalated DPH fluorescent probes.

The whole experiment was repeated twice in different years and the values presented in the Tables are the average values of the two experiments. Deviations from the average values were less than one weight percentage of total for each major component listed in the tables. The presented fluidity curves are the comparisons from one set of experiment, which was chosen as representative. The second set of experiment gave qualitatively the same results.

III.1. Lipid molecular species composition of PC and PE lipid classes

Table III.1 and III.2 show the weight percentage of the major molecular species of phospholipids (PC and PE) extracted from the plasma membranes and mitochondria of livers of fishes seasonally adapted to 5°C and 25°C, and short-term temperature acclimated to 5°C (noted as: ↓5°C) and 25°C (noted as: ↑25°C), respectively.

Table III.1. The effect of the temperature shift on the weight percentage (%) of molecular species of phosphatidylcholine (PC) and ethanolamine (PE) in liver **plasma membranes** of carp.

Molecular species	PC				PE			
	5°C	↑25°C	25°C	↓5°C	5°C	↑25°C	25°C	↓5°C
18:1/22:6	9.4	6.9	4.5	6.7	15.9	7.7	1.4	3.1
16:0/22:6	22.8	16.6	11.3	9.8	12.6	11.8	6.6	7.7
18:0/22:6	5.8	10.6	6.0	9.0	12.5	13.6	7.3	7.6
18:1/20:4	11.3	18.1	4.8	9.7	7.4	9.2	4.6	7.1
16:0/20:4	7.5	12.1	11.2	11.5	13.0	12.9	5.8	9.2
18:0/20:4	6.6	4.7	4.1	2.7	11.9	21.8	12.6	15.2
18:1/18:1	3.3	2.6	4.0	6.7	2.2	1.4	4.0	3.1
16:0/18:1	10.7	12.8	17.6	12.6	2.9	3.7	25.8	18.3
16:0/16:0	1.0	2.7	3.2	1.1	1.7	1.9	3.0	0.4
Σ (Sat/Unsat)	53.4	56.8	50.7	45.6	52.9	63.8	58.1	58
Σ (Mono/Poly)	20.7	25	18.6	16.4	23.3	19.6	6	10.2
Σ (Sat/Unsat)	**2.57**	**2.27**	**2.69**	**2.78**	**2.27**	**3.77**	**9.7**	**5.7**
Σ (Mono/Poly)								

Only the major (>1%) molecular species are listed. Summer- (25°C) and winter- (5°C) adapted carps were exposed to the "opposite" temperature, this is denoted as ↓5°C and ↑25°C for summer and winter carps, respectively. Membranes were prepared from pooled livers of 5 fishes. The values represent the average of two separate experiments, and the deviations were <1% for each species listed.

Table III.2. The effect of the temperature shift on the weight percentage (%) of molecular species of phosphatidylcholine (PC) and ethanolamine (PE) in liver **mitochondria** of carp.

Molecular species	PC				PE			
	5°C	↑25°C	25°C	↓5°C	5°C	↑25°C	25°C	↓5°C
18:1/22:6	10.8	8.8	6.6	6.9	16.7	9.5	6.0	5.6
16:0/22:6	17.7	26.5	15.3	10.6	10.9	12.8	8.5	11.3
18:0/22:6	4.5	5.9	5.9	8.9	11.9	13.5	8.2	8.5
18:1/20:4	6.9	9.3	6.4	10.6	8.0	9.1	7.7	8.9
16:0/20:4	11.4	13.0	12.6	11.5	12.0	14.1	14.0	16.1
18:0/20:4	11.4	4.9	2.3	1.3	12.7	13.8	21.9	19.7
18:1/18:1	3.9	1.8	2.8	5.8	2.9	3.9	1.6	2.0
16:0/18:1	15.7	9.7	10.9	9.7	3.1	3.0	5.2	4.0
16:0/16:0	1.6	2.4	1.6	1.3	2.1	1.9	3.2	2.1
Σ (Sat/Unsat)	60.7	60	47	42	50.6	57.2	57.8	59.6
Σ (Mono/Poly)	17.7	18.1	13	17.5	24.7	18.6	13.7	14.5
Σ (Sat/Unsat)	**3.43**	**3.31**	**3.61**	**2.4**	**2.05**	**3.07**	**4.22**	**4.11**
Σ (Mono/Poly)								

Only the major (>1%) molecular species are listed. Summer- (25°C) and winter- (5°C) adapted carps were exposed to the "opposite" temperature, this is denoted as ↓ 5°C and ↑ 25°C for summer and winter carps, respectively. Mitochondria were prepared from pooled livers of 5 fish, and lipids extracted from the whole organelle. The values represent the average of two separate experiments, and the deviations were <1% for each species listed.

In both plasma membrane and mitochondrial lipids the most important changes occurred in the ratio of *sn*-1 saturated, *sn*-2 unsaturated - to - *sn*-1 monounsaturated, *sn*-2 polyunsaturated molecular species **((Sat/Unsat)/(Mono/Poly))** of PE. This ratio decreased drastically upon cold-adaptation (5°C) and to a lesser extent upon cold-acclimation (↓5°C) too. The same occurred in the PC lipid class but with a much lesser magnitude than in the PE. Upon warm-acclimation (↑25°C) the (Sat/Unsat)/(Mono/Poly) ratio increased in PE, but not in PC where there was a slight decrease. These changes in the PE can be mainly attributed to the variation of the denominator **(Mono/Poly)**, i.e. *sn*-1 monounsaturated, *sn*-2 polyunsaturated molecular species since the change in the amount of Sat/Unsat species was of less magnitude.

The changes of the individual molecular species upon winter-adaptation, short-term cold and short-term warm acclimation was assessed by calculating the percentage of change of their values corresponding to the starting states, i.e.:

1. Winter-adaptation was evaluated in comparison to the percent values obtained for summer-adapted state as: $-100x(X_{25°C}-X_{5°C}) / X_{25°C}$, where X denotes the weight percent value of an individual molecular species.

2. Short-term acclimation to 25°C was evaluated by comparison to the starting, winter-adapted state, as: $-100x(X_{5°C}- (X_{\uparrow 25°C})) / X_{5°C}$.

3. Short-term acclimation to 5°C was evaluated by comparison to the starting, summer-adapted state as: $-100x(X_{25°C}-(X_{\downarrow 5°C})) / X_{25°C}$.

The molecular species displaying the greatest changes were selected and will be discussed in the following section.

During winter-adaptation the amount of **18:1/22:6** PC increased by 108.9% in plasma membrane and by 63.6% in mitochondria when compared to its summer-adapted levels (Fig. III.1, PC).

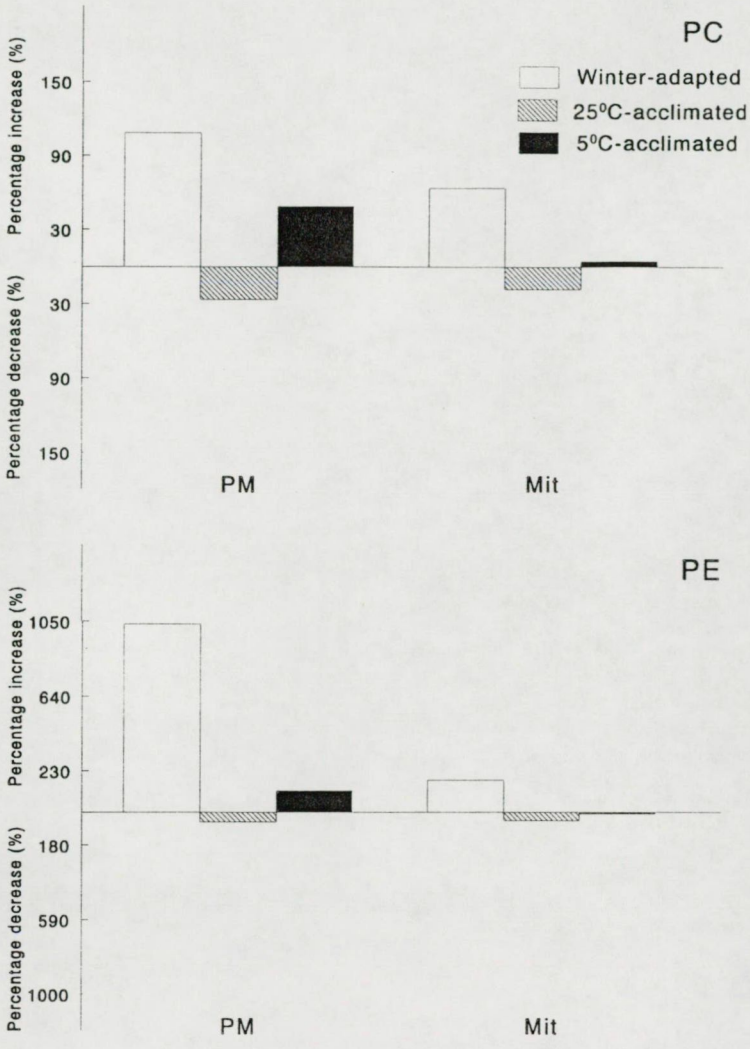


Figure III.1. Percentage of change of **18:1/22:6** molecular species in PC and PE phospholipid classes during winter-adaptation, short-term acclimation at 25°C and short-term acclimation at 5°C in isolated plasma membranes (PM) and in mitochondria (Mit). Values were calculated by assuming summer adaptation as reference (see text).

The trend of change was similar in case of short-term acclimation at 5°C of the summer-adapted fish: 18:1/22:6 increased by 49% in the plasma membrane and by 4.5% in mitochondrial PC. In the reverse acclimation experiments, when winter–adapted fishes were acclimated to 25°C, the level of the 18:1/22:6 PC decreased by 26.6% in plasma membrane and by 18.55 in mitochondria, when compared to the control (winter-adapted state).

The changes in the level of this species were qualitatively similar in the PE as well, but the response was even of higher magnitude (Fig. III.1, PE). Winter-adaptation increased the level of this species more than ten folds in plasma membranes and 1.5 fold in mitochondria. Similarly, during short-term acclimation the level of 18:1/22:6 PE was reduced during temperature up-shift (by 51% in plasma membrane and by 43% in mitochondria). Upon the downshift of the temperature from 25°C to 5°C, 18:1/22:6 PE

increased 1.21 fold in plasma membrane, while in the mitochondria remained almost unchanged.

The level of **16:0/18:1** molecular species varied also upon changes of the adaptation/acclimation temperatures.

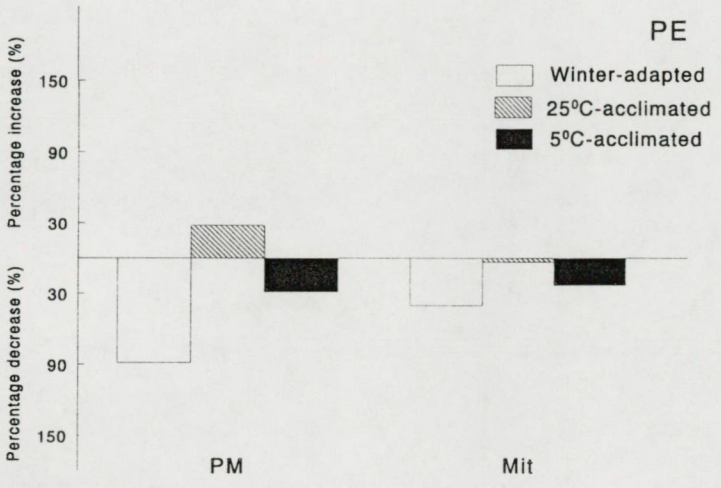


Figure III.2. Percentage of change in the level of **16:0/18:1 PE** molecular species in liver plasma membranes (PM) and mitochondria (Mit) of fish, during winter-adaptation and short-term acclimation at 25 and 5°C. Symbols are as indicated in the figure.

In the PE fraction it has decreased drastically during winter-adaptation: in plasma membrane decreased by 88,7% and in mitochondria by 40% (Fig. III.2, PE). During the short-term acclimation experiments the trend of changes were similar but of much lower magnitude than in the case of seasonal adaptation. In the plasma membrane during 25°C- acclimation 16:0/18:1 accumulated by 27.5% and by 19.6% in PE and PC, respectively (PC not shown) whereas, during acclimation at 5°C decreased by about the same factor (by 29% and 28%, respectively). In mitochondria 16:0/18:1 decreased only by 3.2% in PE during 25°C-acclimation but by 38.2% in the PC. Upon 5°C-acclimation, the amount of 16:0/18:1 decreased by 23% in PE and 11% in PC.

In response to winter-adaptation, the **16:0/20:4** molecular species decreased both in PC and PE (Fig. III.3 PC and PE) of mitochondria and plasma membranes, except in the case of the plasma membrane PE, where a 1.2 fold increase was detected.

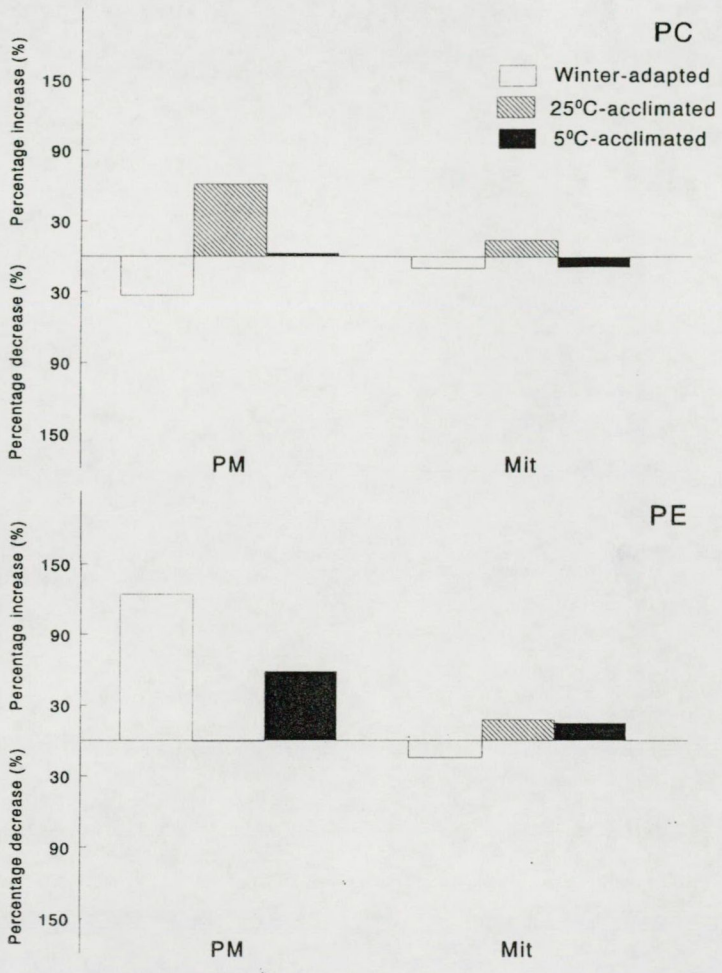


Figure III.3. Percentage of change in the level of **16:0/20:4 PC** and **PE** molecular species in liver plasma membranes (PM) and in mitochondria (Mit) of fish, during winter-adaptation and short-term acclimation at 25 and 5°C. Symbols are as indicated in the figure.

Contrary to winter-adaptation, during short-term cold-acclimation the level of 16:0/20:4 increased among all, except in mitochondria PC. During short-term acclimation at 25°C, its level increased in both lipid class and type of membrane.

