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REVIEW

The cold but not hard fats in ectotherms: consequences of lipid restructuring on susceptibility of biological membranes to peroxidation, a review

Elizabeth L. Crockett

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Abstract The production of reactive oxygen species is a regular feature of life in the presence of oxygen. Some reactive oxygen species possess sufficient energy to initiate lipid peroxidation in biological membranes, self-propagating reactions with the potential to damage membranes by altering their physical properties and ultimately their function. Two of the most prominent patterns of lipid restructuring in membranes of ectotherms involve contents of polyunsaturated fatty acids and ratios of the abundant phospholipids, phosphatidylcholine and phosphatidylethanolamine. Since polyunsaturated fatty acids and phosphatidylethanolamine are particularly vulnerable to oxidation, it is likely that higher contents of these lipids at low body temperature elevate the inherent susceptibility of membranes to lipid peroxidation. Although membranes from animals living at low body temperatures may be more prone to oxidation, the generation of reactive oxygen species and lipid peroxidation are sensitive to temperature. These scenarios raise the possibility that membrane susceptibility to lipid peroxidation is conserved at physiological temperatures. Reduced levels of polyunsaturated fatty acids and phosphatidylethanolamine may protect membranes at warm temperatures from deleterious oxidations when rates of reactive oxygen species production and lipid peroxidation are relatively high. At low temperatures, enhanced susceptibility may ensure sufficient lipid peroxidation for cellular processes that require lipid oxidation products.

Keywords Lipid peroxidation · Temperature · Biological membranes

Abbreviations

ChOOH	Cholesterol hydroperoxide
CoQ	Ubiquinone or coenzyme Q
GPx1	Classic glutathione peroxidase
GPx4	Glutathione peroxidase 4
LOOH	Lipid hydroperoxide
LOX	Lipoxygenase
LPO	Lipid peroxidation
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PLA_2	Phospholipase A_2
PLOOH	Phospholipid hydroperoxide
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid-reactive substance

Introduction

The earth's biosphere hosts a range of temperatures, from the constant chill found at high latitudes and in the deep sea, with relatively warm conditions characterizing most tropical and subtropical habitats. In contrast to these areas, seasonal swings in temperature are the norm in many aquatic and terrestrial habitats in temperate latitudes. Deserts and intertidal zones (including estuaries) cover large areas, and here diurnal fluctuations in temperature are particularly common. In addition to the spatial and relatively short-term changes in temperature, there is abundant

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E. L. Crockett (✉)
Department of Biological Sciences,
Ohio University, Athens, OH 45701, USA
e-mail: crockett@ohiou.edu

E. L. Crockett
Mount Desert Island Biological Laboratory,
Salisbury Cove, ME 04672, USA

evidence for profound and large-scale climatic variation in many of earth's habitats over geologic time while a large and growing database indicates anthropogenic influences on earth's thermal relations (Intergovernmental Panel on Climate Change 2007).

Environmental temperature has an enormous influence on all biota and is a leading factor in biogeographic distributions (Barry et al. 1995; Parmesan and Yohe 2003). Differences in organismal temperatures naturally imply different rates of biochemical reactions, including those that constitute many complex physiological processes and behaviors. The plasma and intracellular membranes, which form barriers as well as interfaces between environment, extracellular milieu, cytoplasm, or lumen of an eukaryotic organelle, represent levels of biological organization that are intimately influenced by temperature. The primary reason for temperature's influence on biological membranes involves principles of kinetic energy. While, we often think of static images of membranes, it is easy to forget that the lipid constituents of membrane bilayers are mobile (i.e., fluid) and the motion of these entropically driven assemblages is anisotropic. Although the timeframes of lipid motions vary depending on the nature of the motion, lipids in biological membranes can vibrate, rotate, isomerize (from trans conformers to gauche conformers and back again), hop laterally, and flip–flop transversely (Gennis 1989). Resistance to these bilayer motions is referred to as membrane viscosity while membrane fluidity represents its inverse (Lee 2004).

Membrane lipid restructuring refers to the compositional shifts in membrane lipids occurring with temperature acclimation, seasonal acclimatization, or adaptation over more long-term (e.g., evolutionary) time scales (Hazel and Williams 1990). Lipid restructuring will affect several physical and chemical properties that govern the structure, integrity, and function of cell and intracellular membranes. Because the consequences of lipid restructuring are multiple, the benefits are likely to involve more than a single attribute of membrane structure/function. The most frequently described outcome of membrane restructuring is its effect on membrane fluidity. In the nearly three and a half decades since Sinensky's (1974) description of homeoviscous adaptation in *E. coli*, numerous examples of partial (Cossins et al. 1980; Roy et al. 1997), and even complete (Behan-Martin et al. 1993; Crockett and Hazel 1995) homeoviscous efficacy have been described in a variety of organisms, yet striking examples exist of membranes lacking a homeoviscous response (Cossins et al. 1978; Lee and Cossins 1990), and some membranes even display inverse compensation with respect to temperature variation (Crockett and Hazel 1995). Although some degree of homeoviscous efficacy is often associated with membrane restructuring, the growing number of exceptions, and the nature of some of the specific lipid changes has led some authors to question the generality of homeoviscous adaptation (see Lee 1991; Hazel 1995). Other physical attributes of the membrane besides fluidity are also likely to be influenced by differences in lipid composition, including membrane phase behavior, membrane thickness, and membrane permeability (Hazel et al. 1998; Lee 2004; Hulbert and Else 2005). The function of many membrane-associated proteins, particularly integral proteins with multiple membrane-spanning α-helices (e.g., the P-type ATPases including Ca^{2+}-ATPase of the sarcoplasmic reticulum and Na^{+}/K^{+}-ATPase), can also be affected by lipid composition either directly (i.e., function may require or be modulated by specific lipids) or indirectly (i.e., via membrane chemical and/or physical properties) (Wu et al. 2001; Starke-Peterkovic et al. 2005; Lee 2003). As a result, lipid restructuring may influence the function of membrane proteins.

