TEMPORAL CHANGES IN THE FATTY ACID COMPOSITION AND FLUIDITY OF GILL AND HEMOCYTE MEMBRANES DURING THERMAL ACCLIMATION OF THE SEA SCALLOP, PLACOPECTEN MAGELLANICUS

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by

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Abstract

The fatty acid composition and fluidity of gill phospholipids and hemocyte membranes of a eurythermal bivalve, *Placopecten magellanicus*, were investigated following a 10°C reduction in acclimation temperature. The time course of the acclimation response from 15 to 5°C was also monitored over a 21-day period. Membrane physical properties were measured by means of an electron spin resonance (ESR) spin-labeling technique, using 5-doxyl stearic acid (5-SASL) as a probe. Changes in the fluidity of gill membranes were analyzed by incorporating 5-SASL into vesicles made from total gill phospholipid (PL). Membrane fluidity measurements showed that cold-acclimated scallop membranes were more fluid than those of warm-acclimated animals. However, the order parameter of 5-SASL was greatest during the first six days of the reduction in temperature, indicating a more rigid gill membrane structure. The fluidity of gill and hemocyte membranes were negatively correlated with the proportion of 20:5n-3 (r = -0.714, $P < 0.001$, n = 24) and the unsaturated to saturated fatty acid ratio (r = -0.775, $P < 0.05$, n =8) respectively in the lipids of these tissues. Acclimation to reduced temperature for a prolonged period resulted in an increase in polyunsaturated fatty acid and a decrease in monounsaturated fatty acid content, in particular 20:5n-3 and C_{20} monoenoic fatty acids. Maximum changes in PL fatty acid levels required 15-21 days following acclimation to 5°C. Scallop gill and hemocyte membranes underwent progressive changes in their membrane fluidity and fatty acid composition, an observation which is consistent with homeoviscous adaptation following a reduction in acclimation temperature.

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List of Abbreviations

$2T_{\perp}$	inner hyperfine splitting value
$2T_{\parallel}$	outer hyperfine splitting value
5-SASL	5-doxyl stearic acid spin label
AMPL	acetone-mobile polar lipid
ANCOVA	analysis of covariance
DG	diacylglycerol
DHA	docosahexaenoic acid
DPH	diphenylhexatriene
EDTA	ethylenediaminetetraacetic acid
ESR	electron spin resonance
FAMEs	fatty acid methyl esters
FFA	free fatty acid
FID	flame ionization detection
G	gauss
GC	gas chromatography
GF/C	glass fibre / coarse
H_{II}	hexagonal phase
HVA	homeoviscous adaptation
L_{α}	liquid-crystalline fluid phase
L_{β}	liquid-crystalline gel phase
MAS	modified anti-aggregate Alsever solution
MUFA	monounsaturated fatty acid
ND	not detectable
PC	phosphatidylcholine
PE	phosphatidylethanolamine

PG	prostaglandin
PI	phosphatidylinositol
PLFA	phospholipid fatty acids
PL	phospholipid
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
S	order parameter
SASL	stearic acid spin label
SEM	standard error of the mean
SFA	saturated fatty acid
ST	sterol
SW	seawater
TAG	triacylglycerol
TLC	thin layer chromatography
T_m	transition temperature
UFA	unsaturated fatty acid
UI	unsaturation index

1. Introduction

Temperature is one of the most important environmental variables affecting the metabolic activities of ectothermic organisms. Generally, changes in temperature induce an acclimatory response in which an animal's physiological functions are altered. The significance of these alterations is that the animal maintains its metabolic functions at a relatively constant rate in spite of varying environmental temperatures (Widdows and Bayne, 1971). At the cellular level, one important mechanism of this acclimatory response is the restructuring of biological membranes. The primary constituents of biological membranes are lipids and proteins held together through relatively weak electrostatic and hydrophobic interactions (Hazel, 1995; Hazel and Williams, 1990). Although this arrangement of constituents is considered an integral component of membrane function (Singer and Nicolson, 1972), it is particularly sensitive to changes in environmental temperature.

The normal functioning of biological membranes is highly susceptible to changes in the overall packing arrangement of membrane constituents, resulting in an increase in membrane order in response to a decline in temperature (Williams and Somero, 1996). Ectotherms are known to counteract the ordering effects of reduced temperature through changes in the structural composition of their cell membranes. Alterations in membrane lipid composition are used by bacteria (Sinensky, 1974), plants (Raison et al., 1982) and animals (Behan-Martin et al., 1993) to ensure 'optimal' cell functions in response to thermal stress. Ectotherms use differences in phospholipids to maintain membrane physical properties during temperature changes. Thus, membrane order is frequently similar at the animals' respective

acclimation temperatures. This preservation of a particular membrane order, despite different environmental temperatures, is believed to be responsible for regulating the activity of membrane proteins and therefore membrane function over wide ranges in environmental temperature (Sinensky, 1974).

1.1. Physical properties of membrane phospholipids

Ectotherms that inhabit regions experiencing seasonal, diurnal and/or short term fluctuations in environmental temperature must contend with the perturbation of membrane organization when cell or body temperatures change. Such changes in membrane organization can significantly compromise membrane structure and ultimately membrane function. Depending on thermal conditions, membrane phospholipids can exist in two very distinct lamellar physical states, known as the L_α and L_β forms (Houslay and Stanley, 1982). At physiological temperatures, a relatively fluid, liquid-crystalline fluid phase (L_α) predominates due to the presence of a number of gauche rotomers. The introduction of gauche rotomers, due to the cis double bond, produces a 'kink' in an acyl chain which results in a pronounced lateral expansion of the lipid bilayer (McElhaney, 1994), thereby reducing the increase in packing order of membrane phospholipids caused by a drop in temperature. Below the physiological range, acyl chains adopt an all-trans conformation and pack tightly to form a highly ordered, less fluid gel phase (L_β) (Caffrey, 1985) in which acyl chain mobility is restricted and lateral motion in the plane of the bilayer is impaired (Hazel, 1988).

In model systems consisting of a single species of phospholipid (i.e. homoacyl

saturated phospholipids) it is possible to identify a single, sharply defined region of phase transition separating gel and liquid crystalline states (Hazel, 1988). In contrast to model systems, biological membranes exhibit much broader phase transitions and more complex phase behaviours due in part to the increased diversity of phospholipid molecular species. Therefore, a zone of phase separation consisting of coexisting domains of fluid and gel lipids may exist (Hazel, 1988). The temperature at which this phase transition occurs depends primarily on the chemical structure and length of the hydrocarbon chains and to a lesser degree on the structure of the phospholipid headgroup (McElhaney, 1994). Although a reduction in chain length may modestly reduce the transition temperature (T_m), its effectiveness is secondary to that of the introduction of a double bond. However, the effectiveness of lowering T_m decreases considerably with the introduction of additional double bonds (Coolbear et al., 1983). Therefore, lipids containing highly unsaturated acyl chains may have higher T_m values than their less unsaturated analogues of comparable length (McElhaney, 1994).

When temperature exceeds the physiological maximum at some defined temperature, acyl chains undergo an additional transition from the L_α to a non-lamellar phase that results in a loss of bilayer integrity due to the formation of hexagonal (H_{II}) structures. Hexagonal membrane structures result from change in the phospholipid molecular geometry from a cylindrical to a conical shape due to an imbalance between the size of the polar headgroup and the volume occupied by the acyl chains. The formation of conical lipids, in particular

phosphatidylethanolamine (PE), is known to compensate for increased disorder, but this process alone cannot maintain bilayer integrity (Hazel, 1995).

1.1.1. Phase transitions in biological membranes

Phase transitions and separations are frequently reported in membranes of homeothermic organisms, for example in the brush border membranes of rat (Brasitus et al., 1980), plasma membranes of rat liver (Schroeder, 1983), and cell membranes of hamster lung (Lepock et al., 1983). This is generally not true for membranes of multicellular ectotherms. For example, measurements of the fluorescence polarization of diphenylhexatriene (DPH) did not detect a phase transition in brain synaptosomal (Cossins and Sinensky, 1986; Cossins et al., 1981; Cossins, 1977) and sarcoplasmic (Cossins et al., 1978) reticular membranes of goldfish, brush border membranes of rainbow trout enterocytes (Di Costanzo et al., 1983) and mitochondrial and microsomal membranes of green sunfish liver (Cossins et al., 1980). However, evidence of a phase transition was detected through discontinuities in Arrhenius plots of the order parameter for 5-doxyl stearic acid embedded in phospholipid vesicles of two species of planktonic crustacean, *Cyclops vicinus* and *Daphnia magna* (Farkas et al., 1984). This general lack of a phase transition in multicellular ectotherms may be attributable to the lower average temperatures in which these animals live and the preponderance of lower melting membrane constituents compared with those found in homeotherms (Hazel, 1988).

1.2. Homeoviscous adaptation

Many studies have shown that ectothermic organisms exploit phospholipid structure to remodel their membranes, with those molecular species with the appropriate physical properties being able to offset the effects of the prevailing environmental temperature. Such adaptive changes in the chemical composition of the membrane are believed to result in the thermal compensation of membrane function. The maintenance of similar physical properties under extreme and variable temperatures is frequently referred to as homeoviscous adaptation (HVA). This hypothesis is based on observations of membrane lipids of *Escherichia coli* acclimated at 43°C and 15°C, which displayed similar physical properties at their respective acclimation temperatures (Sinensky, 1974).

Genotypic comparisons of membrane order between various vertebrates provide the most compelling evidence in support of HVA. When membrane order is measured at a common temperature, those vertebrates adapted to cold environments, like Antarctic fish (*Notothenia neglecta*), produce membranes that are much less ordered than those of homeothermic vertebrates, such as mammals and birds (Hazel, 1995). Temperate species generally produce membranes of intermediate order between the extreme values for polar fish and homeothermic vertebrates, but when membrane order is compared at their respective body or habitat temperatures, values are generally similar between species. This conservation in membrane order despite differences in temperature clearly illustrates the principle of HVA. Comparable trends in membrane order have been shown in Arctic and tropical copepods (Farkas et al., 1988), sarcoplasmic reticular membranes of rabbit and winter flounder (Vrbjar

et al., 1992) and mitochondrial membranes of various species of Pacific abalone (Dahlhoff and Somero, 1993). HVA is also a common acclimatory response of eurythermal temperate species. Similar trends in membrane order in response to changes in temperature have been reported for plasma membranes from liver (Hazel et al., 1992), erythrocytes (Dey and Farkas, 1992) and basolateral (Crockett and Hazel, 1995; Williams and Hazel, 1994) membranes of rainbow trout and for phospholipid vesicles from gills of the intertidal mussel *Mytilus californianus* (Williams and Somero, 1996).

The response of different membrane preparations to changes in temperature can be better assessed by comparing the shift in assay temperatures required to produce equivalent levels in membrane order (Wodtke and Cossins, 1991). This quantitative measure of the acclimatory response to changes in ambient temperature has frequently been called the efficacy of HVA (Cossins, 1983). Homeoviscous efficacy is calculated by first determining the difference in assay temperatures that result in membranes of equal order. This difference in assay temperatures is then expressed as a percentage of the difference in acclimation temperatures. A value of 100% indicates a complete response, while a value of zero indicates no response. The effect of compositional changes in membrane order is generally not perfect and may vary in magnitude depending on the membrane type and the metabolic activity of the membrane fraction. Mitochondrial membranes generally exhibit the highest efficacies of HVA per unit metabolic activity. For example, mitochondrial membranes of frog epidermal cells show a higher efficacy of HVA than their microsomal membrane fractions (Lagerspitz and Laine, 1984). Similarly, in a tropical air-breathing teleost, *Clarias batrachus*, the ranking in

fluidity compensation of brain membrane fractions is as follows: mitochondria > synaptosomes > myelin (Roy et al., 1997).