In response to cold-adaptation, the amount of **18:0/20:4** species has increased in PC (by four fold in mitochondria and by 61% in plasma membranes) and decreased in PE (Fig. III.4, PE and PC). This species is among the major components in PE (Table III.1 and 2). Cold-acclimation initiated opposite changes and in the same direction (decrease) as warm-acclimation, (except in PE of mitochondria, where it increased by 8,6%). The changes of the level of this species in mitochondria were in general much less, in both PC and PE, than in plasma membranes.

The trend of the variation of **18:0/22:6** molecular species was similar in both membrane fractions within PC and PE (Fig. III.5, PE and PC). As a response to short-term acclimation the level of this species increased irrespective of the direction of temperature change and the magnitude of variation was less in PE than in PC. During seasonal adaptation 18:0/22:6 species increased by 71% in PE and remained almost unchanged (decreased by 3,3%) in PC of plasma membrane. In mitochondria the level of this species decreased in PC by 23,7% and increased by 45% in PE.

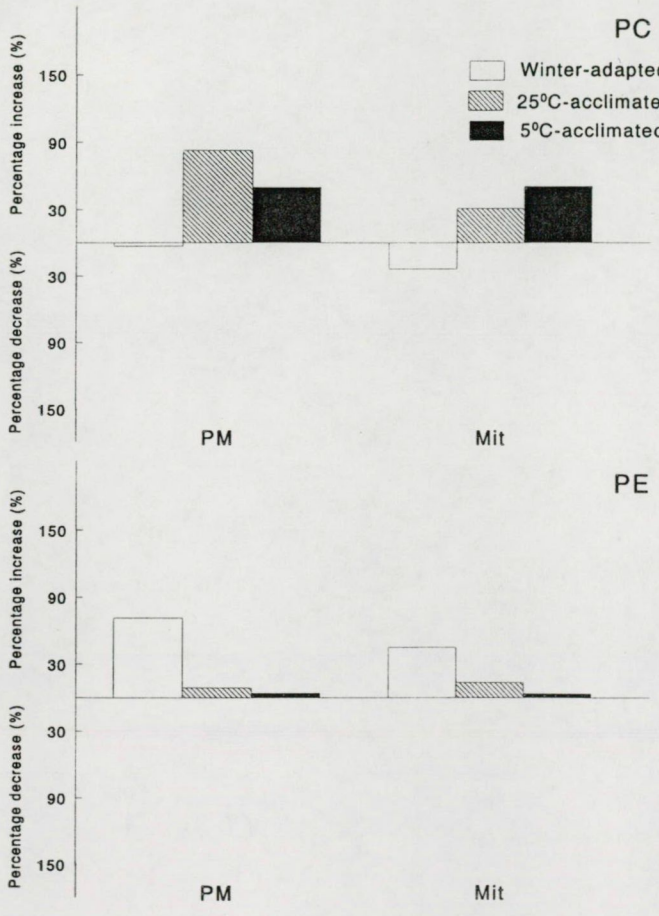


Figure III.4. Percentage of change in the level of **18:0/22:6 PE** and **PC** molecular species in liver plasma membranes (PM) and mitochondria (Mit) of fish, during winter-adaptation and short-term acclimation at 25 and 5°C.

It is to be noted, that some molecular species such as **18:1/20:4**, **16:0/22:6** and **16:0/20:4** always increased, irrespectively of the direction of the short-term acclimation. Therefore these species may be responsive to the temperature "stress" and may not be directly related to cold- or warm-acclimation.

III.2. Membrane fluidity assessed by DPH steady-state fluorescence anisotropy

The steady-state fluorescence anisotropy values of DPH measured either in the liver plasma membranes or in the mitochondria, were lower in seasonally winter-adapted fishes, than in the summer adapted ones (Fig. III.5).

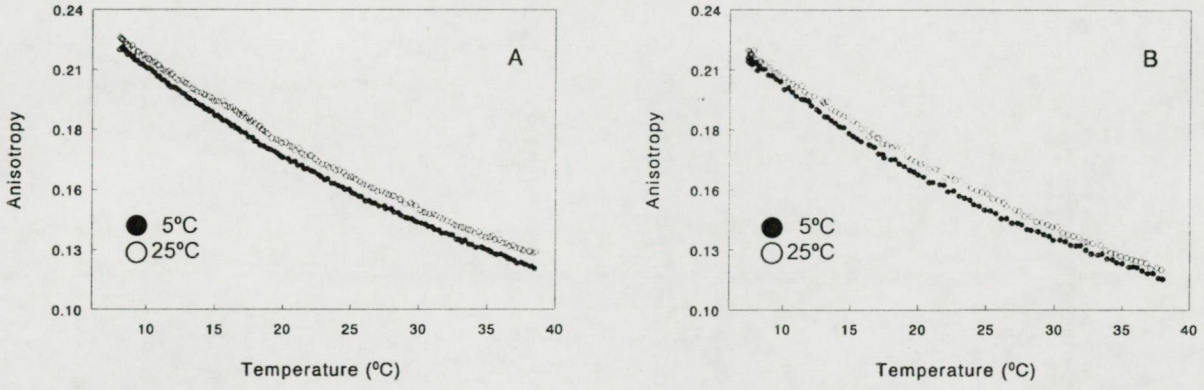


Figure III.5. The steady-state fluorescence anisotropy of DPH incorporated into membranes isolated from livers of fishes seasonally adapted to summer and winter, showing higher fluidity (lower anisotropy values) at winter-adaptation for both types of membranes. **A: plasma membranes**; **B: mitochondria**. Open symbols represent 25°C (summer-), filled symbols 5°C (winter-) adaptation, respectively.

When summer and winter adapted fishes were subjected to temperatures opposite to their adaptational temperature a change in the fluidity, as measured by DPH steady-state fluorescence anisotropy, was detectable in their liver membranes (Fig.III.6 and 7).

The anisotropy values measured in plasma membranes isolated from temperature up-shifted fishes were higher than the control (5°C-adapted). This increase, however, was considerable only up to 25°C in the thermotropic responses of the anisotropies, above 25°C the two curves merged (Fig.III.6.A). The same changes occurred in the fluidity of plasma membranes during the temperature downshift experiments (Fig. III.6.B), being the membranes more fluid (lower anisotropy values) at low acclimation temperatures.

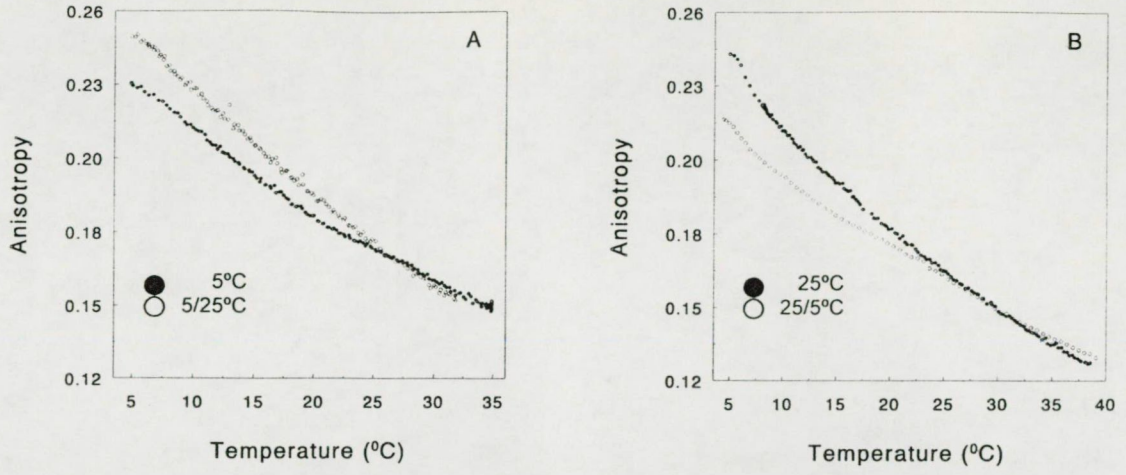


Figure III.6. Steady-state anisotropy temperature profiles of DPH in the **plasma membrane** extracts of livers of seasonally adapted and short-term acclimated fishes. A: winter-adapted (filled symbols), temperature up-shifted (open symbols) fish; B: summer-adapted (filled symbols), temperature downshifted (open symbols) fish.

Mitochondrial membranes showed similar and more pronounced alterations in the membrane fluidity during short-term acclimation than the plasma membranes (Figure III.7). The DPH steady-state anisotropy values were higher in mitochondria isolated from

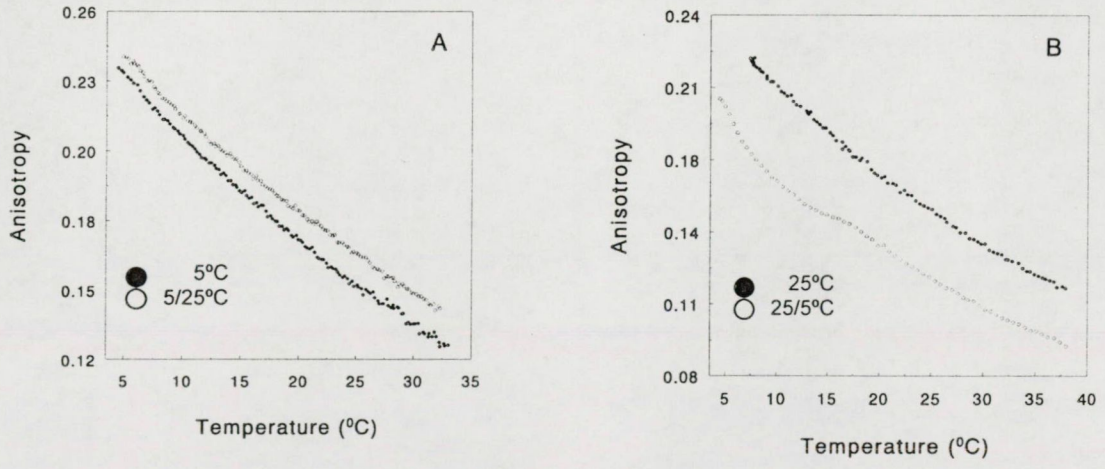


Figure III.7. Steady-state anisotropy of DPH incorporated into **mitochondria** of livers of seasonally adapted or short-term acclimated fishes. A: winter-adapted (filled symbols), temperature up-shifted (open symbols) fish; B: summer-adapted (filled symbols), temperature downshifted (open symbols) fish.

livers of temperature up-shifted fishes than those measured in the control i.e. winter-adapted fish membranes (Fig. III.7.A). In contrast, the downshift of the temperature resulted the fluidization of the membranes (Fig. III.7.B).

IV. ENVIRONMENTAL TEMPERATURE AND DIETARY FATTY ACIDS IN CONTROLLING MEMBRANE FLUIDITY

Experiments aimed to reveal the effect of the ambient temperature on membrane lipid composition and biophysical properties are generally carried out on fish kept on diets of constant lipid composition with the temperature being the only variable (Cossins, 1977; Wodtke, 1978; Hazel, 1979). Under natural conditions, however, different fish species in the same habitat consume foods differing in their fatty acid composition. Thus, during the process of homeoviscous adaptation (HVA) the dietary fatty acids and the ambient temperature may exert a combined effect on the composition of structural lipids and hence on the "fuidity" of membranes.

To study the impact of the diet on these parameters four fish species of different feeding habit, living in same habitat during the course of adaptation to low temperatures were chosen. The fish species investigated were the silver carp, *Hypopthalmichthys molitrix*, V., which feeds mostly on phytoplankton, the big head carp, *Aristichthys nobilis*, R., feeding mostly on zooplankton, while the grass feeding carp, *Ctenopharyngodon idella*, feeding on aquatic weeds. The common carp, *Cyprinus carpio* L. is fed in fishponds with corn and cereals but in its natural environment also consumes organisms in the zoobenthos. Analysis of gut contents of silver carp showed rests of Diatomaceae, Scendesmus, Crucigenia, Euglena, Phacus, Keratella, Glenoidium but also postabdomens and segments of different Cladocerans. The digestive tract of big head carp has Rotifers and postabdomens and segments of different Cladocerans. (Farkas *et al.*, 1980). At the time of sampling the water temperature was at 5±2°C for one month, thus the fish could be regarded as cold adapted or adapted to 5°C.

Data on the fatty acid composition of the food ingested by these fish species (given in Table IV.1) were collected from the literature (Farkas, 1970, 1979; Sebedio and Grandgirard, 1983; Ahlgren *et al.*, 1992) except for the diatoms which were sampled from the Lake Balaton and analysed in our laboratory.

Table IV. 1. Fatty acid composition of the foods of the four fresh water fish.

Fatty acid	GREEN ALGAE[1] (*C. idella*)	CORN OIL[2] (*C. carpio*)	DIATOMS[3] (*H.molitrix*)	CLADOCERA[4] (*A. nobilis*)	COPEPODA[5] (*A. nobilis*)
14:0	-	-	4.9	4.4	3.0
16:0	46.6	10.4	12.2	11.8	18.5
16:1	2.6	-	15.3	11.6	8.8
18:0	6.3	2.0	3.8	4.8	5.9
18:1n-9	9.7	26.9	5.3	12.9	11.0
18:2n-6	13.2	58.5	3.7	5.7	6.7
18:3n-3	21.5	0.9	1.4	7.6	7.2
20:2n-6	-	-	0.3	0.2	0.2
20:3n-6	-	-	0.2	0.9	1.0
20:4n-6	-	-	1.5	3.0	3.4
20:4n-3	-	-	tr.	0.5	0.8
20:5n-3	-	-	15.3	9.1	5.3
22:4n-6	-	-	0.3	0.8	0.9
22:5n-6	-	-	0.3	0.6	1.9
22:5n-3	-	-	0.3	0.3	1.2
22:6n-3	-	-	2.1	1.5	5.2
Σ LcPUFA	-	-	**20.4**	**16.8**	**19.8**
S/U	**1.17**	**0.14**	**0.45**	**0.38**	**0.51**

[1]: average of 3 species (Ahlgren *et al.*, 1992), [2]:average of 200 species (Sebedio and Grandgirard, 1989) [3]: average of 8 species (Farkas, 1979),[4]: average of 15 species (Farkas, 1979), [5]: average of 5 species (Farkas, 1979). Note: "n" is identical with "ω" in the symbols of the fatty acids.

We have no data on the fatty acid composition of aquatic weeds, the food of *C. idella*, but it might be similar to the green algae. Both green algae and corn seeds, the food of *C. idella* and *C. carpio*, do not contain long-chain polyunsaturated fatty acids (LcPUFA). Their fatty acids range from palmitic, 16:0, to linolenic, 18:3, acids. Lipids of green algae are rich in 16:0, 18:2n-6 and 18:3n(ω)-3, the typical fatty acids of chloroplasts, while corn (and wheat) are rich in linolenic acid.

Both Diatoms, the dominant species of phytoplankton, and Cladocera and Copepoda, the food of *H. molitrix* and *A. nobilis*, besides short chain fatty acids, contain

LcPUFAs, especially arachidonic, 20:4, eicosapentaenoic, 22:5 and docosahexaenoic, 22:6, acids.

IV. 1. The fatty acid composition of the liver triacylglycerols, of the total phospholipids, and of the phospholipids extracted from the plasma membranes isolated from fish liver.

The fatty acids present in triacylglycerols and phospholipids from fish livers are shown in Table IV. 2 and the fatty acid composition of liver plasma membranes are given in Table IV. 3.

Table IV. 2. Fatty acid composition of **triacylglycerols** and **phospholipids** in livers of cold-adapted fresh water fishes as weight percentages (%).