Lipid restructuring is unquestionably one of the most common responses to temperature variation in ectotherms as it has been documented in all major taxonomic groups. Despite the occurrence of this phenomenon in a variety of taxa, there is more uncertainty about the driving force(s) underlying membrane restructuring than about the physiological underpinnings associated with some of the less common features of temperature adaptation. Given the ubiquity of membrane restructuring during temperature variation, it is almost certainly a response to a change in a fundamental property (or properties) of membranes—a property(ies) of membranes that is (are) inherently sensitive to temperature. Regardless of what underlies this phenomenon, membrane lipid restructuring should have profound influences on a relatively overlooked, albeit important characteristic of biological membranes. The intent of this review is to lay the framework for how membrane restructuring during temperature variation is likely to affect the susceptibility of the membrane to potentially damaging lipid peroxidation (LPO). Preserving the susceptibility of the membrane to LPO at physiological temperatures represents a consequence of membrane restructuring not previously considered within the context of temperature adaptation. Although investigation of LPO in cells and biological membranes is a growing research area in biomedicine (e.g., Murray et al. 2005; Zieger et al. 2006), and aging biology (e.g., Hulbert et al. 2007), membrane susceptibility to LPO with its accompanying biophysical and functional ramifications, has as yet, received relatively little attention by comparative physiologists interested in temperature.

The diversity of membrane lipids

The lipids that compose biological membranes are enormously diverse (Stillwell and Wassall 2003; Lee 2004) and this assortment of lipids provides the ingredients necessary

for membrane restructuring. The most familiar membrane lipids are the polar lipids, including the phospholipids typically depicted in diagrams of membrane bilayers. Although these images of membranes often illustrate the phospholipid bilayer as balls (headgroups), with two straight lines (fatty acid chains) emanating from the ball, the phospholipids that constitute biological membranes are about as varied as these illustrations make them look identical.

Despite their name, polar lipids are actually amphipathic and can be grouped into two major categories: the glycerophospholipids and the sphingolipids (Fig. 1). The most abundant lipids in animal membranes are the glycerophospholipids (i.e., phospholipids with glycerol backbones). Glycerophospholipids can be categorized into several classes (based on the composition of the polar headgroup) including the relatively abundant phospholipids–phosphatidylcholine, or PC for short (with a choline headgroup), and phosphatidylethanolamine or PE (with an ethanolamine headgroup). The glycerophospholipid classes branch into subclasses depending on the nature of the bonds (i.e., ester or ether) between the non-polar tails and the glycerol backbone. The most common, and frequently-characterized, glycerophospholipids are the diacyl glycerophospholipids, those containing two fatty acids esterified (one each) to the *sn*-1 and *sn*-2 positions on the glycerol backbone. A second subclass of the glycerophospholipids known as the "plasmalogens" contain a vinyl-ether link in the *sn*-1 position on the backbone. Although less familiar, these lipids in ectotherms can represent up to 30% of the total PE contents in muscular tissues (Ohshima et al. 1989; Kikuchi et al. 1999). Functional roles of plasmalogens are not clearly defined although there is mounting evidence that plasmalogens can serve as membrane antioxidants (Nagan and Zoeller 2001; Gorgas et al. 2006). Within each glycerophospholipid subclass, many combinations of fatty acids (or fatty alcohols in the case of the plasmalogens) may be present on the lipid molecule. Each of these pairings of hydrophobic chains represents a different molecular species of that particular phospholipid.

Sphingolipids differ from the glycerophospholipids described above in several respects. The feature that unifies the sphingolipids is the sphingosine backbone, and is therefore characteristic of both phosphosphingolipids (e.g., a type of phospholipid of which sphingomyelin is the most common in vertebrates), and glycosphingolipids (sphingolipids devoid of phosphate but possessing carbohydrate headgroups). Although sphingolipids are important, particularly in membrane microdomains, there are relatively few reports (an exception in Zehmer and Hazel 2005) on whether and to what extent alterations in sphingolipids may be involved with temperature adaptation.

In addition to polar lipids, neutral lipids represent a second group of lipids found in biological membranes (Fig. 1). In contrast with the polar lipids, which are often charged at physiological pH and, as a result somewhat hydrophilic, the neutral lipids are generally uncharged and relatively non-polar. For the purposes of this review it is important to consider several neutral lipids that can influence physical properties of membranes, while also possessing antioxidant properties. The most common neutral lipids fitting this bill are cholesterol, homologues of ubiquinone (CoQ), and the vitamin E derivatives. Cholesterol stabilizes fluid-phase membranes by limiting movement within the bilayer (Yeagle 1985). Although not the membrane's most potent antioxidant, cholesterol may provide some protection to membrane lipids against oxidative damage (Parasassi et al. 1995; Stillwell et al. 1996). Ubiquinone exists as several homologues, varying

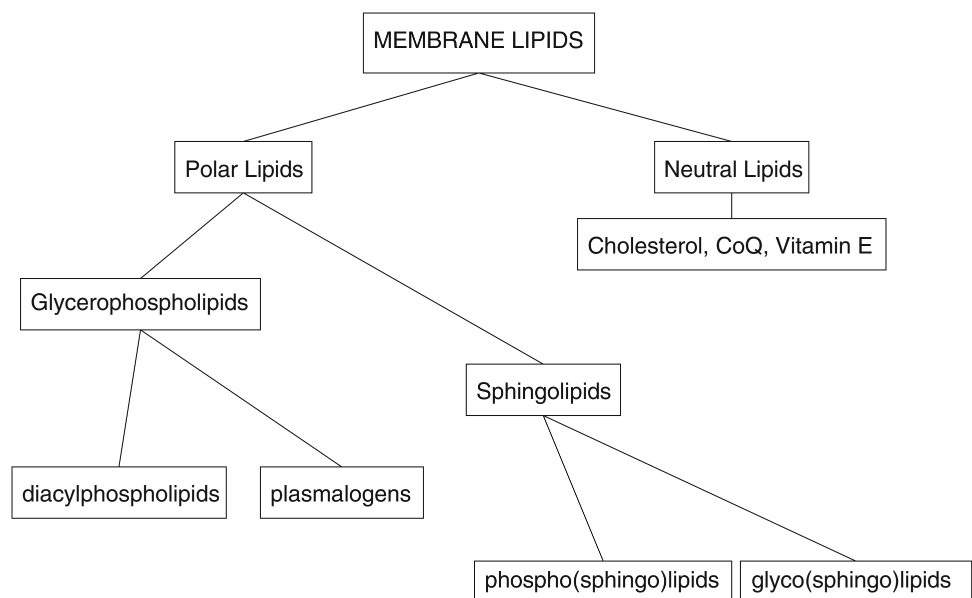

Fig. 1 Classification of membrane lipids

in the number of isoprenoid units; the most common homologues in vertebrates are the long-chain forms (e.g., CoQ_9 and CoQ_{10} with nine and ten isoprenes, respectively) (Ramasarma 1985). Ubiquinone is best-known for its role as a mobile electron carrier in the mitochondrial respiratory chain. This lipid has also become the subject of increasing interest in membrane biology because of its contributions to membrane fluidity (Jemiola-Rzeminska et al. 1996; Skowronek et al. 1996), its roles as lipid-phase antioxidant (Ernster and Dallner 1995; Turunen et al. 2004), and in the recycling of α-tocopherol (Sohal 2004). The vitamin E derivatives (α-tocopherol being the most common) provide the membrane's first line of defense against damaging oxidative reactions (Yoshida et al. 2003) while its putative non-antioxidant functions remain controversial (Azzi and Stocker 2000; Ricciarelli et al. 2001; Traber and Atkinson 2007).