Collectively, the effectiveness of HVA from genotypic comparisons ranges from 70 to 100% (Cossins and MacDonald, 1989) compared with the generally lower efficacies (20-50%) of phenotypic comparisons (Cossins and MacDonald, 1989; Cossins and Sinensky, 1986). Values less than 20% are rarely observed, although efficacies of 7-10% have been detected in a comparison of several species of cold (5-10°C) and warm (20-27°C) adapted teleost fish (Dey et al., 1993). The reason for such low efficacies presumably lies in the reduced ability of the fish to regulate membrane dynamic structure in response to changes in environmental temperature. In contrast, species that live in relatively constant thermal environments (e.g. polar seas and thermal springs) generally demonstrate a more complete adaptation of membrane structure due to the degree of specialization required for survival at such temperatures (Cossins and Prosser, 1978). However, the high degree of adaptation exhibited by these species may incur a considerable cost due to their inability to tolerate even small changes in their ambient temperature (Somero et al., 1996).

1.2.1. Inconsistencies in the homeostatic response

Although there is much evidence in support of HVA there are also many examples of membrane responses that are inconsistent with or difficult to explain in terms of HVA. Certain membranes do not display HVA, such as sarcoplasmic reticular and apical membrane domains of thermally acclimated goldfish (Ushio and Watabe, 1993; Cossins et al., 1980;

Cossins et al., 1978), brush border membranes of carp intestinal mucosa (Lee and Cossins, 1990), and plasma membranes of trout spermatozoa (Labbe et al., 1995). Furthermore, the apical membranes isolated from trout enterocytes display a significant inverse compensation in membrane order following acclimation from 20 to 5°C (Crockett and Hazel, 1995). An inverse relationship between membrane order and acclimation temperature was also reported for two species of bacteria symbiotically associated with entomopathogenic nematodes (Fodor et al., 1997). Although the capacity for HVA seems to represent a basic cellular response, it is hardly a universal response to changes in temperature. Collectively, the above evidence shows that there are no consistent relationships between the direction, occurrence and magnitude of HVA in response to thermal stress, suggesting that mechanisms other than the conservation of lipid order may contribute to the thermal compensation of membrane function.

1.2.2. Rapid adjustments in membrane order

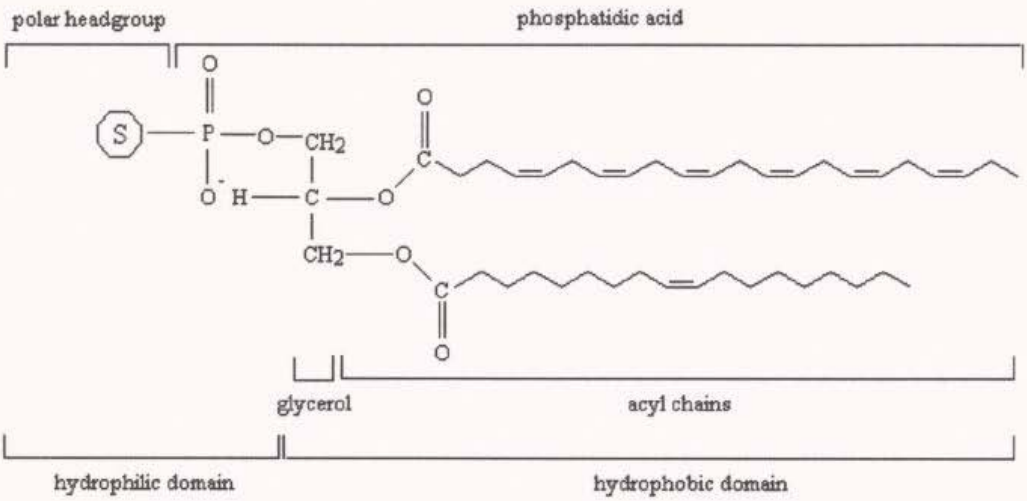
Changes in the thermal environment of aquatic ectotherms can occur rapidly. Therefore, it has been hypothesized that species that experience such conditions probably possess some means of rapidly counteracting changes in membrane order. Adaptive changes in membrane orders in response to changing environmental temperature are well known in ectotherms; however, the time course of this response has received little attention. Although the time course of adjustments in membrane order has been reported in membranes of several multicellular ectotherms, including the rough and smooth endoplasmic reticular membranes

of carp liver (Wodtke and Cossins, 1991), carp erythrocytes (Dey and Farkas, 1992) and plasma membranes of rainbow trout hepatocytes (Williams and Hazel, 1994).

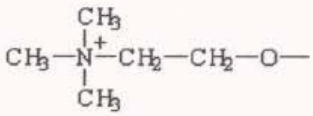
The rate of change in membrane fluidity generally varies according to the thermal direction of the acclimation time course. In goldfish brain synaptosomes, the transition from 5 to 25°C requires 10-14 days, while cold acclimation requires a total of 30-40 days to make similar adjustments in fluidity (Cossins et al., 1977). More recent studies suggest that fluidity adjustments may occur over periods varying from a few hours to several minutes. For example, endoplasmic reticulum membranes of carp liver adjust membrane fluidity within 24 h following cooling from 30 to 23°C (Wodtke and Cossins, 1991) while 25°C-acclimated carp erythrocytes require only 40-60 min to alter the fluidity of their membranes after a 20°C drop in temperature (Dey and Farkas, 1992). Comparable values for microorganisms and protozoans are generally on the order of a few hours (Martin and Thompson, 1978).

1.3. Composition of biological membranes

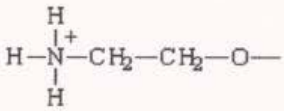
The main structural elements of biological membranes, the phospholipids, represent a diverse family of structurally related molecules that occur in a variety of forms. This structural diversity arises from their heterogenous nature at both i) the hydrophilic domain (i.e. polar head groups), which defines a particular phospholipid class; and ii) the hydrophobic domain (i.e. fatty acyl chains), which designates a unique molecular species within a given phospholipid class (Fig. 1.1).



Structure of polar headgroup (S) — Phospholipid



Phosphatidylcholine (PC)



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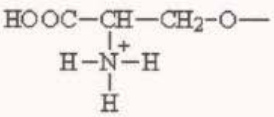
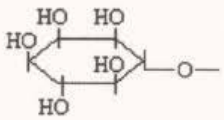


Phosphatidylserine (PS)



Phosphatidylinositol (PI)

Fig. 1.1 The molecular architecture of membrane phospholipids.

Ectotherms are known to exploit this diversity in phospholipid forms to restructure their membranes in response to changes in temperature. Temperature-induced alterations in the chemical composition of membrane structure may include one or more of the following: i) molecular species restructuring, ii) alterations in phospholipid class composition, iii) changes in acyl chain unsaturation and iv) modulation of sterol levels to offset the disruptive effects of temperature change.

1.3.1. Molecular species

The restructuring of the acyl chain composition of membrane phospholipids is a common adjustment used by ectotherms in response to temperature change. However, gross changes in the acyl chain composition do not provide a complete picture of alterations in membrane structure because the molecular species composition is not specified. In fact, molecular species may be altered independently of the fatty acid composition through the reshuffling of existing acyl chains. Phospholipases play a key role in this restructuring of membrane phospholipids through the cleavage of acyl chain moieties and subsequent re-acylation to produce new membrane phospholipids (Evans et al., 1997). Therefore, since rates of membrane lipid biosynthesis are severely depressed at low temperatures, such alterations in membrane lipid composition provide a mechanism for rapid adjustments in membrane physical properties in response to changes in environmental temperature (Evans et al., 1997; Williams and Hazel, 1994). Such a mechanism was first proposed by Ramesha and Thompson (1983) to account for changes in membrane fluidity that preceded changes in acyl

chain unsaturation of *Tetrahymena pyriformis* membranes following cold acclimation from 39.5 to 5°C. Similarly, the green alga *Dunaliella* displays an acclimatory response to low temperature by increasing the diunsaturated constituents of its phosphatidylglycerol molecular species at the expense of molecular species having one saturated acyl chain (Lynch and Thompson, 1984).

Changes in membrane physical properties may be accounted for by the adjustment in the levels of relatively few molecular species. For example, it has been observed that the 1-monounsaturated-2-polyunsaturated-PE molecular species are the predominant phospholipids in the liver and brain of freshwater and marine fish (Buda et al., 1994; Dey et al., 1993) seasonally adapted to their thermal environments. Furthermore, the determination of the molecular species composition of PC and PE revealed a 4- to 5-fold and 10-fold increase in the level of 18:1/22:6 and 18:1/20:5 respectively in cold adapted fish liver membranes (Dey et al., 1993).

1.3.2. Phospholipid class composition

Membranes of cold-acclimated ectotherms normally possess higher proportions of PE and lower proportions of PC than those acclimated to higher temperatures (Hazel and Williams, 1990). Furthermore, alterations in the PC/PE ratio of biological membranes are believed to contribute to the thermal adaptation of membrane function by mediating the balance between bilayer-stabilizing and bilayer-destabilizing lipids in response to thermal change (Hazel and Williams, 1990). Due in part to the bulky hydrophobic domain of PE

compared with that of PC, PE assumes a conical rather than a cylindrical geometry and, consequently, destabilizes the lamellar phase of the bilayer by increasing the interval between the ambient temperature of the animal and the gel/fluid transition temperature. An increase in PC levels compared with PE has the opposite effect. Therefore, adjustments in phospholipid class composition, in response to thermal acclimation, ensures that the ambient temperature of the organism is maintained within a suitable interval above the T_m yet below the H_{II} phase transition (Hazel, 1995). Thus through regulating membrane physical properties an animal prevents its membranes from undergoing a phase transition which would otherwise compromise membrane integrity and as a consequence membrane function.

Adjustments in phospholipid class composition are known to change rapidly in response to temperature changes. For example, Hazel and Landrey (1988a) showed that rainbow trout, acclimated from 20 to 5°C, decreased the level of PC and PE in their renal plasma membranes after only 8 and 16 h respectively. Similar alterations in the ratio of PC to PE have also been reported in gill membranes of trout (Hazel and Carpenter, 1985) and microsomal membranes of Sonoran desert teleosts (Carey and Hazel, 1989). These patterns of change in phospholipid class composition are consistent with the theory of thermal compensation of membrane function, which states that rapid adjustments in phospholipid headgroup composition may aid in stabilizing membrane structure during the initial onset of a thermal stress. Interestingly, initial changes in phospholipid composition of trout kidney and gill membranes are not sustained in fully acclimated animals, suggesting that initial

adjustments in headgroup composition may be superseded by other mechanisms of temperature acclimation later in the acclimation time course (Hazel, 1995).

1.3.3. Acyl chain unsaturation

The fatty acid composition of structural lipids is regarded as one of the most important factors controlling the physical state of biological membranes. An inverse relationship between the content of unsaturated fatty acids (UFA) and temperature has been reported for several ectothermic animals (Hazel, 1988; Williams and Hazel, 1994), including crustaceans (Kashiwagi et al., 1997; Pruitt, 1990), molluscs (Viarengo et al., 1994; Napolitano and Ackman, 1993; Napolitano et al., 1992) and fish (Roy et al., 1997).

One universal response to a decline in temperature is a decrease in the proportion of saturated fatty acids and a corresponding increase in the proportion of UFA. Although saturated fatty acids are usually reduced in response to a decrease in temperature, the nature of the UFA that replace them is usually variable. The physiological importance of such changes remains unclear, although it has been suggested that this differential pattern of replacement may relate to the activity level of the organism at low temperatures (Hazel, 1988). The importance of UFA in maintaining physiological functions at low temperatures has been shown by Farkas et al. (1981), who observed that a winter-active crustacean (*Cyclops vicinus*) acquired higher PUFA levels compared with the elevated monounsaturated fatty acid levels of an inactive crustacean (*Daphnia magna*). Increased levels of monoenes

have also been reported in membranes of goldfish and related species that tend not to be very active at low temperatures (Schunke and Wodtke, 1983; Wodtke, 1978; Cossins, 1977).

1.3.4. Sterol content

Cholesterol is generally the dominant sterol of marine crustaceans and fish, although some marine sponges, echinoderms and molluscs may possess 20 or more different sterols, with cholesterol present at relatively low concentrations (Napolitano et al., 1993). For example, 17 different sterols have been identified in the sea scallop *Placopecten magellanicus* (Napolitano et al., 1993; Idler and Wiseman, 1971).