	TRIACYLGLYCEROLS				TOTAL PHOSPHOLIPIDS			
Fatty acid	*H.mol*	*A.nob.*	*C.ide.*	*C.car.*	*H.mol.*	*A.nob.*	*C.ide.*	*C.car.*
14:0	4.2	3.1	2.4	1.0	0.4	0.2	0.2	0.3
i16:0	1.0	0.4	nd	nd	0.6	0.4	0.5	0.3
16:0	17.9	17.6	23.8	19.2	14.5	18.2	21.9	21.8
16:1	15.7	17.6	23.8	9.5	2.7	2.9	7.4	6.2
18:0	3.1	2.1	1.5	5.2	4.7	4.9	5.6	4.2
18:1	27.0	21.0	39.6	52.5	11.4	15.1	12.0	16.2
18:2n-6	2.9	4.0	6.6	7.2	0.9	0.9	3.4	3.4
18:3n-3	5.1	5.7	1.0	2.9	1.2	tr.	0.8	0.7
20:2n-6	0.3	tr.	1.5	0.6	1.1	tr.	1.2	2.4
20:3n-6	0.4	0.1	0.5	0.8	0.5	tr.	0.8	4.1
20:3n-3	0.7	0.4	tr.	0.3	0.3	tr.	2.1	1.8
20:4n-6	2.5	2.8	0.5	0.4	6.1	5.0	9.6	9.2
20:5n-3	4.8	8.5	0.4	tr.	10.1	7.4	5.4	3.7
22:3n-6	0.4	tr.	0.6	tr.	tr.	tr.	0.6	0.1
22:4n-6	2.1	1.7	0.4	tr.	2.1	tr.	0.4	0.7
22:5n-6	0.8	1.2	0.4	tr.	4.1	1.7	2.4	0.6
22:5n-3	3.3	2.4	0.4	tr.	2.9	6.7	5.1	4.7
22:6n-3	7.8	8.9	0.1	0.1	36.2	36.1	20.6	19.6
Σ LcPUFA	**23.1**	**26**	**8.4**	**2.2**	**63.6**	**57.4**	**48.2**	**46.9**
S/U	**0.35**	**0.31**	**0.36**	**0.34**	**0.25**	**0.31**	**0.39**	**0.36**

H.mol.: *Hypopthalmichthys molitrix; A.nob.*: *Aristichthys nobilis*; *C.ide.*: *Ctenopharyngodon idella*; *C.car.*: *Cyprinus carpio*. S: saturated fatty acids; U: unsaturated fatty acids; LcPUFA: long chain polyunsaturated fatty acids. Σ LcPUFA: the sum of long-chain (>20:1) polyunsaturated fatty acids. nd: not detected; tr.: trace amount.

Table IV. 3. Fatty acid composition of total phospholipids extracted from isolated **plasma membranes** of livers of the four cold-adapted fresh water fishes as weight percentages (%).

Fatty acid	*H.mol.*	*A.nob.*	*C.ide.*	*C.car.*
14:0	1.2	3.0	1.8	1.2
15:0	0.4	tr.	0.3	0.1
16:0	15.6	23.0	23.0	19.4
16:1	4.5	7.5	7.0	6.0
16:2	2.5	tr.	0.7	0.7
16:3	0.7	tr.	tr.	0.2
18:0	4.7	5.4	6.0	7.8
18:1n-9	15.8	16.5	18.4	18.5
18:2n-6	1.5	2.4	4.3	2.6
18:3n-3	1.3	1.4	2.5	3.0
18:4n-3	0.3	0.8	tr.	0.2
20:1n-9	1.1	0.5	1.1	0.3
20:3n-6	0.6	0.9	3.2	3.9
20:3n-3	0.3	tr.	1.6	1.5
20:4n-6	6.3	5.7	5.8	9.5
20:4n-3	0.9	tr.	0.8	0.5
20:5n-3	5.5	6.7	1.4	1.5
22:4n-6	1.4	1.2	1.4	1.8
22:5n-6	1.4	1.3	6.1	1.2
22:5n-3	4.8	2.2	2.8	3.3
22:6n-3	29.2	21.5	11.8	16.8
Σ LcPUFA	**50.4**	**39.5**	**34.9**	**40.0**
S/U	**0.28**	**0.45**	**0.45**	**0.39**

H.mol.: *Hypopthalmichthys molitrix; A.nob.*: *Aristichthys nobilis*; *C.ide.*: *Ctenopharyngodon idella*; *C.car.*: *Cyprinus carpio*. S: saturated fatty acids; U: unsaturated fatty acids; LcPUFA: long chain polyunsaturated fatty acids. tr.: trace amount.

The fatty acid composition of the liver triacylglycerols resembles that of the potential foods shown in Table IV. 1. Note that 18:2 and 18:3, the major fatty acids in green algae, corn and diatoms, were present only in low amounts in the phospholipids of *C. idella*, *C. carpio* and *H. molitrix*. *A. nobilis* has also low amounts of these fatty acids in comparison to their cladoceran food (Table IV. 1). In all species the levels of long-chain polyunsaturated fatty acids are much higher than those available in their food indicating their capacity to elongate and desaturate the precursor linolenic and linoleic acids. The amounts of docosahexaenoic acid, are almost two times higher than in the liver phospholipids of *H. molitrix* and *A. nobilis* as compared to *C. idella*, and *C. carpio* (Table

IV. 2). Another characteristic difference between the two pair of species: *C. idella, C. carpio* and *H. molitrix* and *A. nobilis,* was the content of arachidonic acid: its level was three times higher in *C. idella* and *C. carpio* than in *H. molitrix* and in *A. nobilis*.

The fatty acid composition of plasma membranes (Table IV. 3) was more uniform among the four fish species than that of total phospholipids (Table IV. 2). Only docosahexaenoic acid decreases from the 29% of the *H.molitrix* to 11% of the *C. idella*.

IV. 2. The fluidity of plasma membranes

Figure IV. 1 shows the steady-state fluorescence anisotropy values obtained for the fluorescent probe DPH, incorporated into isolated plasma membranes from the livers of the fish species.

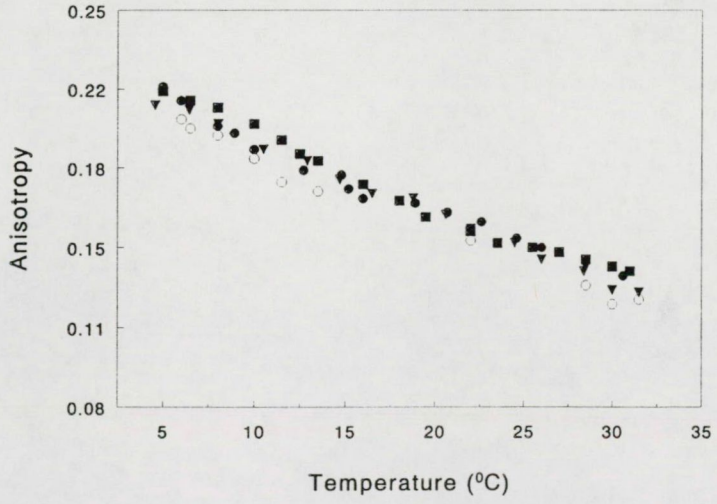


Figure IV. 1. The temperature dependence of the steady-state fluorescence anisotropy of DPH embedded in plasma membranes isolated from livers of fresh water fish of different feeding habit. Open circles: *A. nobilis*; filled circles: *C. idella*; filled squares: *H. molitrix*; filled triangles: *C. carpio.*

The DPH fluorescence polarization technique did not detect differences in the fluidity of the plasma membranes of the different fish species. The rotational correlation time of the 16-doxylstearic acid was shorter in the plasma membranes of *H. molitrix* than

in *C. carpio* below about 25°C (Fig. IV. 2) indicating that plasma membranes in the former are more fluid below this temperature.

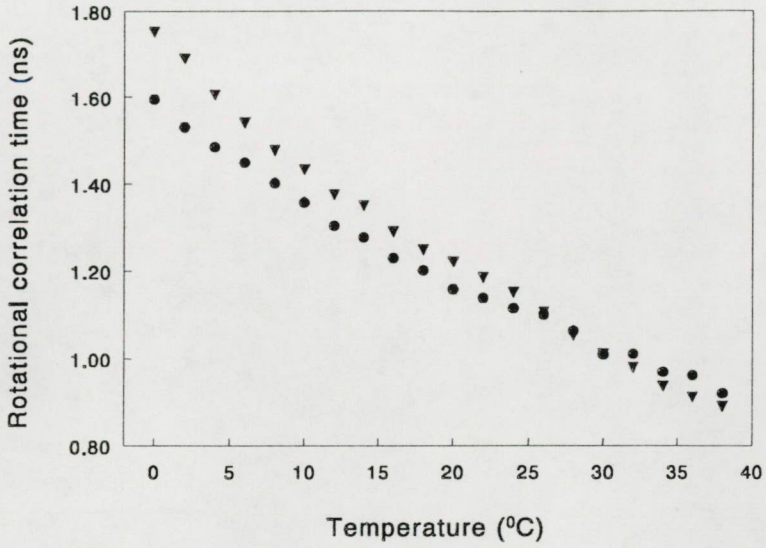


Figure IV. 2. The temperature dependence of the rotational correlation time of 16-doxylstearic acid embedded in liver plasma membranes of carp *C. carpio* and silver carp, *H. molitrix*. Triangles: *C. carpio*; circles: *H. molitrix*.

IV. 3. Molecular species composition of diacyl phosphatidylcholines and phosphatidylethanolamines extracted from the isolated plasma membranes.

The molecular species were separated from both diacyl phosphatidylcholines and diacyl phosphatidylethanolamines of plasma membranes (Table IV. 4).

Although there were differences in the amount of individual molecular species of the four fish species investigated, the total amount of Sat/Unsat species was rather similar in both PE and PC of the four fishes investigated (Table IV. 4). *H. molitrix* and *A. nobilis* contained higher amounts of monounsaturated/polyunsaturated species than *C. idella* and *C. carpio,* which may explain the higher mobility of 16-SASL probe in the plasma membranes of *H. molitrix* when compared to *C. carpio*.

Table IV. 4. The molecular species composition of diacyl PC and diacyl PE of liver **plasma membranes** of cold-adapted fresh water fishes of different feeding habit. The values represent the weight percentages (%) of molecular species.

Molecular species	PC				PE			
	H.mol.	*A.nob.*	*C.ide.*	*C.car.*	*H.mol.*	*A.nob.*	*C.ide.*	*C.car.*
16:0/16:0	1.3	0.7	1.1	0.9	0.7	1.7	1.2	3.4
16:0/18:0	nd	nd	0.5	nd	nd	nd	nd	0.5
Σ(Sat/Sat)	1.3	0.7	1.6	0.9	0.7	1.7	1.2	3.9
16:0/22:6	30.0	34.7	16.0	24.9	17.5	22.1	8.0	9.9
16:0/22:5	7.5	5.5	3.5	2.1	7.3	6.6	nd	nd
16:0/20:5	7.1	6.3	2.5	11.5	10.4	nd	nd	nd
16:0/20:4	2.4	4.5	12.0	5.9	3.7	7.5	14.9	10.2
16:0/18:2	nd	nd	7.2	nd	nd	nd	4.9	7.1
16:0/18:1	8.1	5.3	7.4	12.0	0.5	3.5	2.7	2.2
18:0/22:6	5.1	4.5	6.8	9.2	13.5	11.4	7.9	9.9
18:0/22:5	1.8	1.1	4.5	nd	2.6	1.4	nd	nd
18:0/20:5	nd	nd	7.6	1.5	7.3	nd	7.5	3.2
18:0/20:4	3.7	4.3	2.0	2.7	2.5	2.3	14.7	16.5
18:0/16:1	nd	0.7	1.3	nd	3.3	nd	5.3	nd
Σ(Sat/Unsat)	**66**	**67**	**71**	**70**	**68.6**	**55**	**66**	**59**
16:1/20:5	0.1	nd	nd	nd	0.2	0.1	nd	nd
16:1/20:4	2.3	2.0	nd	2.0	1.5	1.3	nd	1.3
18:1/22:6	16.8	16.1	9.2	9.5	23.0	20.1	8.4	11.7
18:1/22:5	8.7	6.2	1.5	0.7	10.1	6.7	nd	4.0
18:1/20:5	3.2	3.4	2.7	nd	nd	nd	2.7	2.2
18:1/20:4	4.5	3.7	6.7	11.6	6.9	7.1	15.7	11.7
Σ(Mono/Poly)	**35.6**	**31.4**	**20.1**	**23.8**	**41.7**	**35.3**	**26.8**	**30.9**
20:5/22:6	0.1	0.1	2.6	nd	0.1	0.1	3.9	nd
22:6/22:6	0.1	1.3	0.5	0.3	0.1	0.1	0.3	0.2
20:4/20:4	4.1	5.0	3.5	4.9	3.1	3.4	1.1	2.2
18:1/18:1	1.9	0.8	3.0	1.3	1.4	2.1	0.6	0.4
Σ(Sat/Unsat) / **Σ(Mono/Poly)**	**1.8**	**2.1**	**3.5**	**3.0**	**1.64**	**1.55**	**2.45**	**2.0**

H.mol.: *Hypopthalmichthys molitrix; A.nob.*: *Aristichthys nobilis*; *C.ide.*: *Ctenopharyngodon idella*; *C.car.*: *Cyprinus carpio*. PC: phosphatidylcholine, PE: phosphatidylethanolamine. nd: not detected.

V. CHANGES IN THE LIVER LIPID COMPOSITION OF THE FROG *Rana esculenta* DURING ITS SEASONAL ADAPTATION TO LOW TEMPERATURE

The body temperature of frogs follows the changes in the ambient temperature but during winter they undergo hibernation. In the studies presented below the lipid composition of the liver and the fluidity of the phospholipid membranes were compared on active and hibernating frogs in order to see whether they utilise the same adjustments in the lipid composition and membrane fluidity as the winter-active poikilotherms or not.

V.1. Fatty acid analysis of the total phospholipid extract of liver

More than 20 different fatty acids, ranging from 14:0 to 22:6, were found in frog liver phospholipids. The major (>1%) fatty acids in total phospholipid (TPL) extracts, in phosphatidylcholines (PC) and in phosphatidylethanolamines (PE) are shown in Table V.1. The percentage of changes occurring in the level of individual fatty acids when frogs were turning from active state to hibernation are shown in Fig. V.1.

In the total phospholipids levels of 18:0, 18:1, 18:2, 20:5 and 22:5 increased during hibernation, while 16:0, 16:1, 16:2, 18:3, 20:4, 22:6 decreased. As a consequence the saturated-to-unsaturated fatty acid ratio decreased only by 9% during hibernation (Figure V.1.A). In both lipid classes there was a pronounced decrease of 16:0, 20:4 and 22:6 fatty acids during hibernation. On the other hand, there was an increase in the level of several fatty acids like 18:1 (40% increase in PE and 13% in PC), 18:2 (30% in PE and 33% in PC) 20:5 (73% in PE and 78% in PC). The amount of the linolenic acid increased significantly in PC but not in PE.

Table V.1.: The fatty acid composition of phospholipids extracted from livers of active and hibernating frogs.