Patterns of phospholipid restructuring with temperature variation

The most widespread trend associated with membrane restructuring involves changes in the degree of unsaturation (reviewed in Hazel and Williams 1990). In vertebrate taxa, the percentage of unsaturated fatty acids in both of the two major phospholipid classes (PC and PE) is inversely correlated with body temperature (Logue et al. 2000). This same trend is apparent from intraspecific studies in ectotherms (e.g., in the common carp, *Cyprinus carpio*, acclimated to either 10 or 30°C) (Tiku et al. 1996). Relative proportions of unsaturated fatty acids in membranes rise at low temperature in a variety of tissues and biological membranes including plasma membranes of polarizied epithelia (Lee and Cossins 1990; Crockett and Hazel 1995;), liver mitochondria (Wodtke 1978; Cossins et al. 1980), skeletal muscle mitochondria (Van den Thillart and de Bruin 1981), and brain synaptosomes, (Logue et al. 2000). Interestingly, in rainbow trout liver the non-raft portion of the plasma membrane does not fit the pattern described above while rafts increase their percentage unsaturation and polyunsaturated fatty acids (PUFA) at cold temperature (Zehmer and Hazel 2005). The sarcoplasmic reticulum of skeletal muscle also does not display an increase in unsaturation at low temperature (Cossins et al. 1978; Carey and Hazel 1989; Vornanen et al. 1999). In the sarcoplasmic reticulum of the goldfish, *Carassius auratus*, the membrane experiences ≈30% rise in PUFA in choline and ethanolamine glycerophospholipids, while the content of the most abundant monounsaturated fatty acid, 18:1, declines by approximately 50% in PC at low temperature (Cossins et al. 1978). Given these results, it is not surprising that there is no evidence for homeoviscous adaptation in sarcoplasmic reticulum (Cossins et al. 1978).

One might expect that changes in phospholipid unsaturation are distributed randomly amongst various phospholipids but this is not the case. Instead, restructuring of acyl chains within glycerophospholipids is highly specific. Several patterns are worth emphasizing here. First, restructuring of acyl chains is more evident in PE than in PC (Logue et al. 2000; Farkas et al. 2000; Trueman et al. 2000; Brooks et al. 2002). Although the class phosphatidylinositol typically represents a lower proportion of total phospholipid (often less than 10%), this class can also experience significant restructuring (e.g., more than a 40% drop in content of 18:0/20:4 with a 20°C reduction in temperature in liver microsomes from carp) (Brooks et al. 2002). Second, while PE is generally more unsaturated than PC (e.g., Logue et al. 2000), the nature of restructuring is fundamentally different for PC and PE. The average number of double bonds in PC increases at cold temperatures compared with those found in PC at warm temperature, while there is an increase in the proportion of fatty acyl chains that are unsaturated (with no change in the average number of double bonds) in PE at low temperatures (Tiku et al. 1996; Logue et al. 2000; Zehmer and Hazel 2005). Third, adjustments in acyl chain pairs are distinct within a phospholipid class and may, in fact, be independently maintained (Brooks et al. 2002). The development of more sophisticated techniques (such as electrospray ionization mass spectrometry—see Brooks et al. 2002) has allowed researchers to examine more precisely the changes in compositions of molecular species within phospholipids, and in particular, the combination of different fatty acids on specific positions on the glycerol backbone. These studies have revealed that relatively few molecular species of phospholipids are actually altered with temperature. An increase in the pairing of monounsatured fatty acids at *sn*-1 and PUFA at *sn*-2 positions occurs in a variety of different phospholipids at low body temperatures. Two relatively abundant pairings of acyl chains in PC and PE are18:1/22:6, and 18:0 (or 16:0)/22:6. These two different combinations, both with 22:6, respond in an opposite manner to low temperature; the former increases while the latter decreases (Dey et al. 1993; Fodor et al. 1995; Brooks et al. 2002; Buda et al. 1994; Farkas et al. 2000).

A second common response to temperature variation involves modulation of proportions of the two most common phospholipid classes, PC and PE (reviewed in Hazel and Williams 1990; Hazel 1995). In membranes from ectotherms, the content of PE relative to PC (expressed as a ratio PE/PC) typically rises at low temperatures, and as a result, PE/PC is inversely correlated with body temperature. In contrast, membrane rafts prepared from livers of 5°C-acclimated trout have elevated PC and reduced

phosphatidylserine contents compared with rafts prepared from 20°C-acclimated animals (Zehmer and Hazel 2005).

Although less well-studied than the diacylglycerophospholipids, shifts in plasmalogen content with temperature have also been documented. Plasmalogen levels rise with temperature in nervous tissue from fishes (Roots and Johnston 1968; Selivonchick and Roots 1976; Matheson et al. 1980; Yeo et al. 1997) while a similar trend has been observed in mitochondrial membranes prepared from oxidative muscle (Wodtke 1981; Kraffe et al. 2007).

The subject of restructuring of neutral lipids is important to consider since cholesterol, α-tocopherol, and ubiquinone all have effects on membrane physical properties, including fluidity and phase behavior (Yeagle 1985; Jemiola-Rzeminska et al. 1996; Stillwell et al. 1996). Accordingly, there may be involvement of neutral lipids in preserving membrane physical and/or chemical properties during variations in body temperature. Yet, only cholesterol has been systematically examined in this regard (see Crockett and Hazel 1995; Robertson and Hazel 1995; Zehmer and Hazel 2003). Since cholesterol orders fluid-phase membranes, particularly those with more saturated phospholipids (Pasenkiewicz-Gierula et al. 1990) one would predict a rise in cholesterol levels at higher temperature if animals use cholesterol to counter the effects of temperature (Hazel and Williams 1990; Crockett 1998). In some membranes, cholesterol responds to temperature in the manner expected (e.g., Robertson and Hazel 1995; Zehmer and Hazel 2003), while in other membranes there is either no change in cholesterol content (Crockett and Hazel 1995; Labbe et al. 1995; Robertson and Hazel 1995) or cholesterol contents may even be elevated at low body temperatures (e.g., Crockett and Hazel 1995).