The primary function of cholesterol is believed to be an architectural component of biological membranes although it may also serve to alter the physical state of the lipid bilayer (Nes, 1974). For example, cholesterol influences the activity of membrane proteins at low temperatures (Raynard and Cossins, 1991; Yeagle et al., 1988), presumably by increasing the fluidity of the lipid bilayer. In contrast, sterols may also counteract this increase in fluidity by preventing the formation of a loosely arranged lipid bilayer, which would otherwise occur as the temperature is raised (Yeagle, 1985).

Membranes from animals acclimated to high temperatures typically contain higher levels of cholesterol than in cold-acclimated animals (Robertson and Hazel, 1995; Wodtke, 1978). However, higher levels of cholesterol have been reported in brush border membrane fractions of intestinal epithelia of cold-acclimated trout compared with equivalent membranes from warm-acclimated fish (Crockett and Hazel, 1995). Such differences in sterol levels are

believed to minimize both a decrease and increase in membrane fluidity which may otherwise occur in response to fluctuations in temperature.

1.4. Objectives

The objective of this study was to examine the effects of reduced acclimation temperature on the acclimatory response in membrane composition and structure of a bivalve mollusc. The sea scallop, *Placopecten magellanicus*, was chosen for study because it is a temperate water bivalve that normally experiences large seasonal and short-term fluctuations in environmental temperature. Sea scallops inhabit a region of the Northwest Atlantic ranging from Cape Hatteras, North Carolina to the north shore of the Gulf of St. Lawrence, where water temperatures may vary from below 0°C in winter to above 23°C in summer (Boghen, 1995). In this study the effects of a change in the acclimation temperature in this range (15 to 5°C) was investigated.

Alterations in fatty acid composition and membrane order of scallop gill and hemocyte membranes were determined to compare temperature responses with different functions. Therefore one might suspect that temperature induced alterations in phospholipid fatty acid composition and order may aid in the functional adaptation of their membranes that are directly influenced by the animal's thermal environment. Hemocytes also provide a nondestructive method with which to study temporal changes in membrane composition and structure because they can be easily obtained from hemolymph sinuses without unduly harming the animal (Fisher, 1988). Changes in the fatty acid composition and membrane

order were also analyzed at regular intervals over a 21-day period of acclimation to characterize the time course of the adaptation to a rapid reduction in environmental temperature.

Changes in the biophysical properties of gill and hemocyte membranes of *P. magellanicus* were investigated by means of an electron spin resonance (ESR) spin-labeling technique. The spin label selected to study the effect of reduced temperature upon the fluidity of gill and hemocyte membranes was 5-doxyl stearic acid. Changes in the fluidity of gill membranes were assessed by incorporating 5-doxyl stearic acid into phospholipid vesicles formed from the gills of thermally acclimated scallops.

Relatively few studies have investigated the effect of a reduction in temperature on the activity of bivalve hemocytes. Fisher (1987) observed that a reduction in the environmental temperature reduced the defense-related activities (i.e. aggregation, cell spreading and locomotion) of bivalve hemocytes. Although these activities were reduced at low temperatures, they were partially restored after a period of acclimation (Fisher, 1988). Many studies have examined changes in hemocyte physiology following thermal adaptation; however, studies that detail the effects of temperature acclimation on the fatty acid composition and order of hemocyte membranes have not been investigated, to my knowledge.

2. Materials and Methods

2.1. Animals

Sea scallops (*Placopecten magellanicus*, 8-12 cm shell height) were obtained by SCUBA divers from North Harbour, Placentia Bay, Newfoundland in November 1997 (approximate water temperature 10-12°C). After collection, scallops were transported live to the Ocean Sciences Centre, Memorial University of Newfoundland, St. John's, Newfoundland and maintained in flowing seawater (SW) in a 192-l fibre glass tank for no less than four weeks before experimentation. Submersible heaters (Aquarium Systems) were used to maintain a controlled water temperature of between 14-15°C. Scallops were feed a mixed algal diet of *Isochrysis galbana* and *Nannochloropsis* sp. (see Section 2.2 for details).

2.2. Algal cultures

Scallops were fed four times a week a mixed diet of *Isochrysis galbana* (Clone T-Iso) and *Nannochloropsis* sp., grown separately in 0.5 to 200 l volumes under semi-continuous culture. Cultures were continuously aerated and illuminated with cool white "daylight" fluorescent tubes. pH was maintained between 7 and 8 by bubbling carbon dioxide through the culture twice daily for 2 min.

Algal cultures were grown in f/2 nutrient media (Fritz f/2 Algae Food, Fritz Industries, Inc., Dallas, USA) mixed with filtered SW, pre-screened through a series of in-line (coarse/ 10 μm / 3 μm/ 0.2 μm) and ultraviolet (UV) filters. The recipe for the above nutrient mixture consisted of 0.14 ml f/2 nutrient media per litre of filtered SW. It was not possible to

autoclave volumes greater than 10-l.

Algal cultures were maintained for no longer than 14 days at any one volume, after which a newly prepared culture was initiated using aseptic techniques. Cultures were harvested daily, after which the volume removed was replenished with an equal volume of filtered SW enriched with f/2 medium. Algal cultures ranged in concentration from 2.5 to 5.5 million cells·ml^{-1} and provided a nutrient rich diet high in myristic (14:0), palmitic (16:0), palmitoleic (16:1n-7), oleic (18:1n-9), linoleic (18:2n-6), linolenic (18:3n-3), stearidonic (18:4n-3), eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids (Table 2.1).

2.3. Time course protocol

Twenty-four scallops acclimated at 14-15°C were transferred directly to 80 l temperature-controlled aquaria maintained at 5 ± 0.5 °C for a period of up to 21 days (Fig. 2.1). Two chambers were used due to the limited capacity for holding scallops at this reduced temperature. No significant difference in temperature was observed between the two chambers throughout the experiment. Groups of four randomly selected scallops were killed at 0 (14-15°C), 1, 3, 6, 10, 15 and 21 days (all at 5°C) post-transfer, when hemocytes were isolated and gill tissue was excised for lipid extraction.

Each chamber was supplied with SW regulated at ambient temperatures and pre-filtered through a series of in-line filters (coarse/ 10 μm/ 3 μm/ 1 μm). The flow rate was regulated by controlling the volume of water entering a header tank before emptying into the

Table 2.1 Fatty acid composition of total lipid extracts of *Isochrysis galbana* (T-Iso) and *Nannochloropsis* sp. (mean ± SEM).

Fatty acid (mol%)	*Isochrysis galbana* (n=5)	*Nannochloropsis* sp. (n=4)
14:0	19.9 ± 0.59	7.13 ± 0.05
15:0	0.36 ± 0.02	0.34 ± 0.01
16:0	8.74 ± 0.31	17.3 ± 0.59
18:0	0.15 ± 0.06	0.39 ± 0.06
16:1n-9	0.87 ± 0.23	3.17 ± 0.37
16:1n-7	6.47 ± 0.18	21.0 ± 0.50
16:3n-4	0.37 ± 0.01	0.84 ± 0.01
16:4n-1	0.43 ± 0.02	0.25 ± 0.01
18:1n-9	8.38 ± 0.09	3.38 ± 0.04
18:1n-7	1.65 ± 0.05	0.51 ± 0.01
18:2n-6	19.4 ± 0.13	4.36 ± 0.07
18:3n-6	1.82 ± 0.02	0.43 ± 0.02
18:3n-3	8.79 ± 0.19	ND
18:4n-3	9.77 ± 0.19	ND
20:4n-6	0.19 ± 0.05	3.95 ± 0.07
20:5n-3	0.26 ± 0.03	33.7 ± 1.05
22:5n-6	1.77 ± 0.07	ND
22:6n-3	8.56 ± 0.41	ND
Σ saturates	30.0 ± 0.79	28.4 ± 0.58
Σ monoenes	17.4 ± 0.42	28.1 ± 0.59
Σ polyunsaturates	52.7 ± 0.79	43.5 ± 1.09
Σ n-3	27.4 ± 0.70	33.7 ± 1.05
Σ n-6	23.1 ± 0.21	8.74 ± 0.16
n-3 / n-6	1.18 ± 0.03	3.86 ± 0.12
Saturation index	2.35 ± 0.09	2.53 ± 0.07
Unsaturation index	1.95 ± 0.04	2.26 ± 0.05

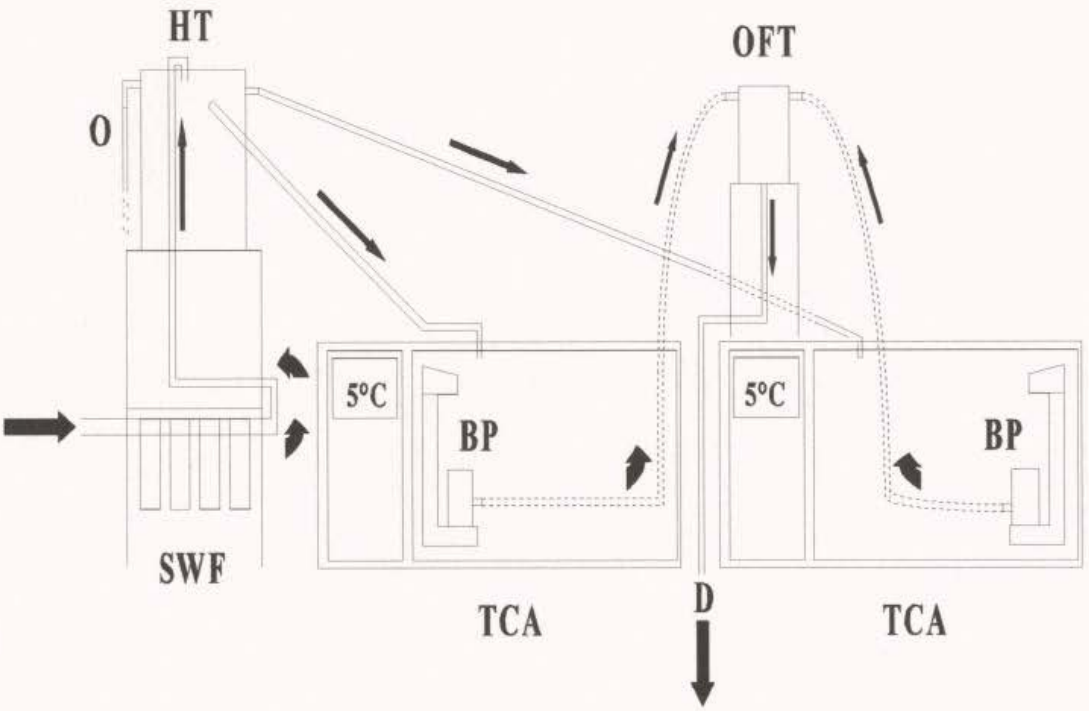


Fig. 2.1 Schematic diagram of acclimation chamber set-up. Arrows indicate direction of water flow through system. Diagram not drawn to scale. BP, bilge pump; D, drain; HT, header tank; O, overflow; OFT, overflow tank; SWF, saltwater filtration (coarse/ 10 μm/ 3 μm/ 1 μm pore size); TCA, temperature controlled aquarium.

refrigerated aquaria. Using this set-up it was possible to maintain a flow rate of 8.4 l/h. Excess water build-up in each tank was removed using a bilge pump (Rule 360 GPH, Rule Industries Inc., MA.) equipped with an automated water level control lever. The total volume of each tank was renewed every 9.5 h without significantly altering the acclimation temperature.

2.4. Hemocyte isolation and preparation

Hemocytes were withdrawn from the adductor muscle sinus of *P. magellanicus* with a 10 ml syringe fitted with a 23-gauge needle. Hemolymph (6-10 ml) was collected and immediately diluted with 3 volumes of a modified anti-aggregate Alsever solution (MAS) consisting of 0.12 M glucose, 0.03 M sodium citrate, 0.01 M ethylenediaminetetraacetic acid (EDTA) and 0.39 M sodium chloride (López et al., 1997) plus 1% sodium azide in distilled water. Negligible aggregation of hemocytes occurred when this procedure was followed as determined by microscopy. Hemolymph samples were centrifuged at 2000 g for 10 min to separate the hemocytes from the plasma (Thompson, 1977). The hemocyte pellet was resuspended in 1 ml MAS and frozen at -20°C for lipid analysis.