Fatty acid	ACTIVE STATE			HIBERNATING STATE		
	TPL	PC	PE	TPL	PC	.PE
	weight %					
16:0	23.6	29.6	18.1	18.8	27.5	13.8
16:1	2.9	2.5	0.7	1.7	2.6	1.0
16:2	1.0	0.8	1.2	0.8	0.7	1.0
18:0	4.2	2.5	11.5	7.7	2.6	11.2
18:1	9.4	9.6	8.9	9.5	10.9	12.5
18:2	18.3	22.6	11.6	20.6	30.2	15.2
18:3	4.5	4.3	3.3	3.6	4.6	3.6
20:4	13.7	7.3	20.3	12.1	5.3	14.4
20:5	6.8	2.7	7.2	11.0	4.8	11.9
22:5	1.4	0.13	1.9	2.2	1.6	2.9
22:6	9.7	5.8	14.9	7.2	4.6	9.3
S/U	**0.41**	**0.57**	**0.42**	**0.38**	**0.46**	**0.35**

S/U: saturated-to-unsaturated fatty acid ratio. The livers of ten frogs were pooled for analysis at each state.

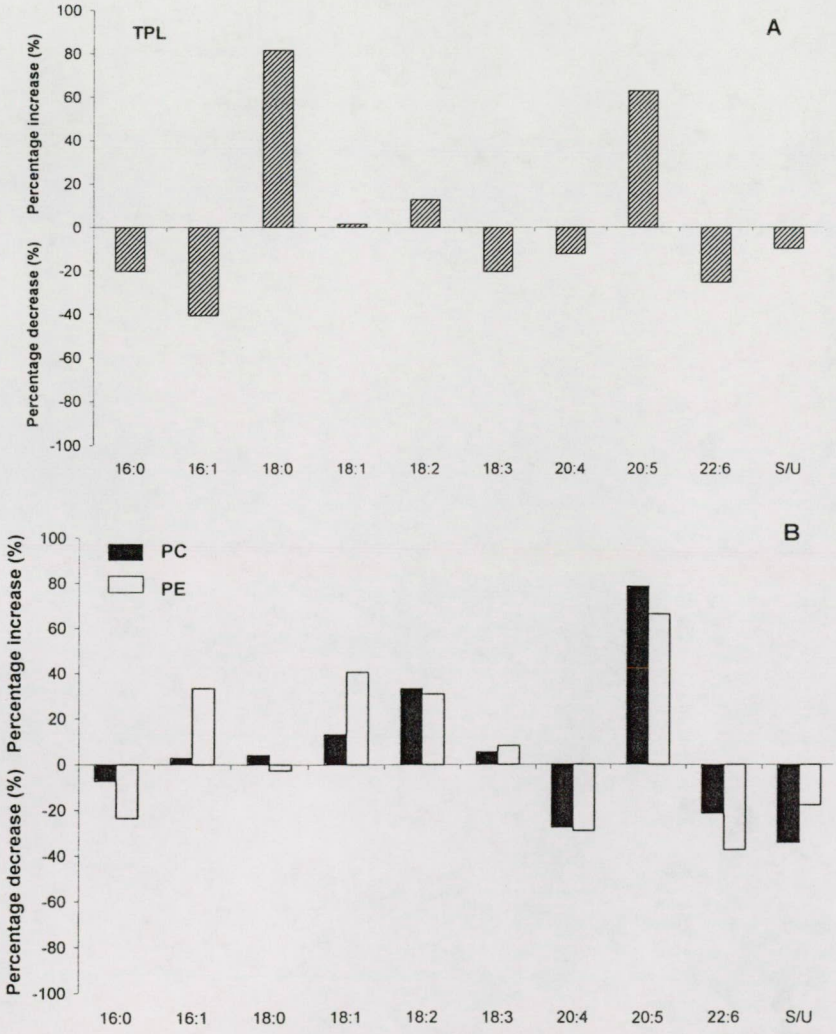


Figure V. 1: The percentage of change of the major fatty acids upon winter-adaptation. The values corresponding to summer-adapted state were taken as reference. A: Total phospholipid extract of livers (TPL); B: Fatty acids of phosphatidylcholines (PC, filled bars) and phosphatidyl-ethanolamines (PE, open bars). S/U represents the saturated-to-unsaturated fatty acid ratio.

V.2. Molecular species composition of diacyl PC and PE lipid classes of liver

The molecular species composition of diacyl PC and PE lipid classes of liver were analysed and compared in the active and hibernating states of the frogs (Table V.2).

Table V.2. The molecular species composition of diacyl PC and PE lipid classes presented as weight percentages (%).

	PC		PE	
	Summer	Winter	Summer	Winter
16:0/22:6	11.3	11.5	16.9	8.8
16:0/20:4	6.0	3.9	10.2	4.4
18:0/20:5	4.4	8.0	0.7	6.7
16:0/18:2	0	0	11.6	4.9
16:0/18:1	0.2	0.9	1.2	1.8
18:0/22:6	35.7	39.8	15.9	11.7
18:0/22:5	0.2	0.4	0.7	1.2
18:0/22:4	1.7	0.7	0.2	0.1
18:0/20:4	3.2	2.3	16.0	9.7
Σ Sat/Unsat	**62.7**	**67.5**	**73.3**	**49.4**
18:1/22:6	4.7	5.4	6.2	6.0
18:1/22:5	4.3	3.5	2.4	1.8
18:1/20:4	1.4	1.7	5.3	5.5
Σ Mono/Poly	**10.3**	**10.5**	**13.9**	**13.2**
20:5/22:6	0.3	1.2	0.9	1.5
20:4/20:4	0.6	2.2	1.2	1.8
ΣPoly/Poly	0.9	3.3	2.3	3.3
16:0/16:0	14.9	10.9	8.0	7.1
16:1/16:1	0.3	0.6	1.5	3.4
Σ Sat/Unsat				
Σ Mono/Poly	**6.1**	**6.4**	**5.3**	**3.7**

Sat: saturated fatty acid. Unsat: unsaturated fatty acid. Mono: monounsaturated fatty acid. Poly: polyunsaturated fatty acid. Σ: represents the sum.

The most pronounced differences were found in the levels of *sn*-1 saturated, *sn*-2 unsaturated (Sat/Unsat) and *sn*-1 polyunsaturated, *sn*-2 polyunsaturated (Poly/Poly) lipid species. In the PE fraction there is a pronounced decrease (32%) in the level of the total Sat/Unsat species (like 16:0/22:6, 16:0/20:4, 16:0/18:2, 18:0/22:6, 18:0/20:4) during hibernation and an increase of Poly/Poly species (by 40%). In PC the Sat/Unsat species were much less responsive than in PE (Fig. V.2).

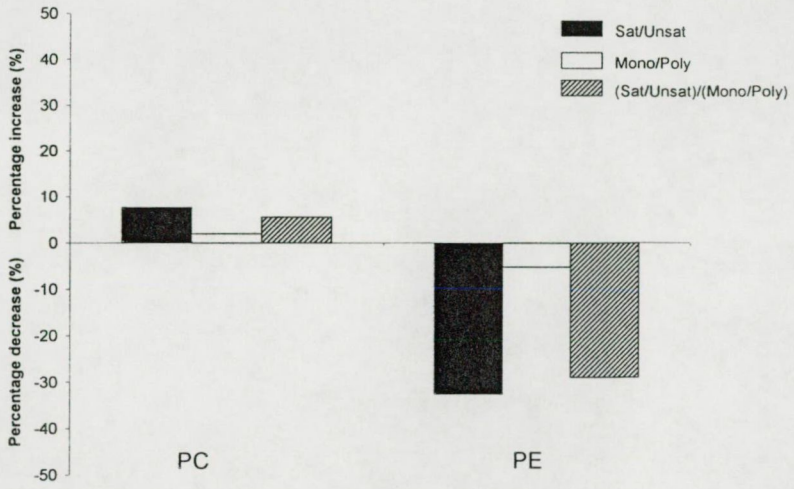


Figure V. 2. The percentage of change of the total saturated/unsaturated (Σ(Sat/Unsat), filled bars); monounsaturated/poly-unsaturated (Σ(Mono/Poly), open bars) molecular species, and of their ratio (Sat/unsat)/(Mono/Poly), (dashed bars), in PC and PE lipid classes.

It is interesting to note that the levels of *sn*-1 monoenoic, *sn*-2 polyenoic molecular species (like 18:1/22:6) which are the major species that increase during cold adaptation in fishes did not change during the hibernating frogs, neither in PC nor in PE (Fig. V.2).

V.3. The fluidity of phospholipid vesicles

The steady-state fluorescence anisotropy values of 2-AS, 12-AS and 16-AP anthroyloxy fatty acids embedded in vesicles made of total phospholipid extracts of livers of both active and hibernating frogs are shown in Fig. V.3.

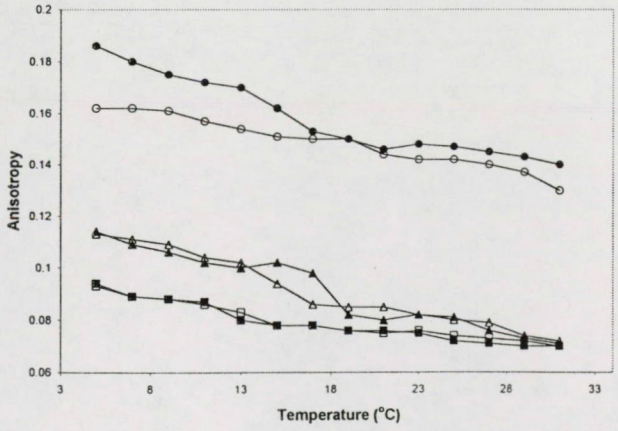


Figure V. 3. Steady state-fluorescence anisotropy of the anthroyloxy fatty acids 2-AS (filled and open circles), 12-AS (filled and open triangles) and 16-AP (filled and open squares) embedded in the vesicles made of total phospholipids of frog liver. Filled symbols stand for hibernation state and open symbols for active state.

At the region of C-12 and C-16 carbon atoms of the acyl chains in the bilayer, the anisotropy parameters assessed by 12-AS and 16 AP, respectively, show no difference

between the physical characteristics of the lipid membranes of active and hibernating states. At the region of C-2 carbon atom, the phospholipid vesicles prepared from lipids of hibernating species seem to have even more rigid structure than those of active state contrary to the classical fluidization at cold temperatures observed in winter-active fish species.

VI. THE POSSIBLE ROLE OF SPECIFIC LIPIDS IN REGULATING THE PHYSICAL PROPERTIES OF THE MEMBRANES DURING THERMAL ACCLIMATION

To understand the role of changes in phospholipid molecular species during thermal adaptation, model experiments on vesicles made from selected synthetic phospholipids were designed. It seemed logical to decide first whether the downshift of the onset of the phase separation, and the lower order of vesicles made either of total phospholipids or of phosphatidylcholines of winter-adapted fishes compared to summer-adapted ones (Fig.II.1 and 2, chapter II) can be related to the molecular species alterations or not.

Figure VI.1 compares the temperature profiles of the outer hyperfine splitting parameters of 5-SASL spin probe incorporated into pure (100%) 18:0/22:6 phosphatidylcholine vesicles with ternary mixtures of synthetic phosphatidylcholines. The composition of the ternary mixtures was chosen based on the data shown in Table II.2, Chapter II of Results, from the major PC components of the fish liver total phospholipids, and the mixing ratios according to the characteristic values present in a cold- and a warm-adapted fish, respectively.

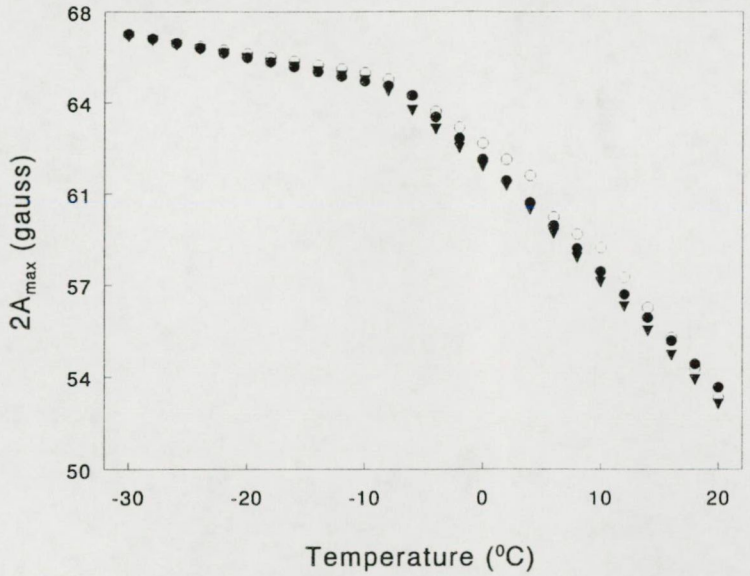


Figure VI.1. Temperature profiles of the outer hyperfine splitting of 5-doxylstearic acid spin label incorporated into vesicles made from 18:0/22:6 PC (▼) or from mixed phospholipid vesicles consisting of either 1% 16:0/16:0 PC, 10% 20:4/20:4 PC and 89% 18:0/22:6 PC (●) or 4% 16:0/16:0 PC, 2% 20:4/20:4 PC and 94% 18:0/22:6 PC (○).

Vesicles made of 4% 16:0/16:0, 2% 20:4/20:4, and 94% 18:0/22:6 phosphatidylcholines were chosen to mimic the phosphatidylcholines of summer-adapted (SA) fishes. The vesicles made of 1% 16:0/16:0, 10% 20:4/20:4 and 89% 18:0/22:6 phosphatidylcholine were chosen to mimic the phosphatidylcholines of winter-adapted (WA) fishes. The temperature value where the change in the slope of the curve (Fig. VI.1) corresponding to the 18:0/22:6 PC coincides with the main, gel-to-liquid-crystalline transition temperature of the lipid. As seen in Fig. VI.1, the addition of 4% 16:0/16:0 phosphatidylcholine along with 2% 20:4/20:4 phosphatidylcholine did not significantly affect the transition temperature of 18:0/22:6 (100%) phosphatidylcholine (-8.8°C vs. - 8.4°C). Increasing the amount of 20:4/20:4 phosphatidylcholine with a concomitant reduction of the amount of 16:0/16:0 phosphatidylcholine (from 2% to 10% and from 4% to 1%, respectively) increased the phase transition temperature from -8.4°C to -6.2°C. The acyl chain order as

assayed by the hyperfine splitting values, remained unaffected in these combinations of phosphatidylcholines.

In other series of experiments, fluorescence spectroscopy was used and the steady-state anisotropy parameters of 2-, 12-AS and 16-AP fluorescent probes were compared in 16:0/18:1, 18:0/22:6 and 22:6/22:6 PC vesicles (Figure VI.2).

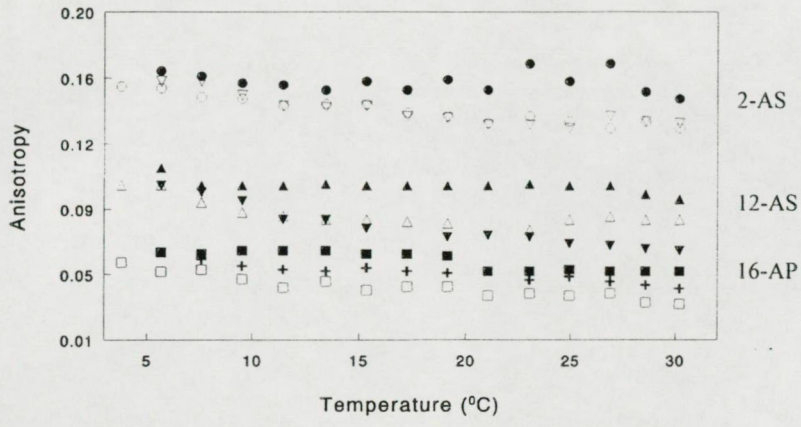


Figure VI.2. Temperature dependence of the steady-state fluorescence anisotropy of 2-AS, 12-AS and 16-AP in 16:0/18:1 (∇ 2-AS, ▼ 12-AS and + 16-AP), 18:0/22:6 (○ 2-AS, Δ 12-AS and □ 16-AP) and 22:6/22:6 (● 2-AS, ▲ 12-AS and ■ 16-AP) PC vesicles.