Although there is little work on whether temperature affects levels of vitamin E or ubiquinone, studies performed on various taxonomic groups shed some light. Mourente et al. (2007) have recently pointed out that fishes generally contain higher levels of vitamin E than mammals; and the blood of Antarctic fishes contains, on average, seven-fold higher contents of α-tocopherol than the blood of temperate species (Gieseg et al. 2000). Whether higher plasma levels of α-tocopherol in these studies corresponds to higher contents of α-tocopherol in membranes is not known. Studies clearly indicate, however, qualitative changes in vitamin E and ubiquinone with temperature variation. A recently described compound, "marine-derived tocopherol"differs from the more common α-tocopherol in having a methylene unsaturation at the terminus of the isoprenoid tail (Yamamoto et al. 1999). Marine-derived tocopherol is distributed to a much greater extent in species of cold temperate (Yamamoto et al. 1999) and Antarctic (Dunlap et al. 2002) fishes than those found in warmer waters. At low temperature, marine-derived tocopherol is nearly three times more effective at inhibiting lipid oxidation than α-tocopherol (Yamamoto et al. 2001). Ubiquinone homologues found in nature vary in the length of the isoprenoid tail (Battino et al. 1990) of which CoQ_{10} (ten isoprene units) is the common homologue in temperate fishes (Battino et al. 1990) while the shorter CoQ_9 is the dominant form in Antarctic fishes (Giardina et al. 1997). The lower crystallization temperature of CoQ_9 may enable this electron carrier/antioxidant to function at very low temperatures (Giardina et al. 1997).

Reactive oxygen species and LPO are natural consequences of life with oxygen

Regular production of reactive oxygen species (ROS) is a natural outcome of life with oxygen. In eukaryotic cells, ROS are generated during mitochondrial respiration, catalysis by peroxisomal oxidases, and electron transport through the cytochrome P450 system of the endoplasmic reticulum (Halliwell and Gutteridge 1999). Although ROS are natural byproducts of metabolism, if uncontrolled, they can trigger oxidative damage to biological molecules (lipids, proteins, DNA) as well as macromolecular assemblages (biological membranes) (Halliwell and Gutteridge 1999; Kühn and Borchert 2002). Lipid peroxidation represents a distinct set of oxidations initiated by ROS that possess sufficient energy to remove a hydrogen atom from a methylene ($-CH_2-$) group within the parent lipid (LH) (Girotti 1985). During initiation, a lipid radical (L·) is produced which then reacts with dioxygen to produce a lipid peroxyl radical (LOO·). Lipid oxidation is propagated as the peroxyl radical reacts with a second lipid (LH) to generate another radical (L·) and a lipid hydroperoxide (LOOH). Lipid hydroperoxides in biological membranes include hydroperoxides of both phospholipids (PLOOH) and cholesterol (ChOOH). Unsaturated fatty acyl groups on glycerophospholipids can be oxidized by direct oxidant attack on the phospholipid or after hydrolysis of the ester bond and release of the fatty acid (catalyzed by phospholipase A_2) (Nigam and Schewe 2000). Decomposition of lipid hydroperoxides can take place by one-electron transfers from LOOH to transition metals or via reactions involving LOOH repair enzymes (see ahead). Secondary LPO products include alcohols, aldehydes, ketones, and lactones, which are generally less damaging than LPO intermediates formed earlier in the chain.

LPO affects membrane physical properties and protein function

Formation of the common products of LPO (e.g., PLOOH) introduces a polar residue within the otherwise non-polar interior of the bilayer and, as a result, can disrupt the non-covalent bonds (e.g., van der Waals interactions) that are

central to membrane stabilization. PLOOH can alter membrane packing, affect lipid–lipid and lipid–protein interactions, and place membrane integrity at risk (Kagan 1988; Kühn and Borchert 2002). LPO may influence phase behavior by raising gel–fluid phase transition temperatures (Coolbear and Keough 1983) and stabilizing the fluid lamellar phase (van Duijn et al. 1984). The effects of LPO on membrane order and/or fluidity have also been reported in numerous studies. There is no consensus, however, as to whether LPO increases or decreases membrane fluidity (e.g., Eichenberger et al. 1982; Wratten et al. 1992; Dinis et al. 1993; Drobnies et al. 1999; Megli and Sabatini 2003a, b). These discrepancies may reflect the use of membranes with varying lipid composition, probes reporting from different regions of the membrane, and/or spectroscopic methods indicating physical properties that may not be directly comparable.

Oxidation of phospholipids within biological membranes may also be deleterious because of influences on membrane physical properties likely to affect protein function. Two groups of membrane-associated proteins have been shown to be affected. Integral membrane proteins, many of which have multiple membrane-spanning helices and hence a significant portion of the protein embedded in the bilayer, are particularly vulnerable to effects of LPO. LPO in brain synaptosomes results in nearly a 90% reduction in Na^{+}/K^{+}-ATPase activity (Chakraborty et al. 2003). LPO also affects kinetic properties of Ca^{2+}-ATPase of sarcoplasmic reticulum. Studies with vesicles prepared from sarcoplasmic reticulum have shown that LPO reduces Ca^{2+} uptake and pump efficiency (i.e., Ca^{2+}/ATP ratio) (Kagan 1988; Dinis et al. 1993). In addition, thermal stability of the calcium pump is inherently sensitive to oxidation of membrane lipids (i.e., the temperature at which thermal denaturation occurs is significantly lowered by LPO) (Kagan 1988). Amphitropic proteins, whose activity depends on translocation to membrane surfaces, are also affected by LPO. CTP: phosphocholine cytidylyltransferase, which catalyzes the regulatory step in phospholipid (PC) synthesis, is activated by LPO and this activation is positively correlated with an LPO-induced disordering of the membrane (Drobnies et al. 1999; 2002). Presumably, disordering from oxidation of phospholipids facilitates insertion of CTP: phosphocholine cytidylyltransferase in the membrane. In contrast, protein kinase C is inhibited by phospholipid oxidation (Drobnies et al. 2002).