2.5. Lipid analysis

2.5.1. Lipid extraction

Hemocyte and algal samples were extracted following the procedure of Folch *et al.* (1957) employing 3 volumes of chloroform-methanol ($CHCl_3$-CH_3OH; 2:1 v/v) and additional quantities of $CHCl_3$ and chloroform-extracted deionized water. Prior to extraction, algal cells were collected on pre-combusted GF/C filters.

Due to the limited quantity of gill tissue per scallop (3-8 g animal $^{-1}$), an alternative method was used to ensure the complete recovery of lipids. Pre-weighed frozen gill filaments were finely ground with a Polytron tissue grinder in four-volumes of $CHCl_3$-CH_3OH (1:2, v/v) according to the method of Bligh and Dyer (1959), as modified by Christie (1982). The homogenate was filtered through a Whatman #1 filter and the filtrate was collected. The homogenized tissue and filter were re-extracted with $CHCl_3$-CH_3OH (1:2, v/v) and the filtrates were combined with the initial filtrate. Additional quantities of $CHCl_3$ and chloroform-extracted de-ionised water were used for washing to increase the recovery of lipids from the organic layer. The combined filtrates were allowed to separate overnight under nitrogen at 4°C in a 60 or 100-ml separatory funnel, after which the lower phase was dispensed and concentrated under a gentle stream of N_2. Concentrated lipid extracts were stored at -20°C under nitrogen prior to lipid class determination and fatty acid analysis.

2.5.2. Lipid class separation and identification

The lipid classes of scallop hemocytes and gill tissue were analyzed by thin layer chromatography with flame ionization detection (TLC/FID) using a MARK V Iatroscan (Parrish, 1987). Between 0.5 and 20 μl of the total lipid extract was separated into individual lipid classes on silica gel-coated SIII Chromarods using four different solvent systems. The rods were initially developed for 25 min in hexane/diethyl ether/ formic acid (99:1:0.05 by vol.), followed by a second development of 20 min, after which the rods were scanned to the ketone peak. The remainder of the neutral lipids, varying in polarity from triacylglycerols to diacylglycerols, were separated with a subsequent 40 min development in hexane/diethyl ether/formic acid (80:20:1 by vol.). The remaining polar fraction was developed twice in 100% acetone for 15 min and twice in $CHCl_3/CH_3OH/H_2O$ (50:40:10 by vol.) for 10 min and then quantified by scanning the entire length of the Chromarod.

The three chromatograms were digitally combined and the individual peaks were integrated using *T*-data scan software (RSS Inc., California, USA). Peak areas were converted to mass values by employing a separate calibration curve for each lipid class in the extract. Lipid standards (Sigma, St. Louis, MO) consisted of the following: n-nonadecane for hydrocarbons, cholesteryl palmitate for steryl and wax esters, hexadecan-3-one for ketones, tripalmitin for triacylglycerols, palmitic acid for free fatty acids, hexadecan-1-ol for alcohols, cholesterol for sterols, glyceryl-1-monohexadecanoate for acetone-mobile polar lipids and DL-α-phosphatidylcholine for phospholipids.

2.5.3. Polar lipid separation

Total gill phospholipids were separated from neutral lipids by passing each extract through a Pasteur pipette filled with 0.5-0.6 g of silica gel (Fisher Scientific). Neutral lipids were eluted from the column with 3 ml of chloroform: methanol: formic acid (9.9:0.1:0.1 by vol.) and the phospholipids removed from the column using 5 ml of 100% methanol. After elution, the purified phospholipids were concentrated under a gentle stream of nitrogen and stored at -20°C.

2.5.4. Derivatization and fatty acid analysis

Fatty acid methyl esters (FAMEs) were prepared by transesterifying each lipid extract with 14% BF_3 in methanol, following the method described by Parrish *et al.* (1996). Lipid extracts were methylated by incubating an aliquot of each extract with 0.5 ml of 14% BF_3/MeOH plus 1 ml hexane at 80°C for 60 min. FAMEs were recovered by centrifuging the mixture at 125 g for 5 min, after adding 0.5 ml of $CHCl_3$-extracted water and 2 ml hexane.

FAMEs were analysed with a Varian Model 3400 gas chromatograph (GC) equipped with a flame ionization detector. The FAMEs were separated on a 30 m x 0.32 mm (i.d.) fused silica capillary column coated with a 0.25 μm film of Omegawax (Supelco, Bellefonte, PA, USA), using hydrogen as the carrier gas. The oven temperature was programmed to warm at 65°C for 0.5 min, followed by an increase to 195°C at a rate of 40°C·min^{-1}, where it was held for 15 min. The final oven temperature of 220°C was reached at a rate of 2°C·min^{-1}, and was held there for 0.75 min. Hydrogen was supplied as a carrier gas at a rate

of 2 ml/min. The injector temperature was programmed to rise from 150°C to 250°C at a rate of 200°C·min^{-1}. Fatty acid methyl esters were identified from their retention times relative to known standard mixtures (PUFA1 and PUFA2, Supelco). The percent fatty acid composition was determined by integration using a Varian Star Integrator.

2.6. Electron spin resonance (ESR) analysis

2.6.1. Hemocyte labelling

The incorporation of a stearic acid spin label (SASL) into a heterogenous mixture of scallop hemocytes was readily accomplished using a method established for human erythrocytes (Kamada and Otsuji, 1983). Hemocytes were isolated and concentrated as described above in section 2.4. An aliquot (500 μl) of the hemocyte suspension was mixed with 25 μg (5-mg/ml in 90% ethanol) of 5-doxyl stearic acid (5-SASL; Sigma, St. Louis, MO) in 500 μl of MAS and incubated at 37°C for 30 min. After incubation, hemocytes were washed three times with 10 volumes of MAS to eliminate free spin label. After the final wash the labelled hemocytes were resuspended in 500 μl MAS and centrifuged at 2500 g for 10 min to remove the aqueous supernatant.

2.6.2. Vesicle preparation

Phospholipid vesicles were prepared by evaporating gill phospholipids dissolved in $CHCl_3$ onto a pre-weighed glass test tube using a gentle stream of N_2. The dried phospholipids were weighed, resuspended in 1 ml of $CHCl_3$ and mixed with <1 mol% of 5-

SASL (in 90% ethanol). The lipid mixture was gently vortexed for 10 min prior to solvent removal and resuspended in 1 ml 0.2 M Tris-HCl (pH 7.4) and sonicated in a bath type sonicator (Bransonic® 1200) at 30°C for 20 min to facilitate vesicle formation. Labelled vesicles were isolated from the buffer by centrifuging at 10,000 g for 10 min.

2.6.3. Electron spin resonance spectroscopy

Labelled hemocytes and phospholipid vesicles were transferred to a 100 mm disposable quartz capillary tube (0.8-mm i.d.) which was inserted into a standard quartz ESR tube (Wilmad Glass, N.J. USA). Hemocytes and vesicles were drawn into the capillary tube and the ends sealed with Critoseal® before the capillary was centrifuged at 13,360 g to remove excess water. Spectra were obtained from 0 to 20°C at 5° ± 1°C intervals of the cooling cycle using a computerized ESP-300 ESR spectrometer (Bruker, Billerica, MA) equipped with a programmable temperature controller (Omega Engineering, Inc., CN-2000). Samples were maintained at each assay temperature for 15 min between varying thermal measurements. Optimal signal to noise ratios were achieved using the following acquisition parameters: modulation frequency, 100 kHz; modulation amplitude, 1.01 Gauss (G); field set 3385 G; scanning field, 100 G; receiver gain, 1.60×10^4, time constant, 20.48 sec; sweep time, 163.84 sec; microwave power, 10 dB, and microwave frequency, 9.75 GHz.

2.6.4. Membrane fluidity estimates

Representative ESR spectra of 5-SASL embedded in scallop hemocyte membranes and gill phospholipid vesicles are shown in Fig. 2.2. The observed values of the outer ($2T_{\sqcap}$) and inner ($2T_{\perp}$) hyperfine splitting values (in Gauss) were used to calculate the order parameter (S) of 5-SASL, according to the following relationship:

$$S=\frac{T_{\sqcap}-T_{\perp}-c}{T_{\sqcap}+2T_{\perp}+2c}\times 1.723$$

where $c = 1.4G - 0.053(T_{\sqcap} - T_{\perp})$. The correction term (c) accounts for deviations between calculated and experimental first approximation values of $2T_{\sqcap}$ and $2T_{\perp}$ (Gaffney, 1975). The spectral estimate of the order parameter of 5-SASL was interpreted as a singular measure of the extent to which the membrane lipids were arranged in the hemocyte and gill membrane. The smaller the order parameter estimate the less ordered the membrane interior and therefore the more fluid the membrane structure.

2.7. Chemicals

All chemicals were purchased from either Fisher Scientific, Inc. or Sigma Chemical Company (St. Louis, MO) and were of the highest analytical grade available.

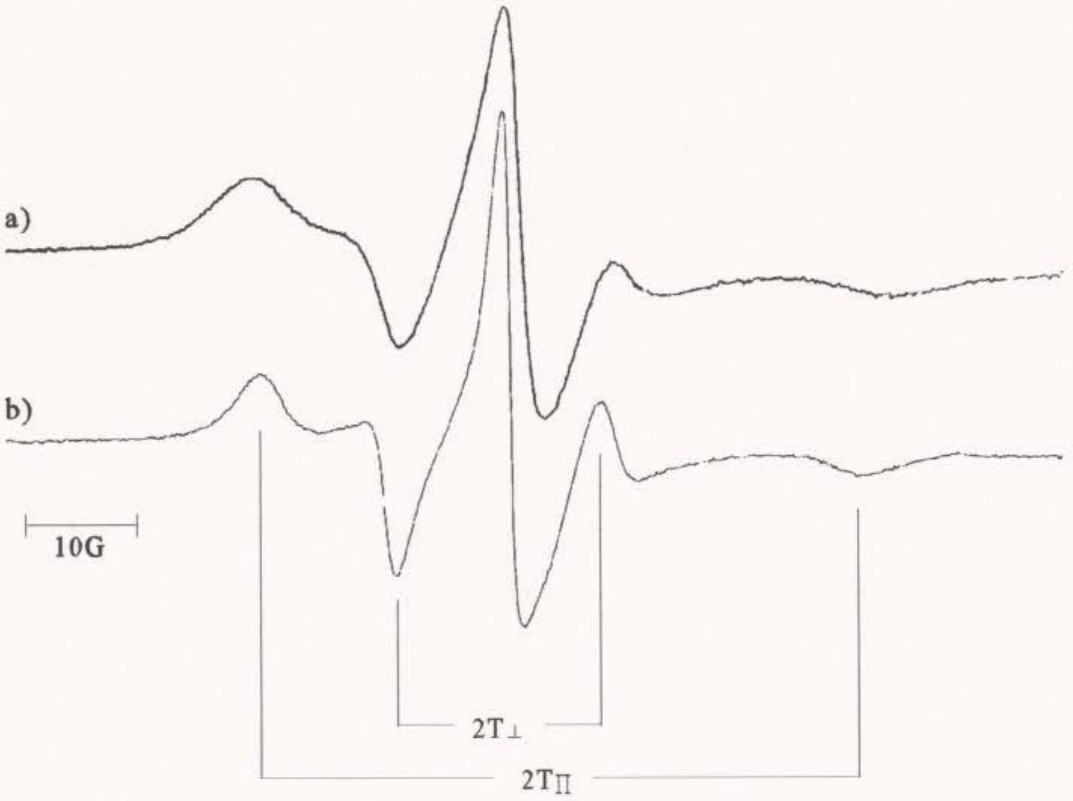


Fig. 2.2 ESR spectra of 5-doxyl stearic acid in a) hemocyte membranes and b) hydrated gill phospholipid vesicles obtained from thermally acclimated scallops. G, Gauss; $2T_{\perp}$, inner hyperfine splitting; $2T_{\parallel}$, outer hyperfine splitting.