The data presented in Figure VI.2 shows (in agreement with those in Fig. VI.1) that the anisotropy values obtained in these phospholipids were very similar in the corresponding membrane segments: that is, the presence of 11 extra double bonds in the 22:6/22:6 PC molecule does not render the vesicles more disordered as compared to a single Δ-9 *cis* double bond in the sn-2 position of 16:0/18:1 PC. On the contrary, 22:6/22:6 phosphatidylcholines were slightly more ordered at higher temperatures (above 15°C).

The total amounts of Sat/Unsat and Mono/Poly species showed the most characteristic changes in thermally adapted fish (Table II.2). Therefore, experiments were carried out on binary mixtures of phosphatidylethanolamines (16:0/18:1 PE or 18:1/22:6 PE) and phosphatidylcholines (18:0/22:6 PC). Figure.VI.3 shows the comparison between the $2A_{max}$ parameters of the 5-SASL embedded in the vesicles made from (1) pure 18:0/22:6 PC, (2) vesicles made of binary mixtures of 18:0/22:6 PC (75%) and 16:0/18:1

PE (25%) and (3) vesicles made of binary mixtures of 18:0/22:6 PC (75%) and 18:1/22:6 PE (25%).

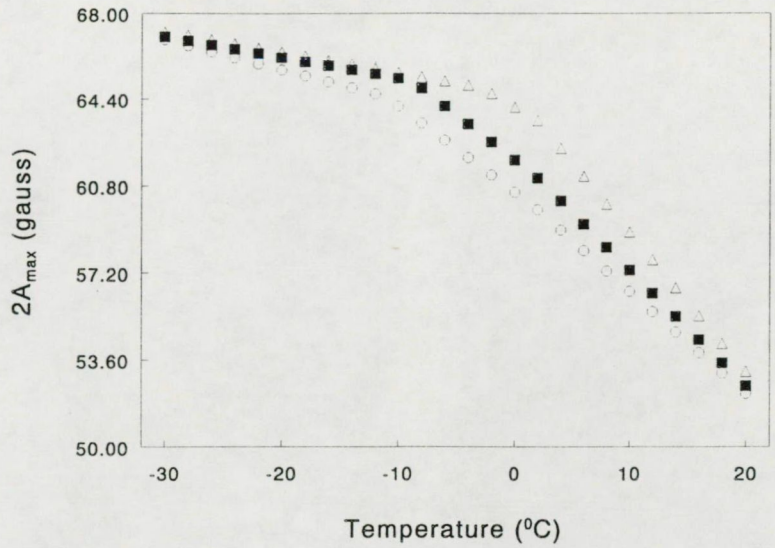


Figure VI.3. Temperature profiles of the outer hyperfine splitting of 5-doxylstearic acid spin label incorporated into vesicles made from 18:0/22:6 PC (100%) (■), from binary mixtures of 16:0/18:1 PE (25%) + 18:0/22:6 PC (75%) (△), and from 18:1/22:6 PE (25%) + 18:0/22:6 PC (75%) (○).

The addition of 18:1/22:6 PE to the 18:0/22:6 PC decreased the melting temperature of the latter from -8.8°C to -11°C. The acyl chain disorder also decreased especially in the middle range of the temperature interval. On the contrary, the presence of 16:0/18:1 PE in the vesicles shifted the melting temperature to higher temperature, from -8.8 to -3°C.

In the experiment shown in Fig. VI.4, the structural effects of mixing 25% 16:0/18:1 or 25% 18:1/22:6 PE with 75% 16:0/22:6 PC (Fig. VI.4.A and B) was tested by fluorescence anisotropy measurements of intercalated anthroyloxy fatty acids. It can be seen on the figures that while 16:0/18:1 phosphatidylethanolamine rendered the mixed vesicles more ordered in the uppermost segment, the 18:1/22:6 PE species increased the disorder (Fig. VI.4.B) when compared with the pure 16:0/22:6 PC vesicles.

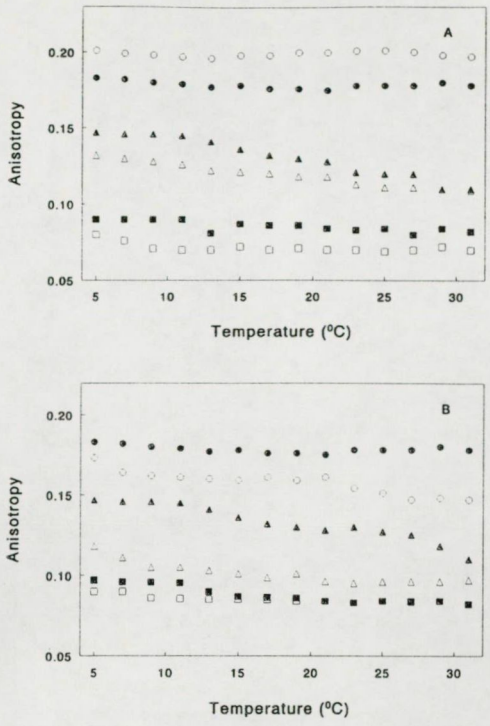


Figure VI.4. The temperature dependence of steady-state fluorescence anisotropy of 2-AS, 12-AS and 16-AP embedded either in A: 100% 16:0/22:6 phosphatidylcholine (● 2-AS, ▲ 12-AS and ■ 16-AP) or 75% 16:0/22:6 phosphatidylcholine and 25% 16:0/18:1 phosphatidylethanolamine (○ 2-AS, △ 12-AS and □ 16-AP) or in B: 100% phosphatidylcholine (● 2-AS, ▲ 12-AS and ■ 16-AP) or 75% 16:0/22:6 phosphatidylcholine and 25% 18:1/22:6 phosphatidylethanolamine (○ 2-AS, △ 12-AS and □ 16-AP).

In the deeper layers, the ordering effect of 16:0/18:1 phosphatidyl-ethanolamine was reversed and a slight fluidization was observed (Fig. VI.4.A).

A similar picture is seen (Fig. VI.5) when the anisotropy parameters of anthroyloxy

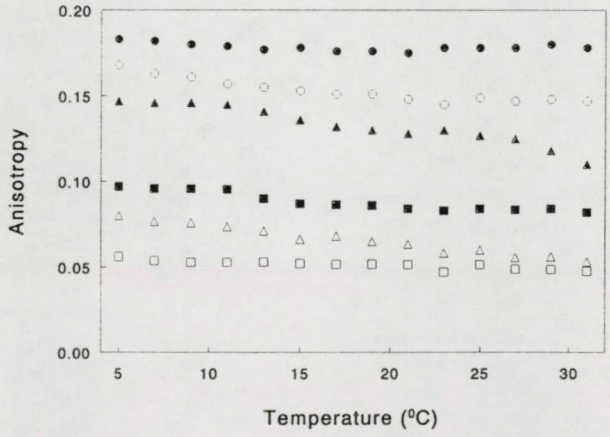


Figure VI.5. Comparison of fluorescence anisotropy of 2-AS, 12-AS and 16-AP embedded either in 100% 16:0/22:6 (● 2-AS, ▲ 12-AS and ■ 16-AP) or 100% 18:1/22:6 phosphatidylcholine (○ 2-AS, △ 12-AS and □ 16-AP).

labels embedded in 18:1/22:6 and 16:0/22:6 phosphatidylcholine vesicles are compared, with the exception that the 18:1/22:6 phosphatidylcholine vesicles were more fluid also in the C-16 segment, than their 16:0/22:6 analogues.

4. DISCUSSION

An inherent property of prokaryotes and eukaryotes is the ability to maintain the structural and functional integrity of their membranous structures under a variety of external conditions. The change in the ambient temperature is one of the most important factors, which can disturb the integrity of the cell membranes as it has direct effect on the rates of molecular motions and also on chemical reactions. The ability of rapid and reversible control over the membrane physico-chemical characteristics under such circumstances is of indispensable survival value for the organisms.

Organisms capable of living at varying thermal conditions without having the possibility to maintain a constant body temperature are able to offset the direct effects of the temperature change and maintain an optimal "fluidity" of their membranes, together with undisrupted functioning of the membrane-related processes. This phenomenon of "homeoviscous adaptation", after being described initially on *E. coli* (Sinensky, 1974) was confirmed on many other organisms like cyanobacteria (Murata, 1989), plants (Quinn *et al.*, 1989), protozoan (Thompson, 1983) and fish (Behan-Martin *et al.*, 1993). The compensation of temperature variation (homeoviscous efficacy) was found to be varying from organism to organism, and from the type of membrane and technique used for estimating membrane fluidity (Cossins *et al.*, 1978; Lee and Cossins, 1990; Behan-Martin *et al.*, 1993). As membrane lipids are the most sensitive molecules against temperature changes and constitute the backbone of the cell membranes, they are considered to be the major candidates of keeping the membrane physical characteristics in a "functional window".

Various studies were conducted on bacteria, plants and animals to understand the mechanism of the thermal adaptation of membranes and the possible regulation of

membrane function can be regulated trough adjustments of lipid composition. Despite a considerable amount of data, a composite study and a generalised perspective of lipid-membrane adaptation in response to temperature changes have not yet been accomplished.

It is often assumed that between membrane fluidity and fatty acid unsaturation there is a simple and direct correlation, in such a way that, an increase in unsaturation implies increased membrane fluidity (Sinensky, 1974; Hazel, 1988; Wada and Murata, 1990; Williams and Hazel, 1993; Vígh *et al.*, 1994; Nishida and Murata, 1996). However, the relationship is frequently not straightforward (Stubbs *et al.*, 1981; Coolbear *et al.*, 1983). The role of lipids in controlling membrane function has not yet been unambiguously established, and the need for lipid diversity in eukaryote membranes remainsed unresolved.

The experiments presented in this thesis were designed to study the role of individual lipids, especially phospholipids in thermal adaptation of the membranes in different bacterial and poikilothermic organisms. Although these evolutionarily diverse organisms inherit difficulties to conclude a "universal adaptation mechanism", our results, nevertheless, unravelled some common aspects of temperature regulation in the organisms studied.

Studies on bacterial species

Absence of direct relation between fatty acid unsaturation, membrane fluidity and growth temperature in bacterial species

Studies of two symbiotic bacteria *P. luminescens* and *X. nematophilus* residing in entomopathogenic nematodes revealed fundamental differences in their lipid metabolism and in the biophysical properties of their lipid membranes (Table I.1, Fig. I.3). One major difference between the primary forms of the two species was that under identical growth conditions, *X. nematophilus* produced a fatty-acid population that gave rise to less ordered

(more fluid) membranous structures, at low measuring temperatures, than *P. luminescens* (Fig. I.3). This result may explain the observation that the *X. nematophilus-Steinernema* symbiotic complex is more cold tolerant than the *Photorhabdus-Heterorhabditis* symbiotic complex (Molyneoux, 1986; Blackshow and Newel, 1987). However, the higher fluidity of *X. nematophilus* can not be unambiguously correlated with the differences in the fatty acid compositions of the two bacteria. At both growth temperatures the total S/U fatty acid ratio of *X. nematophilus* was higher than that of *P. luminescens* (Table I. 1), on the base of which we could expect the contrary, i.e., less fluid structure for the lipids of *X. nematophilus* than *P. luminescens*. However, this was not the case. The lipids of the two bacterial species were also different concerning their cyclopropane fatty acid (cp17:0) content (Table I. 1). However, this can not explain the fluidity differences found either. The molecular packing of lipids containing cyclopropane fatty acids is very similar to those containing monounsaturated fatty acids (Cullen *et al.*, 1971). Monounsaturated fatty acids are known to increase membrane fluidity due to their lower melting temperatures and greater cross-sectional areas compared to saturated ones. If we consider cp17:0 among the monounsaturated fatty acids the more fluid structure of the lipids of *X. nematophilus* could be explained in 18°C but not in the case of 28°C cultures, where the amount of cp17:0 is much higher in *P. luminescens* than in *X. nematophilus*.

Both bacterial species were similar in their inability to compensate the physical properties of their lipid membranes in response to the change of growth temperature. Both species formed lipid populations giving rise to more-rigid membranous structures when grown at lower temperatures (Fig. II.2). This means that the "homeoviscous adaptation" (H.A.) theory is not valid for these bacteria or, at least at the level of their lipid membranes. In this respect the two bacteria differ from *Escherichia coli,* where the theory of H.A. was first proposed (Sinensky, 1974). It was shown that the outer membrane proteins of *X.*

nematophilus are thermally regulated and at increasing growth temperatures several of these are induced (Leisman *et al.*, 1995). This may give an explanation why at high growth temperatures the lipid extracts displayed more fluid structure. Since the presence of proteins exerts an ordering effect on the membranes, more fluid lipid structures may be needed to preserve membrane integrity and also to accommodate these proteins.

Still remains intriguing that the differences in the fluidities, can not be explained solely by the changes in the fatty acid compositions of the bacterial lipids upon changing the growth temperature. Although the S/U fatty acid ratios decreased upon growing the cells at lower temperatures (Fig. II.1), which is a common adaptation mechanism of many organisms, the lipid-membrane order was increased for both species of bacteria. Beside the S/U ratio, the fatty acid cp17:0 showed large changes with the change of growth temperature. If we consider this fatty acid among the monounsaturates, the more ordered structure of *P. luminescens*' lipids may be explained by the virtual increase of S/U ratio, but it can not explain the case of *X. nematophilus*, where the change of cp17:0 would virtually decrease the ratio of saturated-to-unsaturated fatty acids.

On the basis of these experiments it can be concluded that the gross amount of fatty acid unsaturation is not always a good indicator of membrane fluidity characteristics. There may be more important factors for example the head group or molecular species composition of lipids that regulate membrane physical properties. For *E. coli* and *P. fluorescence* that have similar lipid class composition with the studied symbiotic bacteria, it was shown that the growth temperature did not alter the levels of different lipid classes (Cullen *et al.*, 1971; Lugtenberg and Peters, 1976). However, the concentrations of di-unsaturated molecular species of PE and PG increased in inner and outer membranes of *E. coli* at lower growth temperatures (Ishinaga *et al.*, 1979). Therefore, it may be possible that the changes in the molecular species composition of specific lipids, rather than the

headgroup or fatty acid unsaturation, could explain the fluidity changes seen in our experiments.

Cyanobacterial cells resemble chloroplasts of higher plants in terms of thylakoid membrane structure and glycerolipid composition (Kenyon and Stanier, 1970). Therefore, they were widely investigated, (especially the *Synechocystis* PCC 6803, and *Anacytis nidulans*) and were used as model systems for studying the mechanisms of stress responses and adaptation, including temperature adaptation (Murata, 1989; Wada *et al.*, 1992; Vígh *et al.*, 1994; Glatz *et al.*, 1999).

Cylindrospermopsis raciborskii, as well as *Synechocystis* sp. are the members of Group 4 cyanobacteria (Murata *et al.*, 1992) that contain all the four fatty acid desaturases: Δ-9, Δ-12, Δ-15(ω3), and Δ–6, and has high level of polyunsaturated, especially octadecatetraenoic acid, 18:4ω3 (Table I.3).