Cells possess a broad defense against reactive oxygen

To protect against unwanted oxidations, organisms utilize a defense system, including low molecular weight antioxidants and enzymatic antioxidants, which is generally matched to the production of ROS (Halliwell and Gutteridge 1999). Membranes possess their own antioxidants, with the lipophilic chain-breaking, free radical scavengers—the vitamin E derivatives, tocopherols and tocotrienols, constituting the first line of defense (Ricciarelli et al. 2001). As membrane lipids undergo oxidation, several enzymes can participate in repair. Glutathione peroxidase-4 (GPx4, also known as phospholipid hydroperoxide glutathione peroxidase) catalyzes the reduction of LOOH to its corresponding alcohol at the expense of reduced glutathione (Ursini et al. 1982). GPx4 has broad substrate selectivity and can reduce hydroperoxides of either phospholipids or cholesterol (Thomas et al. 1990). GPx4 works effectively within the membrane itself and does not require prior hydrolysis of the fatty acyl hydroperoxide from the parent phospholipid (Thomas et al. 1990). Phospholipase A_2 (PLA_2) can also service the repair of PLOOH (Rashba-Step et al. 1997); its preferred phospholipid substrates are the oxidized forms (van den Berg et al. 1993; Kambayashi et al. 1998). Fatty acid hydroperoxides released by PLA_2 may subsequently be reduced by classic glutathione peroxidase (GPx1).

LPO can play roles in cell processes

Although production of LOOH is an inevitable consequence of life with oxygen, and in many cases may be deleterious, a controlled rate of LPO balanced by antioxidants (to keep a steady state of LPO or what has been called "peroxide tone") appears to be required for a variety of cellular functions including eicosanoid biosynthesis, cell signaling, cell maturation and differentiation, and membrane remodeling associated with vesicular trafficking (Brigelius-Flohé 1999; Kühn and Borchert 2002). Beneficial roles of LPO also may include essential steps in apoptotic signaling via oxidation of the phospholipids phosphatidylserine and cardiolipin (Kagan et al. 2004). LPO under enzymatic control is carried out in a variety of cells by lipoxygenases (LOX) (Kühn and Borchert 2002). The lipoxygenase family, widely distributed in plants, fungi, and animals (Brash 1999), includes isoforms that can catalyze the formation of lipid hormones. 12/15-LOX is capable of dioxygenating ester-linked fatty acids (e.g., diacyl glycerophospholipids in biological membranes) to produce PLOOH (Nigam and Schewe 2000; Kühn and Borchert, 2002). This lipoxygenase isoform and its products are considered key elements in the reticulocyte–erythrocyte transition and degradation of mitochondria (Kühn and Thiele 1999).

Susceptibilities to LPO depend on lipid composition

Lipids with greater degrees of unsaturation are more prone to LPO. As explained in a seminal review on this topic

(Holman 1954), a methylene surrounded by double bonds (as is typically found in polyunsaturated fatty acids or PUFA) are 20–40 times more likely to experience oxidation than are monounsaturated fatty acids. This early report compares autoxidation of polyunsaturated fatty esters (measured as rates of oxygen consumption) and indicates that the oxidizability of docosahexaenoic acid (22:6), with six double bonds is nearly 7.5-fold greater than that of a fatty acid with two double bonds (e.g., linoleic acid, 18:2) (Holman 1954). A more recent study (Cosgrove et al. 1987) provides perhaps a somewhat more accurate comparison of rates of oxidation of polyunsaturated fatty acids. In this study, rates of LPO initiation were carefully controlled in order to obtain precise measures of oxidation kinetics. Using the ratio $k_p/(2k_t)^{1/2}$ as an indicator for oxidizability (where k_p is the rate constant for the rate-controlling propagation step while k_t is the rate constant for LPO chain termination), Cosgrove et al. (1987) demonstrate that docosahexaenoic acid undergoes oxidation at a rate that is five times that of linoleic acid (Fig. 2). Regardless of which comparison of PUFA is most accurate, these data indicate that oxidation rates are likely to be far greater in membranes enriched with PUFA with high numbers of double bonds than those membranes containing fewer PUFA, or PUFA with lower levels of unsaturation. Oxidation rates of the two major phospholipid classes, PC and PE, are also not equivalent. With the same fatty acids present, PE is much more likely to undergo an oxidative reaction than is PC (Kawakatsu et al. 1984; Wang et al. 1994). Furthermore, model membranes made with PE are also more sensitive to the influences of LPO on physical properties of membranes including polymorphic phase behavior than are membranes composed of PC (van Duijn et al. 1984).

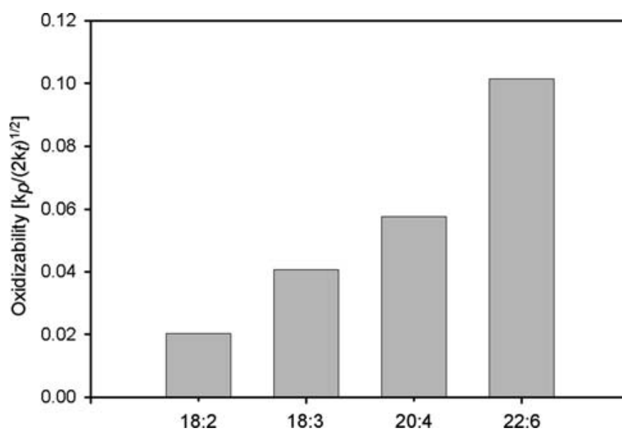

Fig. 2 Fatty acid oxidizability for polyunsaturated fatty acids with different double bond numbers. k_p is the rate constant for the rate-limiting step during LPO propagation while k_t is the rate constant for LPO termination. (Adapted from data presented in Cosgrove et al. 1987)

Temperature variation may alter membrane susceptibility to LPO

Phospholipid restructuring of biological membranes during temperature acclimation/adaptation is likely to have consequences on an organism's susceptibility to LPO. Shifts in relative quantities of PUFA should alter propensities of membranes to LPO. Studies using dietary manipulation of tissue levels of PUFA have shown that LPO is indeed more prevalent in animals with greater proportions of PUFA (Huang et al. 1998; Alvarez et al. 1998; Olsen et al. 1999; Lin and Huang 2007). In addition, Mourente et al. (2007) note that as levels of dietary and tissue PUFA increase in fishes, the requirement for dietary α-tocopherol is heightened, suggesting a need for greater protection in PUFA-enriched animals. Whether temperature-induced changes in PUFA content have a similar effect on LPO as do dietary alterations of membrane unsaturation is currently unknown. A second common feature of lipid remodeling, namely changes in the relative proportions of PE and PC (i.e, the PE/PC ratio), may also modify the nature of the membrane's vulnerability to LPO. PE's greater fatty acid unsaturation than PC may compound the likelihood that membrane susceptibilities to LPO are different for animals at warm and cold body temperatures.