2.8. Statistical analysis

Temporal changes in lipid percentage data and fluidity changes were analysed, over time, using one-way analysis of variance (ANOVA) and followed, where appropriate, by Tukey's multiple comparison post-test when the resulting F statistic was considered significant ($P < 0.05$). Linear regression and Spearman's correlation coefficient (r) were used to assess the degree of correlation between lipid levels and membrane order. Data were statistically tested using either Jandel SigmaStat or Minitab Software. In most cases, the assumptions of parametric tests were satisfied and arcsine or $\log_{10}$ transformations were used to normalize data if required. In the cases where transformations failed to normalize the data, the non-parametric Mann-Whitney Rank Sum test was performed. The criterion of significance was a P value of less than 0.05.

3. Results

3.1. Structural order and lipid composition of scallop gill membranes

3.1.1. The effect of temperature acclimation on membrane order

The structural order of gill phospholipid vesicles isolated from 15 and 5°C-acclimated scallops was measured at five different temperatures extending throughout the physiological range of *Placopecten magellanicus* (Fig. 3.1). The data in Fig. 3.1 clearly illustrate that as the temperature is decreased the order parameter of 5-SASL increases. Secondly, when measured between 0 and 20°C, vesicles prepared from gills of 5°C-acclimated scallops were significantly less ordered than vesicles of 15°C-acclimated scallops after three weeks of thermal acclimation (ANCOVA, $F_{1,29} = 5.05$, $P = 0.032$; Fig. 3.1). The rightward shift in the order parameter curve of warm-acclimated vesicles to lower temperatures shows that the gill membranes of cold-acclimated scallops were distinctly less ordered (i.e. more fluid), than the corresponding membranes of warm-acclimated scallops. The homeoviscous efficacy shown in these membranes was therefore estimated at approximately 14%. This quantitative measure of the acclimatory response to changes in acclimation temperature was determined by calculating the shift in the graph along the temperature axis as a result of acclimation and was expressed as a percentage of the difference in acclimation temperatures (Cossins, 1983).

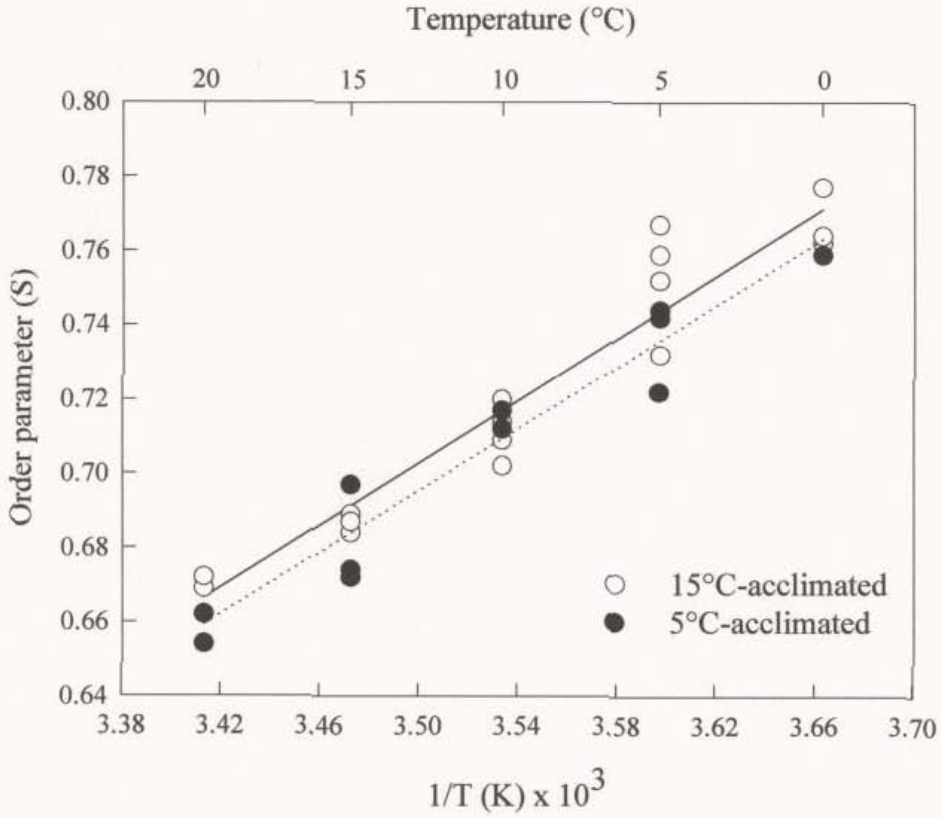


Fig. 3.1 The temperature dependence of the order parameter (S) of 5-SASL embedded in hydrated phospholipid vesicles obtained from gills of 5°C (n = 3) and 15°C (n = 4) acclimated scallops after three weeks of laboratory acclimation. Each data point represents a single animal. Regression plots: solid line - 15°C-acclimated; dashed line - 5°C-acclimated.

3.1.2. Temporal changes in the structural order of gill phospholipids during acclimation from 15 to 5°C

Temporal changes in the order of phospholipid vesicles of warm-acclimated scallops reveal that large changes in gill membrane order can occur during the process of cold acclimation from 15 to 5°C (Fig. 3.2). When measured at a common temperature (20°C), gill phospholipid vesicles of 15°C-acclimated scallops showed an initial increase in molecular order (S) and were significantly ($P < 0.05$) more ordered than warm-acclimated vesicles after 6 days of cold exposure. After an initial rise, the vesicle order gradually decreased and was significantly ($P < 0.05$) lower than in warm-acclimated vesicles after 21 days of cold acclimation.

3.1.3. Effect of temperature on the lipid composition of scallop gill

3.1.3.1. Lipid class composition

Table 3.1 shows the lipid class composition of gills of cold- and warm-acclimated scallops after three weeks of thermal acclimation. Lipids in the gills accounted for about 7.4 ± 1.5 to 8.6 ± 0.79 mg g^{-1} wet weight and showed very similar profiles between acclimation groups (Table 3.1). At both acclimation temperatures, phospholipid (PL) was the dominant lipid class in the gill, contributing between 36 and 43% of the total lipids, followed by free fatty acids (FFA) and sterols (ST). High levels (~8-11%) of ketones (KET) were also

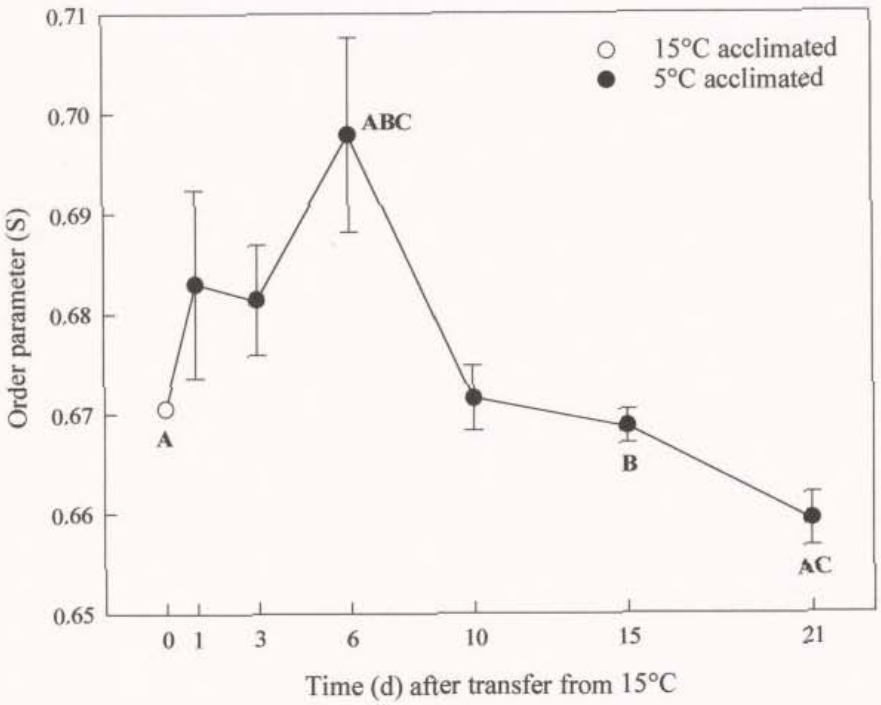


Fig. 3.2 Temporal changes in membrane order of phospholipid vesicles (as determined by 5-SASL) from gills of warm-acclimated scallops following transfer from 15 to 5°C. Data are mean ± SEM of 3-4 different animals measured in duplicate at an assay temperature of 20°C. Pairs of values that are significantly different (ANOVA, $P < 0.05$) are labeled with the same letter.

Table 3.1. Lipid class composition of gill tissue from scallops acclimated for three weeks at 5°C and 15°C expressed as percent of total lipid by weight (mean ± SEM, n=4).

Lipid class	Acclimation temperature	
	5°C	15°C
Hydrocarbon	3.3 ± 1.1 [a]	4.3 ± 2.3 [a]
Ketone (?)	8.4 ± 0.83	11 ± 0.60
Triacylglycerol	2.1 ± 1.2	ND
Free fatty acid	16 ± 1.5	14 ± 1.2
Sterol	15 ± 1.5	15 ± 1.3
Diacylglycerol	6.2 ± 3.7	12 ± 4.9 [a]
Total neutral lipid	52 ± 2.8	55 ± 3.3
Acetone-mobile polar lipid	5.3 ± 0.79	7.2 ± 1.6
Phospholipid	43 ± 2.4	36 ± 2.9
Total polar lipid	48 ± 2.8	44 ± 1.9
Total acyl lipid [b]	72 ± 2.0	70 ± 2.5
Phospholipid/sterol	2.8 ± 0.21	2.5 ± 0.33

[a] Signifies that one or two values expressed in the mean were not detectable (ND) and were therefore included as zeros. [b] Includes triacylglycerol, free fatty acid, diacylglycerol, acetone-mobile polar lipid and phospholipid.

detected. In addition to these neutral lipids small amounts of hydrocarbon (HC), triacylglycerol (TAG) and diacylglycerol (DG) were occasionally detected. The total lipid content and phospholipid/sterol ratio were also not significantly different between acclimation groups (Table 3.1).

During the process of acclimation from 15 to 5°C, gill PL levels showed minor fluctuations ranging from 36 to 43% of the total lipid, although no consistent pattern was evident (Fig. 3.3). In contrast, ST exhibited a gradual increase in levels (from 14.8 to 22.2%) during the initial stages of cold acclimation and were highest after 10 days of cold exposure. However, after the initial rise, sterol levels gradually decreased to those of warm-acclimated scallops after 21 days of cold acclimation (Fig. 3.3).

3.1.3.2. Phospholipid fatty acid composition

Table 3.2 shows the fatty acyl composition of the phospholipid fraction of scallop gills following acclimation at 5 and 15°C. Approximately 48-52% of the total phospholipid fatty acids (PLFA) was accounted for by 16:0, 20:5n-3 and 22:6n-3. Polyunsaturated fatty acids (PUFA) represented ~60% of the total PLFA. Saturated and monounsaturated fatty acids (MUFA) showed similar high levels, of which 16:0 (~13% of total PLFA) and 20:1n-11 (6-9% of total PLFA) were the major components. Additionally, the fatty acids 14:0, 18:4n-3, 20:1n-9 and 22:5n-3 were present in significant amounts, but individually never reached proportions > 2% of the total PLFA.