In case of *Synechocystis* PCC 6803 an increase in ω6 fatty acids, 18:2(6, 9) and 18:3(6, 9, 12), and 18:4(6, 9, 12, 15) were found upon decreasing the growth temperature from 42 to 22°C (Wada and Murata, 1990). An increase in the fluidity of the thylakoid membranes of *Synechocystis* PCC 6803 upon decreasing the growth temperatures was also demonstrated (Horváth *et al.*, 1998) and it could also be seen in Figs. I.5.B and I.6.B. In *C. raciborskii* the levels of ω3 polyenoic fatty acids, 18:3(9, 12, 15) and 18:4(6, 9, 12, 15) increased when exposed to low temperatures while 18:2(9, 12) decreased (Fig. I.4). Despite the massive accumulation of polyunsaturated fatty acids in *C. raciborskii* (Table I.3) the orientational order close to the aqueous interface (measured by 5-SASL spin label) remained unchanged in the cells grown at either 35 or 25°C (Fig. I.6). Although the rotational correlation time of 16-SASL reflected increased mobility in cells grown at 25°C at low measuring temperatures, between 15 to 35°C, above this range the difference

diminished and over all was of much less magnitude than that found for the *Synechocystis* sp. (Fig. I.5). The difference between the two cyanobacteria in the ability to control the fluidity of thylakoid membranes shown in Fig. I.5 and I.6 suggests that *C. raciborskii* may be an exception to homeoviscous regulation of membrane fluidity while growing at altered temperatures. An inevitable conclusion of these experiments is also the lack of correspondence between the fatty acid composition and membrane fluidity in *C. raciborskii* as compared to *Synechocystis* sp.

Exceptions to the classical homeoviscous adaptation theory upon thermal adaptation were found in phsychrotolerant (*Mucor mucedo*) and thermotolerant (*Aspergillius ochraceus*) fungal species, as well (Chavant *et al.*, 1981). It should be noted that for *A. ochraceus*, neither 18:3ω3 nor 18:4ω3 were present. *M. mucedo*, on the other hand, produced a substantial amount of 18:4 when grown at 10°C instead of 20°C. A similar trend was found for the slime mold *Dictyostelium discoideum*: in spite of substantial enrichment of the membranes at low temperatures in dienoic, trienoic, and tetraenoic acids, there was no change in the fluidity of plasma membranes as measured by spin labelling ESR using 5-SASL (Herring and Weeks, 1979).

C. raciborskii is of subtropic origin and was evolutionarily adapted to relatively constant, high temperatures. Thus, it was not forced to adjust membrane properties to changing temperatures. It is tempting to speculate that the few (10-20) years spent in temperate waters were not enough to develop mechanisms necessary to control membrane fluidity.

Studies on poikilotherms: *Cyprinus carpio* L. and *Rana esculenta*

Characteristics of seasonal adaptation to contrasting temperatures as reflected in the total phospholipids of liver of the fresh water fish *Cyprinus carpio* L.

Several lines of evidence showed a compensation of the membrane fluidity for changes in the ambient temperature and parallel alterations taking place at the structural level of membranes during thermal adaptation of poikilotherms (Cossins and Prosser, 1978; Cossins *et al.*, 1980; Abruzzini, 1982; Lagerspetz and Laine, 1984; Lee and Cossins, 1990). Still it is unclear how the individual phospholipid molecules participate in this process.

The fish *Cyprinus carpio* L. was chosen as a model for study, because of its capability of adaptation to a broad range of temperatures (from 5°C to 35°C) being found in temperate, sub-tropical and to some extent in tropical climates. Fatty acid and molecular species compositions of liver phospholipids from winter- and summer-adapted carps were compared. The physical properties of their phospholipid membranes were investigated to look for adaptive changes in fluidity occurred at the level of phospholipids during thermal adaptation.

In the TPL extract of the livers of winter-adapted carps there was a ~25% increase in the long-chain polyunsaturated fatty acids such as 20:4n-6 and 22:6n-3 and a ~25% decrease in the saturated fatty acids such as 16:0 and 18:0. PC and PE showed different responses in winter and in summer adaptation. The level of 16:1 has doubled in amount while 18:1 has increased by 50% during winter adaptation. Cold-induced accumulation of oleic acid in ethanolamine phosphoglycerides is reported in several cases (Dey *et al.*, 1993b). It should be noted that 20:4n-6 showed nearly twofold increase in summer-adapted carps, and 22:6n-3 has also increased but in a lesser amount.

The analysis of the molecular species from extracted PC and PE gives a better understanding of lipid metabolism and reshuffling both in PC and PE. Summer-adapted

carps accumulated more 16:0/22:6, 18:0/22:6 and 16:0/18:1 in PC and 16:0/22:6, 16:0/20:4, 18:0/22:6 and 18:0/20:4 in PE in comparison to winter-adapted carp. In winter-adapted carp there was a substantial increase in *sn*-1 monounsaturated and *sn*-2 polyunsaturated species such as 18:1/22:6 and 18:1/20:4 (from 10.2% to 28.5%) mainly in PE. Significant changes observed in PC of winter-adapted carps were the decrease of the amount of 16:0/18:1 and 18:0/22:6 (by 59%), and of 16:0/16:0 (by 75%) and an increase in the amounts of 20:4/20:4 (by 75%), 16:0/20:4 (by 48%), 18:1/20:5 (by 85%), 18:1/22:6 (by 55%) and 18:1/20:4 (by 52%). Overall, winter adaptation is characterized by 75% decrease in the total saturated/unsaturated-to-total monounsaturated/polyunsaturated species (from 8.1 to 2.2).

If compensation of the membrane fluidity occurs during seasonal adaptation and lipids have the role of adjusting membrane fluidity, it can be expected to see these changes at the level of extracted phospholipids. Using ESR spectroscopy, by comparing the outer hyperfine splitting parameter of 5-SASL in the TPL extracts of the livers of winter- and summer-adapted carps, we detected a less ordered state and an approximately 5°C downshift in the onset of the melting of the gel phase of the phospholipids of cold-adapted carp (Fig II.1). The comparison of the hyperfine splitting found at 5°C for phospholipids from cold-adapted fishes with that of 25°C for lipids from warm-adapted fishes showed a 25-30% compensation of the membrane ordering state for the corresponding change in the temperature. Fluorescence anisotropy measurements, using anthroyloxy fatty acid probes (Fig. II.2) showed a 100% compensation of the lipid order at all depths of the bilayer. These experiments showed that in the two adaptational states of the fish the phospholipid compositions are different and are such that at cold adaptation they provide a more fluid bilayer structure than at warm adaptation. The difference between the extent of compensation in fluidity measured by the two techniques resides in the difference in the parameters

measured and probes used by the two. In addition, perhaps, the outer hyperfine splitting parameter of 5-SASL, which is an indicator of the order parameter is not as sensitive measure of the ordering state of the bilayer as the order parameter itself. Unfortunately the latter was not possible to be determined due to the rigid character of the spectra of 5-SASL at low (<18°C) temperatures.

Both the downshift of the phase-transition temperature and the increase of the acyl chain disorder in the bilayer can be related to the observed changes in the molecular species composition during seasonal adaptation. The most important changes in the molecular species composition are the decrease of the *sn*-1 saturated, *sn*-2 unsaturated species (16:0/22:6, 18:0/22:6) and a concomitant increase of the 1-monounsaturated, 2-polyunsaturated (18:1/22:6, 18:1/20:4) species during adaptation to cold temperature (Table II.2). The phase transition temperatures of 18:0/22:6 PC and 16:0/20:4 PC are rather close, thus, the accumulation of 16:0/20:4 PC and PE and a concomitant decrease of the 18:0/22:6 species (Table II.2) at cold adaptation may not be responsible for the observed changes in the physical properties of the lipid membranes. Accumulation of 18:1/22:6, and to some extent 18:1/20:5 PE, is also reported for marine fishes and shrimps inhabiting polar and subtropic waters (Dey *et al.*, 1993b; Farkas *et al.*, 1994) or in brains of fresh water fishes adapted to low temperature (Buda *et al.*, 1994). In a previous study (Farkas *et al.*, 1994) it was shown that a mixture of PE from salmon liver (containing high amount of 18:1/22:6 and 18:1/20:5) with PC from rat liver phospholipids (75% PC and 25% PE) showed higher fluidity compared with their respective PC alone. This suggests a fluidizing effect of the species 18:1/22:6 and 18:1/20:5 PE. Therefore, based on all these observations it may be proposed that *sn*-1 monounsaturated, *sn*-2 polyunsaturated PE species in membranes play important role in cold- adaptation/acclimation and probably are also responsible for the increase of the fluidity and drop of the melting temperature of the gel phase in order to maintain the

physiological and functional activities of the membranes in cold-adaptation/acclimation.

Short-term-acclimation-induced adaptive changes in the lipid composition and in the physical properties of plasma membranes and mitochondria isolated from carp liver

In vivo experiments were carried out on winter- and summer-adapted carps by acclimating them to the corresponding "opposite" environmental temperature. The aim was to look for similarities found upon seasonal adaptation. Analysis of lipids and physical properties of plasma membranes and mitochondria isolated from livers were investigated. These objects were considered for two reasons: (1) they can be isolated with a purity of >95% and (2) plasma membrane represents the cell boundary through which environmental changes are first perceived and mitochondrion is a representative major cellular organelle.

The most significant change in membrane lipid compositions was the change in 18:1/22:6 molecular species in PC and PE both in plasma membranes and mitochondria (Table III. 1 and 2). This species seems to be the most temperature sensitive, which systematically follows the direction of temperature change: increases at cold and decreases at warm temperatures either during seasonal adaptation or acclimation. The magnitude of the change of this species was higher in plasma membranes than in mitochondria (50% *vs.* 30% in PC and 1.1 fold *vs.* 0.7 fold in PE -summer *vs.* winter adapted), and higher in PE than PC lipid class.

In addition, it may be concluded that plasma membrane is more sensitive to the temperature change than mitochondria. Concerning the phospholipids, PE is more sensitive than PC. It is also noticeable that the magnitude of the change of 18:1/22:6 species during short-term acclimation at 25°C did not reach to that observed upon seasonal adaptation to

summer temperature. In contrast, in summer-adapted carps the temperature downshift causes a drastic increase of the 18:1/22:6 in the PE of the plasma membranes, and this increase is greater than that observed during seasonal adaptation. Wodtke and Cossins (1991) reported a related increment of Δ-9 desaturase activity in carp endoplasmic reticulum during short-term cold acclimation. A change of the 18:1/22:6 species in response to temperature was also reported in red blood cells of fishes (Dey *et al.*, 1993a), in the total phospholipid extracts from the livers of evolutionarily adapted fishes to different temperatures (Dey *et al.*, 1993b) and in the brain cells of fishes (Buda *et al.*, 1994).

Other species responding to temperature changes was the 18:0/22:6. It increased in warm adaptation/acclimation in PC, but remained almost unchanged in PE. The other molecular species investigated had responded to the temperature change in the same manner (increasing or decreasing) irrespective of the direction of the temperature. Therefore, these molecules may be termed as 'Stress Response Molecules' or SRM. The possible mechanism by which SRM molecules may play a role in temperature response is unclear.

The increase of 18:1/22:6 in cold adaptation/acclimation can be explained by fast activation of Δ-9 desaturase activity and molecular reshuffling of the molecular species, because the amount of 22:6 fatty acid does not increase in cold adaptation (Dey *et al.*, 1993, Buda *et al.*, 1994).

The fluidity of the investigated membranes was measured by probing with DPH. Membranes isolated from cold adapted/acclimated fishes showed a more fluid structure than those from the warm adapted/acclimated ones (Fig. III.6 and 7). Mitochondria have responded by hyper-fluidizing their membranes during short-term cold acclimation (Fig. III.7.B). This phenomenon was not observed in plasma membranes.

The fluidization of the membranes in response to cold adaptation or acclimation is reported in several cases such as fish red blood cells (Dey *et al.*, 1992), in carp endoplasmic reticulum (Wodtke and Cossins, 1991), and in plasma membranes of hepatocytes isolated from rainbow trout (Williams and Hazel, 1995).

In search of correlation between the changes in lipid molecular species and the physical properties of the membranes we may say that fluidization upon cold adaptation/acclimation may be due to the increase of the 18:1/22:6 molecular species. It is known that incorporation of a *cis* double bond at the C-9 position of the stearic acid decreases its phase transition temperature from 54°C to -7°C (Silvius, 1982). Moreover, in experiments with reconstituted membranes using a mixture of PE (rich in 18:1/22:6 species extracted from the liver of cold-adapted salmon) and rat PC, fluidization was found compared to the mixture with that of PC of respective animals or with that of a mixture of PC from cold-adapted salmon liver and rat PE (Farkas *et al.*, 1994). Therefore, it may be concluded that 18:1/22:6 or the *sn*-1 monoenoic, *sn*-2 polyenoic species, in general, might be regulatory lipid molecules in response to temperature adaptation.

The 16:0/22:6 species can not be a candidate as far as the influence on the membrane physical properties are concerned, because the phase transition temperature of 18:0/22:6 PC is rather close to 16:0/22:6 PC. Thus, equal but opposite changes of 16:0/22:6 and 18:0/22:6 are not expected to influence membrane physical properties. The same can be said for the species 16:0/20:4 PC and 16:0/22:6 PC, of which the molecular geometries are similar (Applegate and Glomset, 1991; Rabinovich and Ripatti, 1991).

The specific responses of different organelles to the temperature changes may vary in certain extent, but the amount of *sn*-1 monounsaturated, *sn*-2 polyunsaturated phospholipid molecular species seem to be key components in controlling membrane physical properties in temperature adaptation. However, the role of other factors like the

induction of proteins in conjunction with the alteration of lipid molecular species can not be ruled out.

Environmental temperature and dietary fatty acids in controlling membrane fluidity

Beside the temperature, photoperiod, nutrition, and the hormonal state may affect the lipid metabolism of the fish and consequently the lipid composition of its membranes. To investigate the contribution and role of dietary fatty acids in the process of thermal adaptation, different fish species were analysed which live in the same habitat but consume different foods. They were all collected at the same time, when all could be regarded as adapted to cold (5°C) temperatures.

Although the composition of the diet of the four fish species was different, the fatty acid composition of their liver phospholipids did not show significant differences. For instance, neither *C. idella* nor *C. carpio* ingested long chain polyunsaturated fatty acids but still there was ~15% 22:6 in their total phospholipid composition (Table IV. 2). Amounts of n-3 polyenes were also substantially higher in the phospholipids of *H. molitrix* and *A. nobilis* than in their algal or cladoceran food. This was also valid for the isolated plasma membranes (Table IV. 3). These fishes stop eating below ~10°C, therefore, the higher amount of long-chain polyunsaturated fatty acids compared to their food suggests the existence of active chain-elongation and desaturation processes during cold adaptation. The effect of low environmental temperature on liver phospholipid fatty acid composition of *C. carpio*, *H. molitrix* and *A. nobilis* has already been reported (Farkas and Roy, 1989). However, differences might exist between different fish species. Indeed, *H. molitrix* and *A. nobilis* contained twice as much 22:6 in their phospholipids than *C. idella* and *C. carpio* (Table IV.

2). Thus, the adaptation to cold altered the levels of long-chain polyunsaturated fatty acids, and also the ratios of saturated to polyunsaturated fatty acids in structural lipids. In these respects the differences between *H. molitrix* and *C. idella* were greater than observed intraspecifically, for a selected fish species during adaptation to different temperatures where the temperature was the only variable factor (Sellner and Hazel, 1982; Williams and Hazel, 1992).