Any discussion of LPO in ectothermic animals must also consider the influence of temperature on rates of LPO itself and the generation of LPO-initiating ROS. Much of what is known about thermal sensitivities of lipid oxidation comes from the literature concerning preservation of food products. While there is need for caution when applying data from non-living material to biological systems, these studies may be used as starting points for making predictions regarding ectothermic organisms including the fact that thermal sensitivities of lipid oxidation fit an exponential function (Frankel 1998). Studies with model membranes can also be helpful in evaluating temperature-dependence of LPO. Autoxidation of small unilamellar vesicles consisting of 16:0/22:6-PC (a common molecular species found in ectotherms) were monitored over time by quantifying malondialdehyde (a secondary LPO product) at 5°, 15°, and 25°C. Q_{10} values for oxidation rates were found to be 1.5 for every 10°C temperature interval tested (Crockett unpublished). Given the thermal sensitivities of lipid oxidation in non-living material, it is likely that in vivo LPO at warm temperatures outpaces similar reactions in the cold.

Rates of ROS production, which may ultimately set the pace of LPO, are likely to be affected by temperature either directly or because of changes in cell ultrastructure, metabolic fuel preferences, and oxygen solubilities. Increased ROS production accompanies elevated oxygen utilization rates at warmer temperatures (Abele et al. 2002; Keller et al. 2004), however, at higher temperatures there may be a

smaller percentage of oxygen consumption associated with radical formation (Keller et al. 2004). On the other hand, skeletal muscle in fishes (e.g., carp, goldfish, striped bass) living at low temperatures typically has 1.5- to 3-fold higher mitochondrial densities and oxidative capacities than counterparts at warmer temperatures (Johnston and Maitland 1980; Tyler and Sidell 1984; Egginton and Sidell 1989; Guderley 2004). While increases in mitochondrial volume densities facilitate oxygen flux at low temperature (Desaulniers et al. 1996; Sidell 1998), mitochondrial proliferation at low temperature could counter differences in rates of ROS production. A switch in metabolic fuel use with temperature variation may also affect the generation of ROS. Greater reliance on fatty acid fuels at low temperatures has been documented in muscle tissues from teleost fishes (e.g., Rodnick and Sidell 1994; Sidell et al. 1995). Since H_2O_2 production is higher when mitochondria are supplied palmitoyl-carnitine instead of pyruvate (St-Pierre et al. 2002), the shift in metabolic fuel preference could affect ROS production and ultimately LPO. Furthermore, oxygen solubility increases at low temperature due to greater physical dissolution and higher content of intracellular lipid (triacylglycerol droplets and mitochondrial membranes) (Sidell 1998; O'Brien and Sidell 2000). Because oxygen is approximately four times more soluble in lipid than water (Battino et al. 1968), higher lipid contents at low temperature may raise the oxygen availability for ROS-generating reactions.

Is Membrane susceptibility to LPO conserved at physiological temperatures?

Consider two sets of membranes prepared from ectotherms acclimated to either cold or warm temperature (Fig. 3). For each set of membranes, the rates of LPO should increase with temperature because of intrinsic thermal sensitivities of the chemical reactions involved. At any given temperature, however, the membranes most likely to undergo LPO are those with more PE and PUFA (i.e., those membranes from the ectotherm living at low body temperature). When comparison of LPO is made at physiological temperatures—indicated by the position of the arrows (i.e., at low temperature for membranes prepared from the cold-acclimated animal and at elevated temperature for membranes prepared from the warm-acclimated animal) the rates of LPO may, in fact, be quite similar. Membrane restructuring, with its concomitant changes in LPO susceptibility, coupled with differences in mitochondrial contents, oxygen solubility, and metabolic fuel preferenda, could be offset by thermal sensitivities of rates for LPO and ROS generation. It is also plausible that membrane restructuring represents a mechanism for opposing thermal effects on rates of LPO and ROS generation, permitting organisms to maintain a

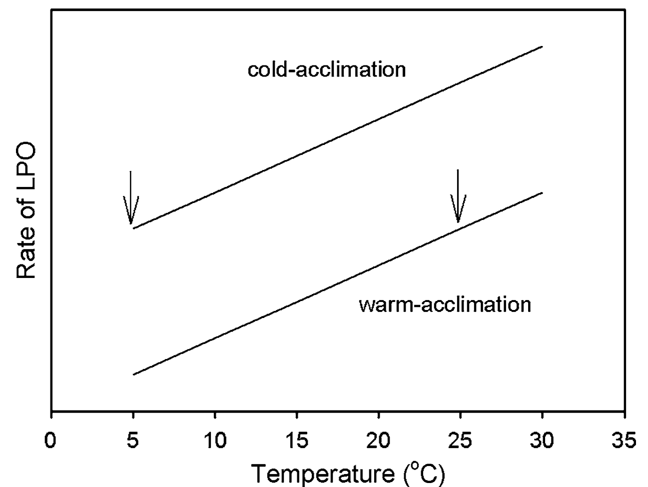

Fig. 3 Predicted (relative) rates of lipid peroxidation (LPO) measured at different temperatures for membranes from cold- and warm-acclimated ectotherms. *Arrows* indicate expected LPO rates are similar for cold- and warm-bodied ecotherms when compared at physiological temperatures

steady state of peroxide tone. I will return to this idea a bit later.

Effects of temperature variation on LPO

There is a growing literature that explores the question of whether LPO is altered by temperature variation in ectotherms. Although there are various ways to quantify LPO with each method possessing pluses and minuses (Dotan et al. 2004), the approach most commonly applied in the temperature literature, involves quantification of an endogenous product of LPO. Many authors have measured thiobarbituric acid-reactive substances (TBARS), which represent contents of malondialdehyde, a secondary albeit non-specific product of LPO while others measure malondialdehyde directly or lipid hydroperoxides (LOOH) (Table 1). A second approach compares rates of lipid radical production in samples of interest. This latter method assesses more directly the oxidation of organismal lipids.