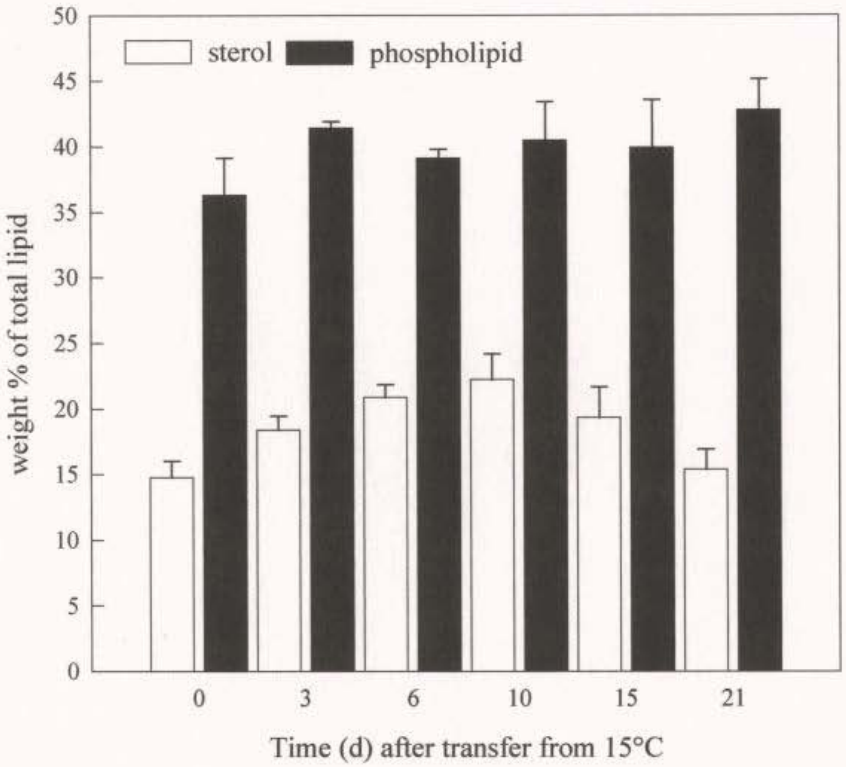


Fig. 3.3 Relative changes in the sterol and phospholipid composition of total lipid of scallop gill following transfer from 15 to 5°C. Values are means ± SEM of 3-4 animals per time period.

Table 3.2. Comparison of the total phospholipid fatty acid composition of gill from scallops acclimated for three weeks at 5°C and 15°C. Values are means ± SEM of 3-4 animals. Minor components identified (< 1%) but not included in the table: iso-15:0, anteiso-15:0, 15:0, iso-16:0, anteiso-16:0, 17:0, 16:1n-5, 16:2, 16:3n-4, 18:1n-11, 18:1n-5, 18:2n-4, 18:3n-3, 20:3n-6 and 21:5n-3. * Significant difference (Student's t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) between acclimation groups. Abbreviations: MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; UFA, unsaturated fatty acid; UI, unsaturation index (mol% x number of double bonds per fatty acid/100).

Fatty acid (mol%)	5°C-acclimated (n = 3)	15°C-acclimated (n = 4)
14:0	0.72 ± 0.02	0.64 ± 0.01 *
16:0	13.1 ± 0.58	13.3 ± 0.09
18:0	3.30 ± 0.17	3.76 ± 0.12
16:1n-7	1.99 ± 0.11	1.50 ± 0.06
16:4n-3	1.00 ± 0.11	1.33 ± 0.12
18:1n-9	3.42 ± 0.31	3.58 ± 0.08
18:1n-7	4.77 ± 0.09	3.95 ± 0.23 *
18:4n-3	0.64 ± 0.06	0.43 ± 0.05 *
20:1n-11	5.84 ± 0.39	8.60 ± 0.27 **
20:1n-9	1.04 ± 0.03	1.41 ± 0.06 **
20:1n-7	0.98 ± 0.09	1.30 ± 0.11
20:2NMID	2.61 ± 0.25	2.98 ± 0.10
20:4n-6	8.57 ± 0.22	9.31 ± 0.18 *
20:5n-3	13.6 ± 0.22	9.02 ± 0.31 ***
22:2NMID	1.54 ± 0.17	1.65 ± 0.13
22:4n-6	1.22 ± 0.15	1.02 ± 0.10
22:5n-6	1.80 ± 0.26	2.24 ± 0.06
22:5n-3	1.13 ± 0.11	0.72 ± 0.07 *
22:6n-3	25.4 ± 1.06	25.8 ± 0.91
Σ saturates	20.4 ± 0.66	20.8 ± 0.24
Σ monoenes	18.5 ± 0.65	20.9 ± 0.48 *
Σ polyunsaturates	61.1 ± 0.18	58.2 ± 0.43 **
Σ n-3	43.1 ± 1.10	38.5 ± 0.57 *
Σ n-6	12.9 ± 0.69	14.1 ± 0.22
n-3/n-6	3.37 ± 0.25	2.74 ± 0.07 *
UFA/SFA	3.90 ± 0.16	3.80 ± 0.05
PUFA/MUFA	3.32 ± 0.11	2.79 ± 0.08 *
UI	3.18 ± 0.03	3.04 ± 0.03 *

Compared with scallops acclimated at 15°C, those acclimated at 5°C accumulated 34% more 20:5n-3 in the total PLFA fraction at the expense of 20-carbon MUFA (Table 3.2). Of all the monoenes, 20:1n-11 showed the greatest response, exhibiting a 32% decrease in levels following a 10°C reduction in water temperature. In contrast, the MUFAs 16:1n-7 and 18:1n-7 increased by 25 and 17 percent, respectively. Overall, these differences represented an increase in the PUFA and a decrease in the MUFA content of the phospholipids from the cold-acclimated scallops. Both changes caused the polyunsaturated to monounsaturated fatty acid ratio (PUFA/MUFA) and unsaturation index (UI) of these lipids to be significantly ($P < 0.05$) greater than the equivalent values for warm-acclimated scallops (Table 3.2).

3.1.4. Temporal changes in gill phospholipid fatty acid composition during acclimation from 15 to 5°C

The net result of changes in PLFA composition was a general reduction in the proportion of total saturated fatty acids (SFA) and a general increase in the total PUFA content of gill phospholipids, although after a transient increase and decrease respectively (Fig. 3.4). Despite the observed differences in the MUFA composition between 15 and 5°C-acclimated scallops (Table 3.2), no significant temporal changes in the total monoene content of gill PL were detected (Fig. 3.4). These changes in fatty acid composition were reflected in the UFA/SFA ratio, which initially declined and subsequently increased to levels of warm-acclimated scallops after 21 days of cold acclimation (Fig. 3.5). The UI did not change

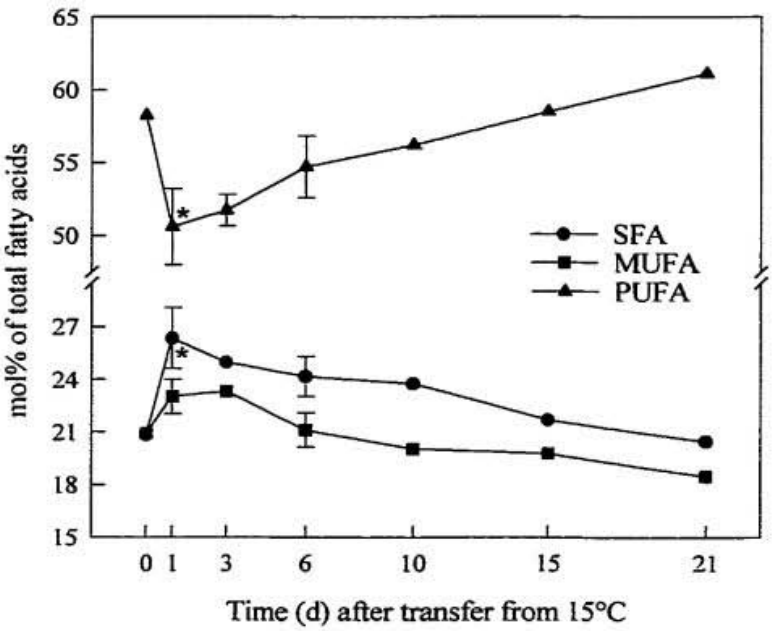


Fig. 3.4 Changes in the proportions of scallop gill total phospholipid saturated, monounsaturated and polyunsaturated fatty acid levels following transfer from 15 to 5°C. Values are means ± SEM of 3-4 animals. * Significantly different (ANOVA, $P < 0.05$) from time zero.

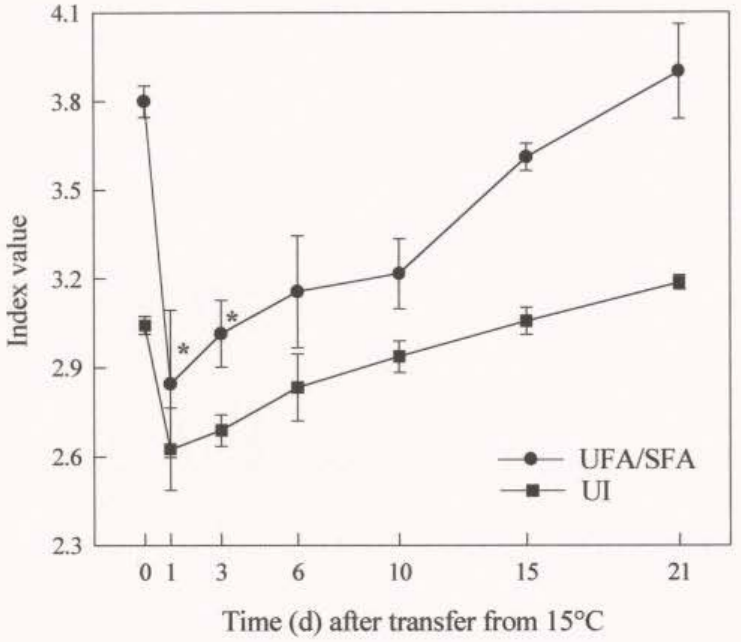
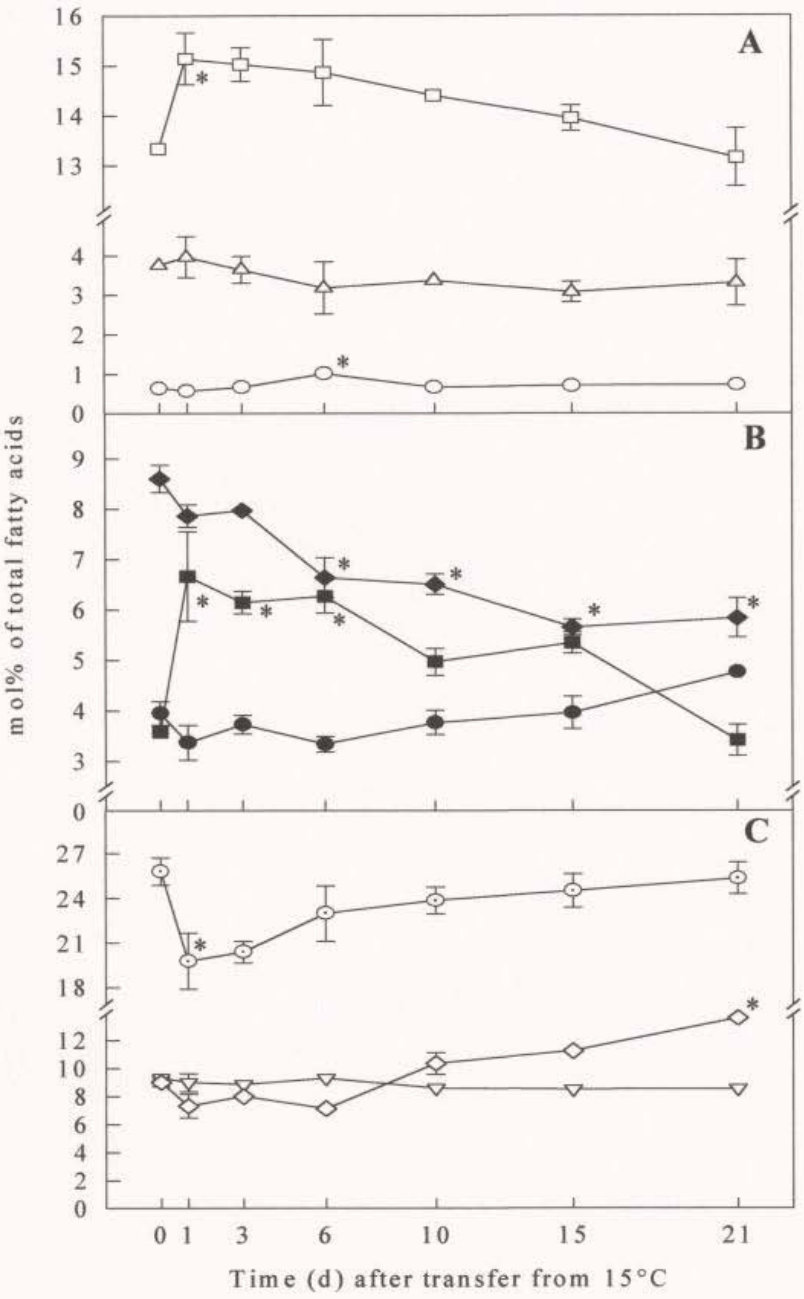


Fig. 3.5 Relative changes in the unsaturated to saturated fatty acid ratio (UFA/SFA) and unsaturation index (UI) of scallop gill total phospholipid following transfer from 15 to 5°C. Values are means ± SEM of 3-4 animals. * Significantly different (ANOVA, $P < 0.05$) from time zero.

significantly during the acclimation period, although a decrease in UI was evident during the initial stages of the acclimation time course (Fig. 3.5).