Accumulation of polyunsaturated fatty acids in response to decrease of ambient temperature accompanied with an increase in fluidity of membraneous structures was reported in several cases (Cossins 1977; Cossins *et al.*, 1980; Lee and Cossins, 1990; Kitajka *et al.*, 1996). On the basis of these results it was expected that differences between phospholipid fatty acids would be reflected in the fluidity of vesicles made of these phospholipids or in purified plasma membranes prepared from the liver of the fishes. Figures IV. 1, and 2, did not show any direct relationship between the fluidity of the membranes and the fatty acid composition of the lipids at temperatures above 15°C. The steady-state fluorescence anisotropy values from phospholipid vesicles and plasma membranes of grass feeding carp (*C. idella*), and carp (*C. carpio*) did not vary in comparison to silver carp (*H. molitrix*), or big head carp (*A. nobilis*), although the former two have lower amounts of unsaturated fatty acids in the total phospholipid extract and have higher saturated-to-unsaturated fatty acid ratios than the latter two. Below 15°C, the vesicles prepared from the phospholipids of *C. carpio* and *C. idella* were relatively more ordered than those of the other two fish species. Although plasma membranes from *C.idella* have more saturated fatty acids than *H. molitrix*, the anisotropy values were almost identical. It is unlikely that ether lipids in plasma membranes had any effect on the fluidity of these structures. Paltauf *et al.* (1971) showed that replacement of the ester linkage by ether links in PC did not affect the molecular packing of monolayers.

The ESR measurements showed more fluid structures for *H. molitrix* than for *C. carpio* (Fig. IV. 2) below ~15°C contrary to the anisotropy values of the fluorescent probe. The discrepancy can be explained by the different locations of the two probes: DPH is randomly distributed in the bilayer and reflects an average fluidity, 16-SASL reports from the region of the 16th carbon atom of the acyl chain.

Based on the available data on the physical properties of polyunsaturated fatty acids like those regarding the surface area (Evans *et al.*, 1987), thermotropic phase transition temperature (Coolebar *et al.*, 1983; Niebielski and Salem, 1994) or fluorescence anisotropy of certain probes embedded in synthetic PC vesicles containing various polyunsaturated fatty acyl chains (Fodor *et al.*, 1995), the observed differences in the amounts of total unsaturated or total polyunsaturated fatty acids are not expected to alter the fluidity of the fish liver plasma membranes.

The results also showed that certain phospholipid molecular species, like 16:0/20:4 and 16:0/22:6 PC, may substitute each other in membranes. In phospholipid extracts or in plasma membranes of *H. molitrix,* the level of the molecular species containing n-3 polyunsaturated fatty acids in the position *sn*-2 was comparable to the level of phospholipids containing n-6 polyunsaturated fatty acids in the same position in *C. idella*, particularly in PE. The molecular geometry of 1-palmitoyl, 2-arachidonyl PC is similar to that of 1-palmitoyl, 2-docosahexaenoyl species (Applegate and Gloomeset, 1991; Rabinovich and Ripatti, 1991). Similar substitution may happen in mammals, as well. Phospholipid vesicles from the livers of rats kept on a fish-oil diet, rich in n-3 polyunsaturated fatty acids, or on a vegetable-oil diet, rich in linoleic acid, exhibited similar fluorescence anisotropy parameters despite their differences in molecular species compositions (Farkas *et al.*, 1994). Stubbs and Kisielevsky (1990) found no difference in the fluidity of muscle sarcoplasmic reticulum membranes of rats fed with either vegetable-oil or fish-oil. Apparently these phospholipid

molecules do not seem to modify the physical properties of the membranes in fishes of different feeding habit.

High levels of molecular species containing a monounsaturated fatty acid in the *sn*-1 position and a polyunsaturated fatty acid in the *sn*-2 position were observed in all four fish species, but the 18:1/22:6 PE of *H. molitrix* and *A. nobilis* was replaced by 18:1/20:4 PE in *C. idella*. The molecular species composition of phospholipids showed higher levels of the above mentioned species in the livers and brains of fishes being either seasonally or evolutionarily adapted to cold (Dey *et al.*, 1993b; Buda *et al.*, 1994; Kitajka *et al.*, 1996).

On the basis of these observations it may be concluded that not the gross amounts of the long-chain polyunsaturated fatty acids but their specific pairing with monounsaturated fatty acids in the position *sn*-1 is the major factor controlling membrane lipid order and physical properties. It is highly probable that the observed differences in the anisotropy parameters of DPH in phospholipid vesicles from *C. carpio* and *C. idella* as well as *H. molitrix* and *A. nobilis* or the differences in the rotational correlation times of 16-SASL in the plasma membranes of *C. carpio* and *H. molitrix* below 15°C are due to the differences in the amount of monounsaturated/polyunsaturated PE molecular species. Since the fatty acid composition of the phospholipids and the fluidity of phospholipid vesicles or isolated membranes seem to be independent of the feeding habit it may be concluded that the environmental temperature but not the dietary fatty acid composition is the major factor controlling membrane physical state. The level of *sn*-1 monounsaturated, *sn*-2 polyunsaturated phospholipid molecular species, however, may depend on a species-specific response of the fishes in response to temperature changes.

Temperature adaptation in frogs at the level of phospholipids of liver

An important difference between the sesonal temperature adaptation of frogs and fishes is that the former spend the winter period in hibernating state when all metabolic processes are switched to minimum while fishes remain active.

Fatty acid and molecular species compositions of the lipids of frog livers did not change significantly during hibernation (Fig.V.1). Surprisingly, certain molecular species containing polyunsaturated fatty acids decreased during this period, but the levels of *sn*-1 monounsaturated, *sn*-2 polyunsaturated species remained unchanged. The increase of these species in fish upon cold adaptation/acclimation caused increased fluidity of vesicles made of liver phospholipid extracts. In case of frogs the absence of the change in the levels of *sn*-1 monounsaturated, *sn*-2 polyunsaturated species combined with the decrease of 16:0/22:6, 18:0/22:6 and 18:0/20:4 species have resulted in an increase of chain ordering at C-2 region. Based on results obtained with synthetic PE (Fodor *et al.*, 1995) it was proposed that the kink in the chain of oleic acid in position *sn*-1 is responsible for the increased disorder in the upper half of the bilayer. These results could serve as further evidence for the importance of *sn*-1 monounsaturated, *sn*-2 polyunsaturated phospholipid molecular species in thermal acclimation processes of winter-active poikilotherms.

Reconstitution experiments with synthetic phospholipids

Although several lines of evidence suggests a compensatory change in the membrane order to the altered ambient temperature (Cossins and Prosser, 1978; Cossins *et al.*, 1980; Abruzzini, 1982; Lagerspetz and Laine, 1984; Lee and Cossins, 1990), little information was available on the structural alterations taking place within the bilayer during thermal

adaptation. It was also unclear how the individual phospholipid molecules participate in this process. In the experiments presented above we found several characteristic changes occuring in the molecular species composition of the two major phospholipid class, PC and PE. On the basis of these characteristics we proposed that the involved molecular species can be the key lipids in determining the actual fluidity of the lipid or native membranes. To confirm this hypothesis, reconstitution experiments were designed with synthetic lipids and with mixtures of the key lipid species.

Experiments on lipid vesicles (Fig.V.1 and 2) made of disaturated (16:0/16:0) PC (high phase transition temperature, above 40 °C) and polyunsaturated (22:6/22:6) (low phase transition temperature, below -40 °C) as well as saturated/polyunsaturated (18:0/22:6) PC species (intermittent phase transition temperatures, around -10 °C) confirmed that these species found in the respective ratios in livers of cold/warm adapted fish (Table II.2, III.1, IV. 2) do not participate in the control of the membrane physical properties upon temperature adaptation. Therefore, these changes alone do not explain neither the increased disorder in the fatty acyl chains (Fig. II.1 and 2, III. 1 and 2) nor the downshift of the onset of the gel phase melting temperature (Fig. II.1) upon cold adaptation. The "non-involvement" of polyunsaturated fatty acids and di-polyunsaturated phospholipid molecular species in controlling membrane physical state was also reported by Stubbs and Kisielewski (1990) on sarcoplasmic reticulum of rats fed either with fish-oil or with vegetable-oil. Similarly, studies made in our laboratory did not reveal any effect of long-chain polyunsaturated fatty acids on the membrane order at the C-2, C-12 or C-16 levels in vesicles made from the phospholipid extracts from livers of rats fed with fish-oil or with vegetable-oil (Farkas *et al.*, 1994).

Figures V. 3, 4A, 4B and 5 show the disordering effect of the 18:1/22:6 PE (Fig. V.3, V.4.B) and PC species (Fig. V.5) in each segment of the bilayer. Previously it was shown that the combination of rat liver PC (75%) with salmon liver PE (25%) rich in 18:1/20:5 and

18:1/22:6 phospholipid species, resulted in a considerable fluidisation as compared to vesicles made from salmon liver PC (75%) and rat liver PE (25%) (Farkas *et al.*, 1994). The fact that 16:0/18:1 PE made the mixed vesicles more ordered in the C-2 segment (Fig. V. 4 and 5) explains the differences found by fluorescence anisotropy between total phospholipids of cold- and warm-adapted fishes and those between the purified PC (Fig. II.2 and Fig. II.3).

The ordering effect of PE has been described by Michaelson *et al.* (1974) and it was attributed to interactions between the positively charged amino group and the negatively charged phosphate ester groups of the adjacent phospholipid molecules. The presence of a *cis* double bond in the position *sn*-1 of PE may overcome these interactions and may increase the disorder in the hydrophobic region of the bilayer. In fact, mixing 18:1/22:6 PE with 16:0/22:6 PC resulted in a drastic fluidization both at the C-2 and C-12 levels. The addition of the 18:1/22:6 PE molecular species to 18:0/22:6 PC vesicles also produced a decrease in the melting temperature as compared to PC alone. The addition of 16:0/18:1 PE produced a reverse effect (Fig. V.3). Therefore, these experiments confirm the above-mentioned hypothesis that the increase of the *sn*-1 monounsaturated, *sn*-2 polyunsaturated PE species in the membranes of marine and fresh-water fishes adapted either evolutionarily or seasonally to cold temperature is responsible mostly for the fluidization of the membranes and also for lowering of the gel-to-liquid crystalline phase transition temperature of the phospholipid extracts of liver.

We may propose that the presence of a monounsaturated fatty acid in position *sn*-1 of a PC or PE is more important in determining the packing properties of membranes than a saturated fatty acid in the *sn*-1 position when both of which is in combination with a long-chain polyunsaturated fatty acid in the position *sn*-2. These long chain polyunsaturated fatty acids adapt a so called "angle iron" (extended) configuration in the

phospholipid molecules (Applegate and Glomset, 1991). Computer modelling studies showed that while the cross sectional area of 18:0/22:0 PC is only 79.6 $Å^2$, that of 18:1/22:6 PC is 102.6 $Å^2$ (Zabelinskii, 1995). The same figures for molecular species containing 20:4 in the *sn*-2 position were 107.1 $Å^2$ and 138.2 $Å^2$, respectively. The condensing effect of the docosahexanoyl chain can be seen when the cross-sectional areas of 18:0/18:1 and 18:0/22:6 are compared: 95.4 *vs.* 79.6 $Å^2$. Since the level of 22:6 increases with decreasing temperatures in most fish (Farkas, 1984; Hazel, 1989; Thomson *et al.*, 1997) **the only mean to maintain proper membrane packing and thus fluidity is to incorporate monounsaturated fatty acid in position *sn*-1 of phospholipids.** Reconstitution experiments using synthetic 18:1/22:6 PE in combination with different PC or PE (Fig. V.3, 4, 5) support this concept, i.e. only phospholipid molecules esterified in the position *sn*-1 with a monounsaturated fatty acid and in position *sn*-2 with a polyunsaturated fatty acid can decrease membrane packing in cold.

Another aspect of the above-mentioned PE is related to their molecular shape. PE are wedge shaped but incorporation of monounsaturated fatty acid in position *sn*-1 further accentuates the conicity of the molecules. Several data showed that to maintain membrane stability in cold an increased number of such conically shaped molecules are required and accumulation of PE in cold adapted /acclimated fish has already been demonstrated (Weislander *et al.*, 1980; Farkas and Nemetz, 1984; Hazel and Carpenter, 1985; Pruit, 1988). **We demonstrated in this study that not only the actual amount of phosphatidylethanolamines but also their molecular architecture plays an important role in thermoadaptational process.**

Further biological significance of accumulation of monounsaturated/ polyunsaturated PE in cold is that they are prone to form so called non-bilayer structures and data are accumulating that at a given temperature a critical ratio of bilayer- and non-

bilayer forming lipids, although at the body temperature no such structures are present, is required for normal membrane functions. Such non-bilayer-prone lipids are required for the activity of several enzymes such as phospholipase A_2, Ca^{+2}-ATPase and others (Yang and Hwang, 1996; Zidovetzki, 1997). 18:1/22:6 PE was shown to exhibit a very low bilayer-to-nonbilayer transition temperature (Giorgione *et al.*, 1995), thus only a small increase in its amount may provide the membranes proper stability in cold and offset the effect of low temperature on activity of certain enzymes. In fact, it was shown, at least for some prokaryotes, that they live in a "window" between the bilayer and non-bilayer state of their membranes and this "window" is kept constant with changing growth temperatures (Morein *et al.*, 1996). It is tempting to speculate, but has to be proven, that one of the functions of monoenoic/polyenoic PE is to keep membranes of fish in such a "window" during acclimation/adaptation to the new thermal environment. This might be a reason why the levels of these molecular species are high in livers of cold-adapted marine and fresh water fishes (Dey *et al.*, 1993b; Fodor *et al.*, 1995, Roy *et al.*, 1999). It is highly probable that accumulation of these phospholipid molecules in cold is a common response of poikilotherms.

CONCLUSIONS

The last decade has seen a significant amount of study aiming to understand the mechanism and the role of the lipid molecules in the process of temperature adaptation of poikilotherms. Although several discoveries had been made on the reorganization of the lipids in the membranes, still there is no any 'global concept' concerning their role that can adequately explain all the aspects of thermal adaptation observed in different organisms at the level of membrane lipids.

The studies presented in this thesis are aimed to refine the existing views on the role of membrane lipids in temperature adaptation. This goal was reached by conducting a series of experiments on different poikilotherms and by relating different lipid-composition patterns and membrane order to the ambient thermal conditions of the organism.

From the studies conducted on bacterial species it can be concluded that:

1. The 'homeoviscous' compensation for the altered growth temperature in lipid-membranes is not universal (as was in the case of *P. luminescens* and *X. nematophilus*), and the extent of the compensation can be very different among species (like in the case of *C. raciborskii* and *Synechocystis* PCC 6803).

2. The gross amount of fatty acid unsaturation of lipids, or the ratio of total saturated-to-unsaturated fatty acids, is not a universal indicator of membrane 'fluidity'.

Studies on the fresh water fish *Cyprinus carpio* revealed characteristic changes in the molecular species composition of liver phospholipids during adaptation and acclimation to different temperatures which were accompanied by the corresponding alteration in fluidity of both lipid- and native-membranes. The following observations and conclus can be made:

1. Both for seasonal adaptation and short-term acclimation to cold the most characteristic changes in the phospholipid composition of liver is the decrease of the saturated/polyunsaturated and the increase of the monounsaturated/polyunsaturated PC and PE lipid species. The 18:1/22:6 PE appeared to be the most responsive lipid molecule to the temperature change both during seasonal and short-term acclimation.

2. Not the gross amount of long-chain polyunsaturated fatty acids, but their specific pairing with monounsaturated fatty acids in the position *sn*-1 is an important factor in adjusting membrane architecture and physical properties to temperature changes in fish.