Regardless of which approach is followed, acute warming of ectotherms is accompanied (in almost all cases studied) by elevated levels of LPO (Table 1). On the other hand, the effects of temperature acclimation/acclimatization on LPO are mixed (Table 1). Some reports indicate similar levels of endogenous LPO products in tissues from cold- and warm-acclimated animals. Contents of malondialdehyde are comparable in glycolytic muscle from zebrafish acclimated for 1 year to either 18°C or 28°C (Malek et al. 2004), and levels of TBARS are equivalent in the gills of temperature-acclimated oysters (Lannig et al. 2006). In contrast, warm acclimation in Antarctic species (limpet and zoarcid fish) is accompanied by elevated levels of LPO

Table 1 Effects of acute, acclimation, acclimatization, or adaptation temperature on indicators of lipid peroxidation

Genera (common names)	Tissue	Temperature effect	Citations
Acute temperature increase			
Carassius (goldfish)	Brain/liver	↑LOOH and TBARS	Lushchak and Bagnyujova (2006)
Carassius (goldfish)	Brain/liver/kidney	↑LOOH	Bagnyukova et al. (2007)
Carassius (goldfish)	Liver (only)	↑ TBARS	Bagnyukova et al. (2007)
Heteropneustes (catfish)	Gill/airsac	↑TBARS	Parihar and Dubey (1995)
Heteropneustes (catfish)	Liver	↑TBARS	Parihar et al. (1996)
Laternula (Antarctic clam)	Digestive gland	↑ L·	Estevez et al. (2002)
Mya (clam)	Digestive gland	↑L·	Estevez et al. (2002)
Mya (clam)	Mantle (mitochondria)	↑ TBARS	Abele et al. (2002)
Rana (frog)	Brain/liver/kidney/muscle	No change in TBARS	Bagnyukova et al. (2003)
Zoarces (eelpout)	Liver	↑TBARS	Heise et al. (2006)
Acclimation/acclimatization			
Carassius (goldfish)	Brain/liver/kidney	No seasonal effect on LOOH	Bagnyukova et al. (2007)
Carassius (goldfish)	Liver	TBARS highest in summer	Bagnyukova et al. (2007)
Crassostrea (oyster)	Gill	No change in TBARS	Lannig et al. (2006)
Danio (zebrafish)	Muscle	No change in MDA	Malek et al. (2004)
Mytilus (mussel)	Digestive gland	MDA highest in winter	Viarengo et al. (1991)
Nacella (Antarctic limpet)	Digestive gland	Warm-acclim ↑ lipofuscin	Abele et al. (1998)
Nacella (subpolar limpet)	Digestive gland	L· lowest in winter	Malanga et al. (2004)
Pachycara (Antarctic eelpout)	Liver	Warm-acclim ↑ TBARS	Heise et al. (2007)
Zoarces (eelpout)	Liver	TBARS lowest in summer	Heise et al. (2007)
Antarctic versus temperate			
Laternula versus *Mya*	Digestive gland	Higher L· in *Laternula*	Estevez et al. (2002)
Pachycara versus *Zoarces*	Liver (microsomes)	Higher L· in *Pachycara*	Heise et al. (2007)
Pachycara versus *Zoarces*	Liver	Lower TBARS in *Pachycara*	Heise et al. (2007)

Species are from temperate regions unless indicated otherwise. Indicators of LPO include lipid hydroperoxides (LOOH), thiobarbituric acid-reactive substances (TBARS), lipid radical (L·), malondialdehyde (MDA). Lipofuscin is an intracellular pigment used as an index of LPO

products (Abele et al. 1998; Heise et al. 2007). Some reports indicate that higher levels of LPO may be associated with reduced temperatures over seasonal time scales. Doubling of malondialdehyde contents occurs in the digestive gland of winter-acclimatized mussels compared with animals collected in warmer months (Viarengo et al. 1991) while in temperate zoarcid fish, TBARS are lowest in livers from summer animals (Heise et al. 2007). Yet, TBARS are nearly fourfold higher in liver of goldfish at summer temperatures (21°C) than those held at 3°C (Lushchak and Bagnyujova 2006; Bagnyukova et al. 2007). Results of two studies comparing Antarctic and temperate poikilotherms (clams and zoarcid fish) (Estevez et al. 2002; Heise et al. 2007) support the idea that animals at chronically low body temperatures may be at greater risk of LPO since lipid radical production is 4–5-fold higher in the Antarctic than in the temperate species when compared at physiological temperatures. Increased lipid radical production in the Antarctic animals may be countered by enhanced antioxidant defense (e.g., α-tocopherol) since the Antarctic species also have lower TBARS (Heise et al. 2007). Estevez et al. (2002) suggest that elevated lipid radical formation in an Antarctic clam may actually reflect higher contents of iron (which can promote formation of the toxic hydroxyl radical) than in the temperate species.

Can membrane susceptibility to LPO be inferred from differences in capacities for antioxidant defense?

A number of studies have sought to determine if body temperature influences an animal's requirements for enzymatic antioxidants (Witas et al. 1984; Viarengo et al. 1998; Regoli et al. 2000; Gieseg et al. 2000; Abele and Puntarulo 2004; Speers-Roesch and Ballantyne 2005; Leggatt et al. 2007; Heise et al. 2007). Unfortunately there is, as yet, no consistent pattern as to whether low (or high) body temperature is associated with fortification of antioxidant enzymes. The fact that consistent patterns do not emerge is probably due, in part, on experimental approach. Much of the literature on antioxidant capacities in the context of

temperature use seasonal acclimatization (Viarengo et al. 1991: Cancio et al. 1999; Ronisz et al. 1999; Lesser and Kruse 2004; Keller et al. 2004; Heise et al. 2007; Malanga et al. 2007), acute shifts in body temperature (Parihar and Dubey 1995; Parihar et al. 1996, 1997; Keller et al. 2004; Heise et al. 2006; Lushchak and Bagnyujova 2006; Bagnyukova et al. 2007), or make comparisons among species from different thermal habitats (e.g., Viarengo et al. 1995; Giardina et al. 1997; Gieseg et al. 2000; Estevez et al. 2002; Speers-Roesch and Ballantyne 2005; Heise et al. 2007). Although temperature variation is clearly a dominant physical feature of seasonal acclimatization in temperate zones, other physical factors (photoperiod or UV irradiance), biotic changes (nutritional and reproductive cycles), and/or seasonal pulses in xenobiotics may also be contributing factors (Winston 1991). Unrelated species from different thermal habitats (polar versus temperate) have often been used, and as a result, phylogeny may confound interpretation (Garland and Adolph 1994). Finally, some ambiguity may arise from the frequent use of tissues (e.g., liver) that are particularly complex in terms of ROS production, with significant ROS generated by mitochondria as well as peroxisomes and reactions of the cytochrome P450 system. It is largely unknown how temperature affects metabolism in peroxisomal and cytochrome P450 systems.