Fig. 3.6 shows the temporal changes in the major PL saturated, monunsaturated and polyunsaturated fatty acids in the gills of warm- and cold-acclimated scallops following acclimation from 15 to 5°C. During the acclimation time course, the majority of the gill PLFA were unaffected by reduced temperature. No significant trends were observed in the major saturated fatty acids, 1 4:0, 16:0 and 18:0, of gill phospholipids (Fig. 3.6A), although there was a significant increase in 16:0 after 1 day and 14:0 after 6 days of cold acclimation. However, levels gradually decreased thereafter to those of warm-acclimated scallops after 21 days of cold acclimation. In contrast, the amount of the fatty acid 20:1n-11 showed a significant ($P < 0.05$) decrease within 6 days of the initial reduction in temperature (Fig. 3.6B). Thereafter, levels of 2:0:1n-11 continued to decline and were significantly lower than in warm-acclimated scallops after 21 days of cold exposure. In contrast to 20:1n-11, the amount of fatty acid 18:1n-7 did not change significantly over the 21 days of cold acclimation, whereas proportions of 18:1n-9 increased significantly on days 1 through 6 before returning after 10 days of cold exposure to levels characteristic of warm-acclimated scallops. Transfer of scallops from 15 to 5°C resulted initially in a significant decrease in the proportion of 22:6n-3 (from 22.8 to 19.8%), a decrease in 20:5n-3 (from 9.0 to 7.2%), and no change in the level of 20:4n-6 (Fig. 3.6C). Thereafter, the proportion of 22:6n-3 gradually returned to a level not significantly different from 15°C-acclimated scallops, whereas the proportion

Fig. 3.6 Temporal changes in the major phospholipid A) saturated, B) monounsaturated and C) polyunsaturated fatty acids in the gill of the scallop following transfer from 15 to 5°C. Values are means ± SEM of 3-4 animals. * Significantly different (ANOVA, $P < 0.05$) from time zero. Symbol index: (○) 14:0, (□) 16:0, (△) 18:0, (●) 18:1n-7, (■) 18:1n-9, (◆) 20:1n-11, (▽) 20:4n-6, (◇) 20:5n-3, (⊙) 22:6n-3.


A
B
C
mol% of total fatty acids
Time (d) after transfer from 15°C
0 1 3 6 10 15 21

of 20:5n-3 increased significantly ($P < 0.05$), resulting in a 48% increase after an additional 15 days of cold acclimation.

3.1.5. Correlations between compositional and fluidity changes

In order to determine the role of individual PL fatty acids in the regulation of gill membrane fluidity, a correlation was sought between fatty acid values and vesicle order. Vesicle order was negatively correlated ($r = -0.714$, $P < 0.001$) with the proportion of 20:5n-3 in gill phospholipids (Fig. 3.7). The increase in vesicle order during the first six days following transfer from 15 to 5°C (Fig. 3.2) was associated with a decline in the proportion of 20:5n-3. Thereafter, the proportion of 20:5n-3 increased as vesicle order decreased. Similarly, the proportion of 18:1n-7 was negatively correlated ($r = -0.625$, $P < 0.001$) with vesicle order (Table 3.3). Significant negative correlations were also detected between vesicle order and the UFA/SFA ratio ($r = -0.465$, $P < 0.05$) and the UI ($r = -0.467$, $P < 0.05$) and vesicle order. In contrast, the proportions of 18:1n-9, 20:1n-9 and 20:4n-6 were positively correlated with vesicle order, as all tended to increase during the initial stages of the acclimation time course and subsequently declined after 6 days of cold acclimation (Table 3.3). No significant correlations were detected between vesicle order and the proportions of 14:0, 16:0, 18:0, 20:1n-11 and 22:6n-3.

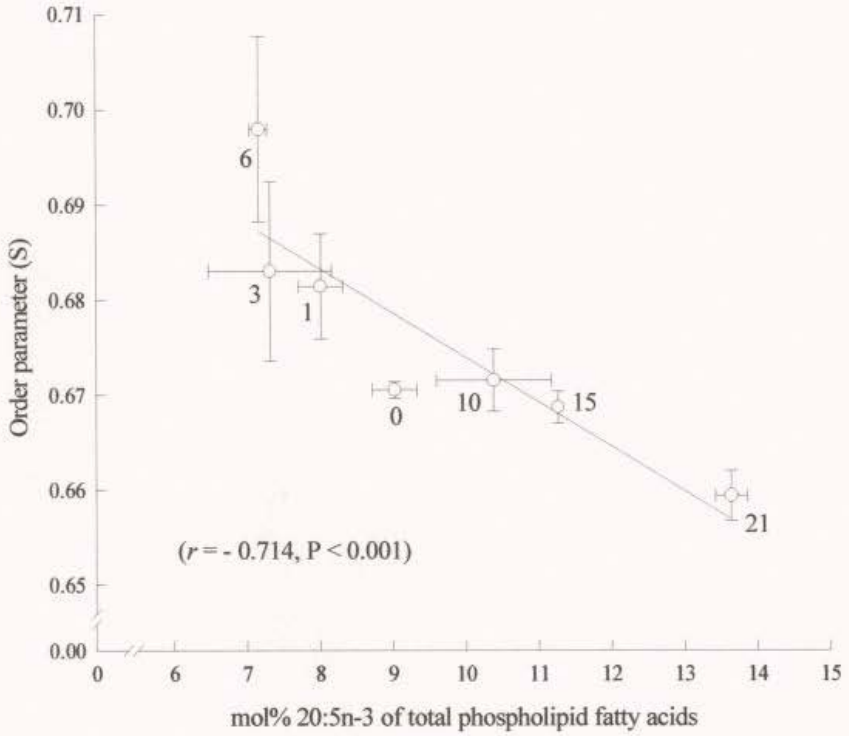


Fig. 3.7 Relative changes in the levels of 20:5n-3 and fluidity of gill membranes during cold acclimation of the sea scallop from 15 to 5°C. Data are mean ± SEM for 3-4 individuals. Membrane fluidity is expressed as an order parameter estimate of 5-SASL incorporated into hydrated gill phospholipid vesicles. Order parameter measurements were made in duplicate at an assay temperature of 20°C. The numbers represent the time, in days, after the change in temperature from 15 to 5°C.

Table 3.3 Summary of significant correlations between scallop gill membrane fluidity and phospholipid fatty acid composition during thermal acclimation of the scallop from 15 to 5°C.

Fatty acid	*r*	P	range (mol%)
20:5n-3	-0.714	<0.001	7.16 - 13.6
18:1n-7	-0.625	<0.001	3.35 - 4.78
20:1n-9	0.588	0.003	1.04 - 1.89
20:4n-6	0.480	0.018	8.53 - 9.32
18:1n-9	0.470	0.021	3.41 - 6.66

r, correlation coefficient for the dependence of membrane fluidity (as determined by 5-SASL) upon changes in gill membrane fatty acid composition.

3.2. Structural order and lipid composition of scallop hemocytes

3.2.1. Effect of temperature on the structural order of hemocyte membranes

The structural order of hemocyte membranes was estimated by determining the order parameter (S) of 5-SASL incorporated into hemocytes isolated from the adductor muscle sinus of *P. magellanicus*. Fig. 3.8 illustrates changes in the order parameter of 5-SASL during the cold acclimation of 15°C-acclimated scallops, measured at an assay temperature of 20°C. From as little as 3 hours after transfer, hemocyte membranes of 5°C-acclimated scallops were significantly ($P < 0.05$) less ordered than hemocyte membranes of 15°C-acclimated scallops. Lower order parameter estimates represent a less ordered, more fluid hemocyte membrane structure.

The order parameter of 5-SASL embedded in hemocyte membranes was correlated with their fatty acid composition to assess which components of the lipid fraction may be regulating the fluidity of hemocyte membranes. The order of hemocyte membranes during cold acclimation from 15 to 5°C was negatively correlated ($r = -0.775$, $P < 0.05$) with the UFA/SFA ratio of total lipid extracts (Fig. 3.9). No other significant correlations were detected.

3.2.2. Effect of temperature on the lipid composition of hemocytes

To explain differences in membrane order between cold- and warm-acclimated scallop hemocytes, the lipid class and fatty acid composition of hemocytes were measured during thermal acclimation of scallops from 15 to 5°C. Owing to low hemocyte levels

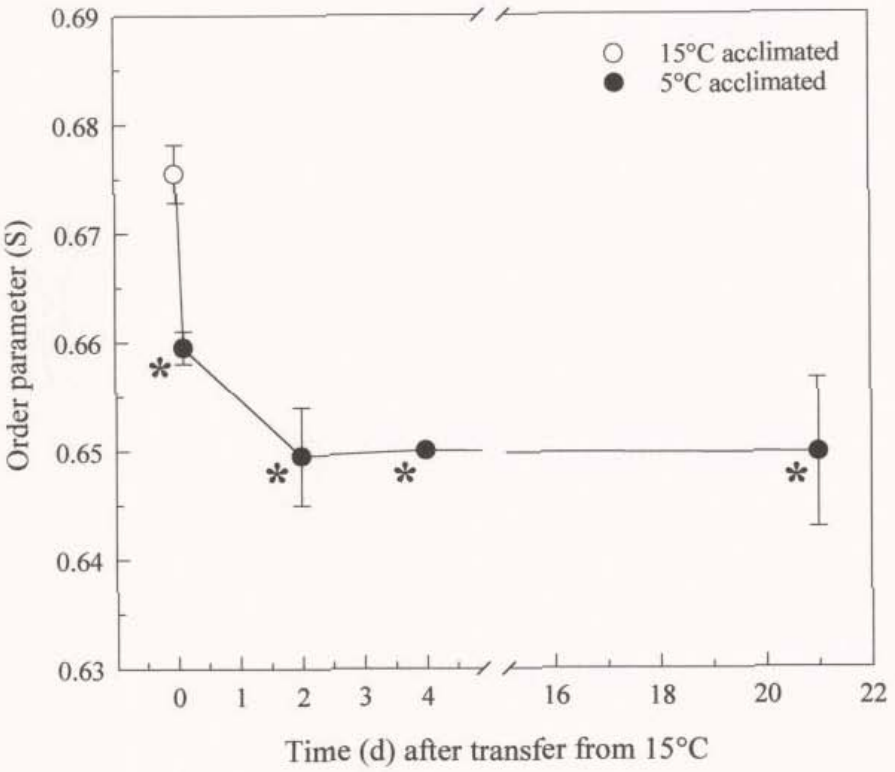


Fig. 3.8 Temporal changes in the order parameter (S) of 5-SASL labeled hemocytes isolated from 15°C-acclimated scallops following a sudden decrease in temperature to 5°C. Values are means ± SEM of 2-10 preparations (except day four: n =1) measured at 20°C. * Significantly different (ANOVA, $P < 0.05$, Student-Newman-Keuls multiple comparison test) from time zero.