Based on the observation that the fatty acid composition of the liver phospholipids did not reflect the differences in the fatty acid composition of diets and there were only small differences in the fluidity of the liver plasma membranes of the cold-adapted fishes investigated (*C. carpio*, *C. idella*, *H. molitrix*, *A. nobilis*) it can be concluded that:

1. The feeding habit does not interfere with restructuring of phospholipids in response to cold adaptation. This means that the environmental temperature and not the fatty acid composition of the diet are the major factor controlling the physical state of the membranes in the studied cases.

In hibernating species, like frogs, levels of molecular species with saturated/polyunsaturated fatty acids decreased while the levels of monounsaturated/polyunsaturated species remained unchanged in response to decrease of temperature. Similarly, the fluidity of the vesicles prepared from the liver phospholipids did not change in a compensatory manner as it can be seen in the case of fish. It is proposed that:

1. The lack of compensation in lipid-membrane fluidity during hibernation of frogs may be related to their inability to alter the level of *sn-1* monoenoic, *sn-2* polyenoic molecular species in the PE and PC lipids.

Experiments made on synthetic phospholipid vesicles seem to support the observations made on the organisms studied concerning the importance of certain lipid molecules during cold-adaptation. The followings can be concluded:

1. Experiments on lipid vesicles made of di-saturated (16:0/16:0), di-polyunsaturated (20:4/20:4) as well as saturated/polyunsaturated (18:0/22:6) PC species mixed at different ratios, showed no difference in the physical properties of their membranes. Therefore these changes alone do not explain the increased disorder and the decrease of the gel-to-liquid crystalline phase transition temperature during cold-adaptation of fishes.

2. Comparison ofthe membrane order of vesicles made of 16:0/18:1 PC, 18:0/22:6 PC and 22:6/22:6 PC showed that the fatty acid unsaturation of the lipids alone is not an indicator of the membrane order.

3. The 18:1/22:6 PE and PC molecular species exerts a disordering effect in each segment of the bilayer and lowers the onset of gel to liquid crystalline phase transition temperature of 18:0/22:6 PC vesicles, while 16:0/ 18:1 PE has just the opposite effect. Therefore it can be concluded that the 18:1/22:6 PE and PC molecular species play major role in fluidizing the membrane structures during cold-adaptation.

On the basis of the presented results we can propose that the *sn*-1-monounsaturated, *sn*-2-polyunsaturated phosphatidylethanolamines and phosphatidylcholines in general and 18:1/22:6 PE in particular are key molecules, that regulate membrane physical properties during thermal adaptation/acclimation in liver, and probably other tissues, like brain membranes of poikilothermic animals like fish.

ÖSSZEFOGLALÁS

A biológiai membránok a sejtek fontos alkotórészei. Bennük nemcsak számos létfontosságú folyamat zajlik, hanem a sejt és sejtorganellumok határolói is, mégpedig úgy, hogy az elhatárolt rész és környezete között állandó, dinamikus egyensúlyt biztosítanak. Nem meglepő tehát, hogy a sejt és környezete közötti kommunikációban fontos szerepet játszanak, különösen a sejt stressz feltételekhez való alkalmazkodásában.

A környezeti hőmérséklet megváltozása az egyik leggyakoribb és legfontosabb stressz faktor ami az élő szervezetekre hat. Hogy veszélyt jelent a biológiai rendszerekre, annak az az oka, hogy közvetlen hatása van a molekuláris mozgásokra és a kémiai reakciók sebességére. A molekuláris hőmozgás energiájának megváltozása veszélyezteti minden olyan molekuláris rendszer integritását melyeket gyenge kölcsönhatások kötnek össze. Ezek között fő helyet kapnak a membránok, melyek egysége a lipidek, fehérjék és más alkotók közötti hidrofób és van der Waals kölcsönhatások eredménye.

A hőmérséklet változásával szemben az élőlények különbözőképen védekeznek. Egyesek képesek minden esetben állandó testhőmérsékletet fenntartani (homeotermek) mások viszont nem (poikilotermek). A poikilotermek egy része a számára megfelelő hőmérsékletű területeket választja életteréül (stenotermek) más része pedig széles hőmérsékleti tartományban képes élni (euritermek). Ez utóbbi csoport rendelkezik azzal a képességgel, hogy membránjaik fizikai-kémiai összetételét úgy szabályozza, hogy a változó hőmérsékleteken zavartalan sejtműködést tartson fenn.

A lipidek hőmérséklet adaptációban betöltött fontos szerepét régóta vallják és számos esetben bizonyították, kimutatva azt, hogy a poikilotermek membránlipid öszetétele változik a környezet hőmérsékletével. Régóta kutatások tárgya annak a megállapítása, hogy hogyan játszanak szerepet a lipidek az adaptációban és melyik az a membrán paraméter vagy alkotó ami a hőmérséklet változását érzékeli és/vagy hatásait kompenzálja. Mindkét szempontból a legalkalmasabb paraméternek bizonyult a membrán "fluiditása". A membrán kutatásokban oly gyakran használt "fluiditás" mint paraméter nem egyezik a folyadékok fizikájában használt viszkozitás reciprokával, hiszen ennek meghatározása biológiai membránok esetében értelmét veszti, hanem egy jóval pontatlanabb fogalom, ami a membrán molekuláinak mozgási szabadságát hivatott jellemezni. Rendszerint egy külső, a membránba ágyazott molekula valamely fizikai paraméterének mérését jelenti. A fluiditás mérésére leggyakrabban spin-jelző vagy

fluoreszcens próba molekula rendparaméterét, rotációs korrelációs idejét vagy anizotrópia paraméterének mérését szokták használni. Ez esetekben a nagyobb rendparaméter, anizotrópia vagy rotációs korrelációs idő rendezettebb, a molekulák mozgását jobban korlátozó, azaz kevésbé fluid, míg ellenkező esetben fluidabb membránszerkezetre utal. Az ilyen értelemben vett membrán fluiditás hőmérséklet adaptáció során való szabályozását régen kimutatták és a vizsgált rendszerek alapján a membrán adaptáció homeoviszkózus jellegét posztulálták.

Különböző szervezetek vizsgálata során kiderült, hogy a homeoviszkózus adaptáció mértéke szervezet és mérési módszer szerint eltérő, azonban jelentősége fontos, olyannyira, hogy ujjabban a membrán fluiditás a hőmérséklet "szenzorának" és a hőmérséklet stressz indukált génexpresszió regulátorának bizonyult egyes szervezetekben.

A poikiloterm állati membránok lipidösszetétele igen változatos, hal esetében például több mint kétszáz különböző lipid molekulafajta mutatható ki. Máig sem ismeretes az, hogy a membránoknak miért van szüksége ilyen sokféle lipid molekulára és, hogy ezek mennyire és hogyan játszanak szerepet hőmérséklet adaptáció során a membrán fluiditásának szabályozásában.

A jelen munkának az volt a célja, hogy egyes poikiloterm szervezetek membránjainak hőmérséklet adaptációja során történő lipidösszetétel és fluiditásbeni változásait tanulmányozva egyes lipideknek a membrán fluiditás szabályozásában betöltött szerepét felderítse.

Megvizsgáltuk a zsírsavak telítetlenségi fokának szerepét a membrán fluiditás szabályozásában különböző hőmérsékleteken nevelt baktériumok esetében. Az eredmények azt mutatták, hogy mind az *X. nematophilus* mind a *P. luminescens* összlipidjeiből készült vezikulák fluiditása magasabb nevelési hőmérsékleten nagyobb volt, ami ellentmondásban van a homeoviszkózus adaptáció elméletével. Ugyszintén a össz zsírsavak telítettlenségi foka sem függött össze a mért membránfluiditással. A két vizsgált cianobaktérium közül a *C. raciborskii* tilakoid membránjának fluiditása, szemben a *Synechocystis* PCC 6803 törzzsel, nem változott jelentősen különböző nevelési hőmérsékleteken, annak ellenére, hogy a politelítetlen zsírsavak mennyisége alacsony nevelési hőmérsékleteken megnőtt mindkét baktérium membránjaiban.

Amennyiben a lipidek fontos szerepet játszanak a hőmérséklet adaptációban, úgy azt a leginkább a széles hőmérséklet tartományban élő poikiloterm állatok, mint például a halak használják ki a legnagyobb mértékben. Pontyok májának foszfolipid összetételét hasonlítottuk össze szezonális, téli és nyári hőmérsékletekhez való adaptáció során. Mind a

zsírsav összetételben, mind a foszfatidilkolin (PC) és foszfatidiletanolamin (PE) molekulaspeciesz összetételében változások voltak. Az össz telítetlen zsírsavak aránya megnőtt téli adaptáció során, úgyszintén a PC és PE lipid molekulaspecieszekben az 1-monoén, 2-polién specieszek mennyisége, különösen a PE-ben. A hidegadaptált halak májának össz foszfolipidjeiből készült vezikulák fluidabbnak bizonyultak a melegadaptált halakénál mind a spin-jelző, mind a fluoreszcencia mérési módszerek alkalmazása során. Az anizotrópia mérésekből kitűnt, hogy az eltérés mértéke nő a lipid kettősrétegben a fejcsoportok felé haladva (C-2 szénatom közelében). A májból tisztított mitokondrium és plazmamembránok fluiditása DPH fluoreszcencia anizotrópia mérések alapján a hidegadaptáció során nagyobb volt mint melegadaptáció során. Ezzel párhuzamosan a PC és PE lipidosztályokban hidegben az 1-monoén, 2-polién lipid molekulaspecieszek szintje megnőtt és az 1-telített, 2-telítetleneké pedig lecsökkent.

Hasonló vizsgálatokat végeztünk halak, akváriumban történő, rövidtávú akklimatizálása során is. Pontyokat (*Cyprinus carpio* L.) gyűjtöttünk télen ill. nyáron és az adaptációs hőmérsékletükkel ellentétes hőmérsékleten akklimatizáltuk őket. Az akklimatizációs periódus végén azt találtuk, hogy a hideghez akklimatizált halak májából tisztított mitokondrium és plazmamembrán frakciók fluiditása, fluoreszcencia anizotrópia mérések alapján, nagyobb volt mint a meleghez akklimatizált halaké, és a fluidizáció mértéke meghaladta a szezonális adaptáció során elért mértékét azonos hőmérsékleteken. Ezzel párhuzamosan alacsony hőmérsékleteken az 1-monoén, 2-polién lipid specieszek mennyisége megnőtt, hasonlóan a szezonális adaptációkor tapasztaltakhoz. Néhány lipidspeciesz mennyisége viszont minden esetben nőtt függetlenül attól, hogy a hőmérsékletet növeltük vagy csökkentettük.

A hőmérséklet adaptáció során a diétának is szerepe lehet a membránok lipid összetételének szabályozásában. Azt, hogy a diéta mennyire befolyásolja a lipidösszetétel szabályozását változó hőmérsékleteken, nem ismert. Ennek kiderítésére négy, téli hőmérsékletekhez adaptált de zsírsav összetételben igen eltérő diétán élő halat hasonlítottunk össze. Azt találtuk, hogy míg a trigliceridek zsírsavösszetétele tükrözte a diétában lévő különbségeket, a foszfolipidek zsírsavösszetétele ettől független volt. Ezzel párhuzamosan a máj össz foszfolipidjeiből készült vezikulák fluiditása, DPH fluoreszcencia anizotrópia mérések alapján, nem különbözött és a májból tisztított mitokondriumok és plazmamembrán frakció fluiditása sem tért el jelentősen a négy halfajta esetében. Ezek az eredmények arra utalnak, hogy a diéta zsírsavösszetétele nem

befolyásolja a membránok lipid összetételét és fizikai tulajdonságait és valószínűleg ebben az adaptációs hőmérsékletnek van döntő szerepe.

Szezonális adaptációjuk során a kétéltűek a vizsgált halakhoz képest abban térnek el, hogy télen nem aktívak. Célunk volt megvizsgálni azt, hogy a lipidösszetétel és membrán fluiditás szabályozásuk eltérő-e a halakétól. A máj zsírsavösszetételben nem találtunk lényeges különbségeket téli és nyári állatoknál. A PC és PE lipidosztályok molekula speciesz összetételében, a halaktól eltérően, az 1-monoén, 2-polién specieszek mennyisége sem változott. A máj foszfolipidekből készült liposzómák fluiditásában, téli és nyári szervezeteket összehasonlítva, nem volt eltérés a lipid kettősréteg C-16 és C-12 szintjén (antroyl-zsírsav jelölők anizotrópia mérése alapján), míg a C-2 szinten a nyári állatok foszfolipidjei fluidabb szerkezetet mutattak mint téli megfelelőjük.

Szintetikus lipidekből modell kísérleti rendszereket terveztünk, hogy eldöntsük egyes molekulaspecieszek szerepét a membránfluiditás szabályozásában. Kiválasztottuk azokat a lipideket amelyekről kísérleteink alapján bebizonyosodott, hogy mennyiségük változik a halakban és melyekről feltételeztük, hogy fontos szerepet játszhatnak a hőmérséklet adaptáció során. Ezekből egykomponensű, illetve olyan keverékű liposzómákat készítettünk amelyek egy téli és egy nyári hőmérséklethez adaptált hal májának lipidösszetételében jelen lévő arányokkal megegyeznek. Ezen rendszerek fluiditás mérése (spin-jelző rendparamétere valamint antroyl-zsírsav fluoreszcens jelölők anizotrópia mérése alapján) alapján beigazolódott az, hogy: (i) az 1-telített, 2-politelítetlen molekulaspecieszek arányának (szezonális mértékű) változása nem befolyásolja a membránszerkezet fluiditását. (ii) az 1-monoén, 2-polién specieszek mint a 18:1/22:6 PE és PC, növeli, az (iii) 1-telített, 2-monoén PE speciesz mint a 16:0/18:1 PE pedig csökkenti a membránszerkezetek fluiditását.

Végezetül megállapíthatjuk, hogy:

1. Baktériumok esetében, a zsírsavak össz telített/telítetlen aránya nem univerzális jelzője az összlipidekből készült membránok fluiditásának, tehát, valószínűleg a lipid molekuláknak létezik más olyan tulajdonsága ami felelős egy adott fluid szerkezet meghatározásáért.
2. Pontyok esetében amelyek széles hőmérsékleti tartományban élnek és aktívak, mind a foszfolipidek mind a májból tisztított mitokondrium és plazmamembránok esetében mérhető a fluiditás kompenzáció. Ezzel párhuzamosan jellemző a a lipidösszetételben

az 1-monoén, 2-polién, különösen PE, molekulaspecieszek növekedése alacsony hőmérsékleteken.

3. Poikilotherm szervezetek membrán lipid összetétele és fluiditásának szabályozásában az adaptációs hőmérsékletnek van döntő szerepe a diétával szemben.
4. A télen hibernáló kétéltűek téli és nyári adaptációja során az 1-monoén, 2-polién foszfolipidek mennyisége nem változik és a foszfolipid membránok fluiditásában sincs egyértelmű különbség. Valószínűnek tartjuk, hogy a két jelenség összefügg és hogy a fluiditás regulációja azért nem lehetséges mert ezen lipid molekulák mennyiségét sem képesek szabályozni. Feltételezhető, hogy ez egyik oka annak, hogy csak inaktív állapotban, ami csökkentett funkciójú membránokat jelent, képesek túlélni a hideg periódust.
5. A szintetikus lipideken készült modell rendszereken igazolást nyert az a feltevésünk miszerint, magasabb rendű poikiloterm szervezetek hőmérséklet adaptációja során az 1-monoén, 2-polién, ezen belül a 18:1/22:6 PE lipid molekulaspeciesz mennyiségének változása játszik főszerepet a (lipid-) membránok fluiditásának szabályozásában.

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