Relatively few studies have utilized controlled laboratory acclimations to examine how antioxidant capacity varies with temperature. Using an oligonucleotide microarray, Malek et al. (2004) report upregulation of several genes for antioxidant enzymes in skeletal muscle from zebrafish held for one year at 18°C compared with control (28°C) animals. A more recent study (Leggatt et al. 2007) examines activities of the antioxidant enzymes glutathione peroxidase and glutathione reductase in livers of killifish *Fundulus heteroclitus* acclimated for three weeks to various temperatures. In the latter study, no differences in enzyme activities are observed among acclimation groups when measured at 28°C. When enzymes are assayed at their respective acclimation temperatures, however, activities are significantly lower at 6°C than 30°C (Leggatt et al. 2007).

While there is no direct evidence for how, or to what extent, the capacity for repair of oxidized phospholipids is affected by temperature variation in ectotherms, some data are indirectly related to this topic. Activity of phospholipase A_2 increases in ectotherms at reduced temperatures indicating enhanced deacylation/reacylation for membrane restructuring (Neas and Hazel 1985; Roy et al. 1997). A second interpretation of these data, however, may be that in order to keep pace with LPO, a boost in the capacity for removing oxidized fatty acyl chains from parent phospholipids is necessary at low temperature.

Why do organisms restructure the lipids in biological membranes?

Although much literature on the topic of restructuring has accumulated over the last several decades, there is still neither a simple explanation nor is there likely to be a single answer for why ectotherms restructure membrane lipids. More than a decade ago, Hazel (1995) questioned why animals at low temperatures fortify their membranes with PUFA (when monounsaturated fatty acids are considered "superior") and why PE contents are elevated (when PE has an ordering effect on membranes). As Hazel points out, these broad-scale restructuring patterns do not fit particularly well with a homeoviscous response, but instead are more consistent with dynamic phase behavior (i.e., adjustment of phase transition temperatures to maintain proximity between phase transition and physiological temperatures). There is indeed evidence to support the idea that phase transitions in animals may be modified with temperature acclimation (Hazel et al. 1998).

A large literature (beyond the scope of this review), however, points to various chemical and physical properties, other than membrane fluidity (and phase transitions), that are key to membrane function (see review by Lee 2004). While there is little question that the ectotherms often protect some physical property (or properties) of biological membranes during thermal change, retaining the membrane's inherent susceptibility to LPO may also be requisite for a structurally and functionally sound membrane. Since LPO can alter membrane physical properties (via lipid–lipid interactions) and membrane function (via lipid–protein interactions), membranes must avoid excess LPO while at the same time tolerating a level of oxidation sufficient for cell processes that rely on LPO products, such as cell signaling. Conservation of membrane susceptibility to LPO may represent a homeostatic response in ectotherms, underlying a portion of the driving force for lipid restructuring.

While the most prominent restructuring patterns (including changes in degree of unsaturation and PE/PC ratios) lend support to the idea that membrane susceptibility to LPO is protected at physiological temperatures in ectotherms, some of the more fine-tuned changes in lipid composition also strengthen this hypothesis. Could the different modes of restructuring within the two major phospholipid classes (PC and PE) reflect the fact that PE is more vulnerable to LPO? Several reports (e.g., Tiku et al. 1996; Logue et al. 2000; Zehmer and Hazel 2005) describe an increase in average number of double bonds in PC at low temperatures while in PE the percent of unsaturated fatty acids is elevated with no accompanying change in the average number of double bonds. Perhaps the best strategy to maximize the physical changes associated with unsaturation, while at the

same time minimizing LPO in the more vulnerable PE, is to replace saturated fatty acids with monounsaturated fatty acids since this form of restructuring should have relatively little effect on LPO status. Since PC is at a reduced risk of LPO compared with PE, increasing the degree of unsaturation in PC may raise the level of LPO by a smaller extent than if those same changes were made in PE.

Several specific questions are germane to the hypothesis that restructuring may reflect, in part, a mechanism to achieve a steady state LPO (i.e., conservation of peroxide tone). Are the few and highly specific changes in fatty acid pairs within the phospholipids those that are least likely to impact LPO susceptibility? Is 22:6 sequestered into microdomains and/or paired with particular fatty acids in ectotherms so that their phospholipids are less likely to be oxidized? Does the increase in plasmalogen content at warm temperatures reflect a greater need for protection when rates of ROS production and LPO are elevated? Are levels of the neutral lipids, vitamin E and CoQ, subject to temperature acclimation, and what part might they play in affecting membrane susceptibility in ectotherms? If changes in the levels of membrane antioxidants do occur with temperature variation, does this mode of restructuring indicate a thermal dependence in the membranes' requirements for antioxidant defense? Does the elevated activity of phospholipase A_2 at low temperature facilitate the repair of damaged phospholipids?

Conclusions

To identify which combination of chemical and physical properties of the membrane represent the driving force(s) for lipid restructuring during temperature variation is a complex and serious challenge for comparative physiologists. Some membranes may have chemical and physical properties that are less sensitive to temperature variation while others may experience thermal disturbances yet possess functions that are relatively resilient. These particular membranes may undergo only modest restructuring. Yet, for the majority of membranes in which extensive restructuring does take place, preservation of both chemical and physical properties of the membrane may underlie restructuring. At the same time, the patterns of restructuring are likely to affect the susceptibility of the membrane to LPO. Whether lipid restructuring in ectotherms conserves peroxide tone by enabling a controlled level of membrane oxidation is not currently known. In any case, reductions in PUFA, PE/PC ratios, and mitochondrial contents at warm temperatures may help minimize unwanted oxidations of biological membranes when rates of ROS generation and LPO are relatively high. Reduced rates of LPO at low temperature may make PUFA and PE enrichment possible without increasing oxidative injury, which could otherwise accompany these modifications. Reduced rates of ROS production and LPO in the cold may also have facilitated the evolution of an enhanced oxidative capacity in ectotherms at very low body temperatures.

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