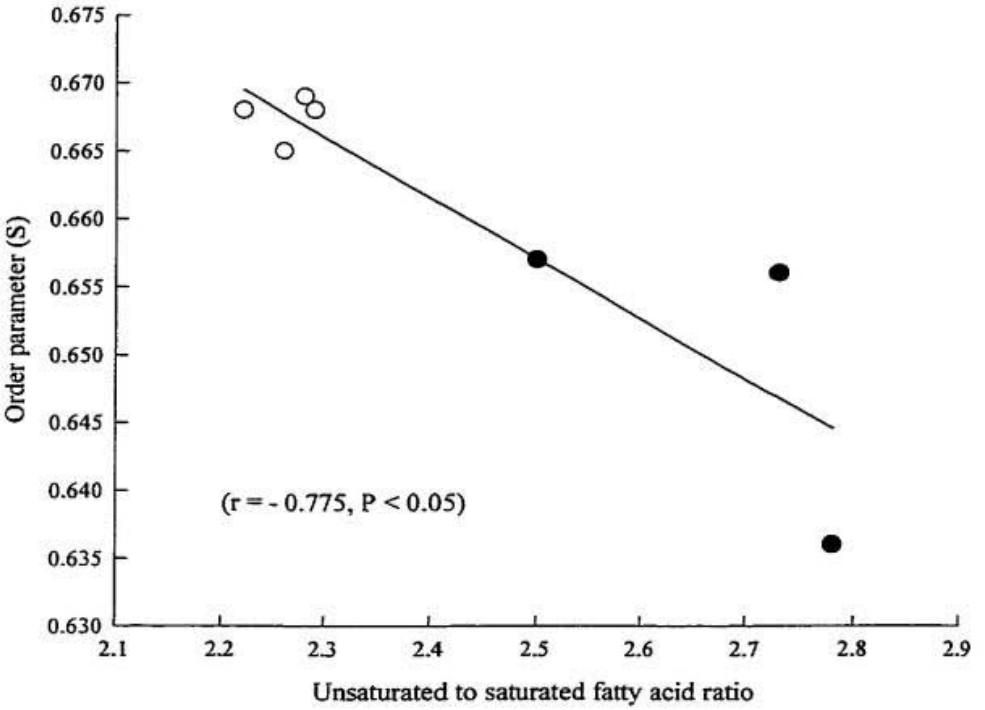


Fig. 3.9 Relationship between the fluidity of hemocyte membranes of the sea scallop acclimated at 5°C (●) and 15°C (○) and the total lipid unsaturated to saturated fatty acids ratio. Each data point represents a single animal. Membrane fluidity is expressed as an order parameter estimate of 5-SASL embedded in hemocytes measured at 20°C.

in the blood, measurements of lipid levels were not made on the same individuals as estimates of membrane order although both were exposed to the same environmental conditions.

3.2.2.1. Lipid class composition

Table 3.4 shows the lipid class composition of hemocytes from scallops acclimated for three weeks at 5°C and 15°C. ST and PL were the major components of scallop hemocytes, accounting for approximately 20 and 60% of the total respectively. Besides PL and ST, small amounts of acetone-mobile polar lipid (AMPL; ~12%) and various neutral lipids were occasionally detected (Table 3.4). There were no significant differences in lipid class composition between cold and warm-acclimated scallops.

3.2.2.2. Fatty acid composition

At both acclimation temperatures, the major fatty acids of scallop hemocytes were 16:0, 18:0, 18:1n-7, 20:1n-11, 20:4n-6, 20:5n-3 and 22:6n-3 (Table 3.5). Of these, 16:0, 20:5n-3 and 22:6n-3 were most abundant and accounted for more than 45% of the total fatty acids of hemocytes. The fatty acid composition of hemocytes differed markedly between scallops acclimated for three weeks at 15 and 5°C. Fatty acid compositional changes in hemocytes from cold-acclimated scallops included a significant increase in 16:1n-7, 20:5n-3, 21:5n-3 and 22:5n-3 and a decrease in 17:0, 20:1n-11 and 20:4n-6 compared with scallops acclimated at 15°C (Table 3.5). Differences in fatty acid composition were reflected in an

Table 3.4 Lipid class composition of hemocytes isolated from scallops acclimated for three weeks at 5°C and 15°C expressed as percent of total lipid by weight.

Lipid class	Acclimation temperature	
	5°C	15°C
Sterol	20.1 ± 1.2	22.3 ± 1.4
Total neutral lipid [a]	25.2 ± 2.4	24.5 ± 1.3
Acetone-mobile polar lipid	13.4 ± 2.9	10.4 ± 1.9
Phospholipid	61.3 ± 4.3	65.0 ± 2.6
Total polar lipid	74.8 ± 2.4	75.4 ± 1.3
Total acyl lipid [b]	79.3 ± 1.4	77.7 ± 1.5
Phospholipid/sterol	2.9 ± 0.04	2.8 ± 0.15

[a] Includes the following lipid classes: hydrocarbon, ethyl ketone, triacylglycerol, diacylglycerol and free fatty acids, although two or three values were not detectable and were therefore included as zeros. [b] Includes triacylglycerol, free fatty acid, diacylglycerol, acetone-mobile polar lipid and phospholipid. Data are mean ± SEM (n = 4)

Table 3.5 Comparison of the fatty acid composition of total lipid extracts of hemocytes isolated from scallops acclimated for three weeks at 5°C and 15°C. Values are means ± SEM (n = 4). The fatty acid in parentheses is a minor component of the value shown. Minor components identified (< 1%) but not included in the table: iso-15:0, iso-16:0, iso-17:0, 16:2, 16:3n-4, 18:1n-5, 18:2n-6, 18:2n-4, 18:3n-6, 18:3n-3, 18:4n-3, 20:1n-7 1nd 22:5n-6. * Significant difference (Student's t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) between acclimation groups. SFA, saturated fatty acid; UFA, unsaturated fatty acid; UI, unsaturation index (mol% x number of double bonds per fatty acid/100).

Fatty acid (mol%)	5°C-acclimated	15°C-acclimated
14:0	2.56 ± 0.29	3.34 ± 0.15
15:0	1.70 ± 0.08	1.79 ± 0.10
16:0	12.8 ± 0.55	13.3 ± 0.21
17:0	0.82 ± 0.02	1.01 ± 0.02 ***
18:0	6.38 ± 0.19	6.41 ± 0.37
16:1n-7	2.26 ± 0.08	1.28 ± 0.16 **
16:4n-3	1.51 ± 0.02	1.63 ± 0.20
18:1n-9	4.04 ± 0.34	3.69 ± 0.25
18:1n-7	7.87 ± 0.27	6.82 ± 0.38
20:1n-11 + (20:1n-9)	4.26 ± 0.09	5.55 ± 0.28 *
20:2NMID	2.16 ± 0.34	1.78 ± 0.05
20:4n-6	4.74 ± 0.09	5.38 ± 0.19 *
20:5n-3	12.2 ± 0.77	9.66 ± 0.12 *
21:5n-3	1.86 ± 0.25	1.15 ± 0.11 *
22:2NMID	2.09 ± 0.14	1.95 ± 0.18
22:5n-6	1.46 ± 0.30	1.36 ± 0.09
22:5n-3	2.08 ± 0.16	1.25 ± 0.09 **
22:6n-3	20.0 ± 0.79	22.4 ± 0.84
Σ saturates	27.7 ± 0.67	31.1 ± 0.36 **
Σ monoenes	19.3 ± 0.46	18.7 ± 0.62
Σ polyunsaturates	52.9 ± 0.58	50.2 ± 0.32 **
UFA/SFA	2.61 ± 0.09	2.22 ± 0.04 *
UI	2.75 ± 0.04	2.66 ± 0.02

overall decrease in SFA and a concomitant increase in PUFA content at low acclimation temperatures. These changes in saturation resulted in a 20% increase in the UFA/SFA ratio of cold-acclimated scallops compared with the equivalent value for warm-acclimated scallops (Table 3.5).

3.2.3. Temporal changes in hemocyte fatty acid composition during acclimation from 15 to 5°C

To examine the time course over which 15°C-acclimated hemocytes exhibit changes in fatty acid composition following a decrease in tempreature, scallops kept at 15°C were transferred directly to 5°C and their fatty acid compositions were measured at regular intervals (see Materials and Methods). PUFA levels showed little change over the first 3 days of cold exposure, after which they increased and were significantly ($P < 0.05$) higher than those of warm-acclimated scallops after 15 days of cold acclimation (Fig. 3.10). In contrast, SFA content gradually decreased and was significantly ($P < 0.05$) reduced after 15 days of cold exposure. Total MUFA did not vary significantly during the acclimation time course. These changes in saturation are reflected in a gradual but significant ($P < 0.05$) increase in the UFA/SFA ratio and no change in the UI after 15 days of cold exposure (Fig. 3.11).

By comparison with lipid class proportions (Table 3.4), the proportion of individual fatty acids varied widely during the acclimation time course (Fig. 3.12). The majority of the changes in hemocyte fatty acid levels were accounted for by changes in the proportion of

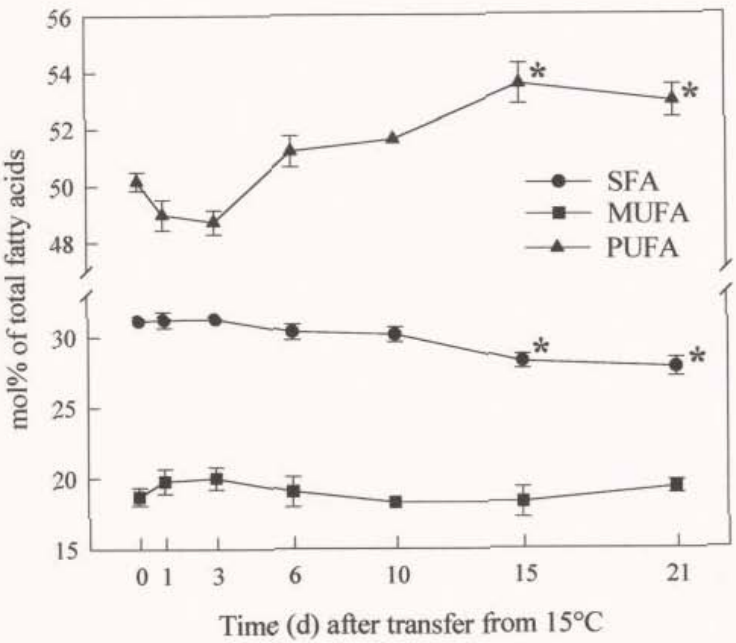


Fig. 3.10 Changes in the proportions of saturated, monounsaturated and polyunsaturated fatty acids of scallop hemocytes following transfer from 15 to 5°C. Values are means ± SEM (n = 4). * Significantly different (ANOVA, $P < 0.05$) from time zero.

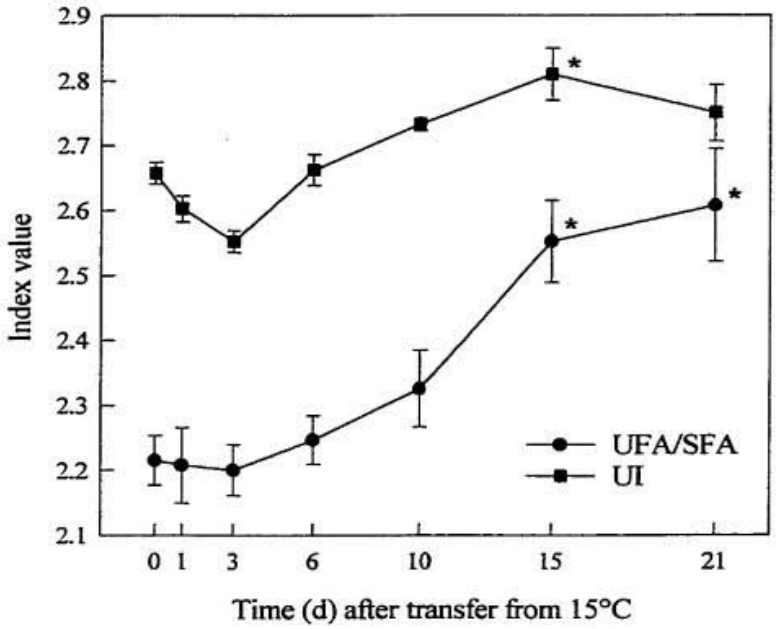
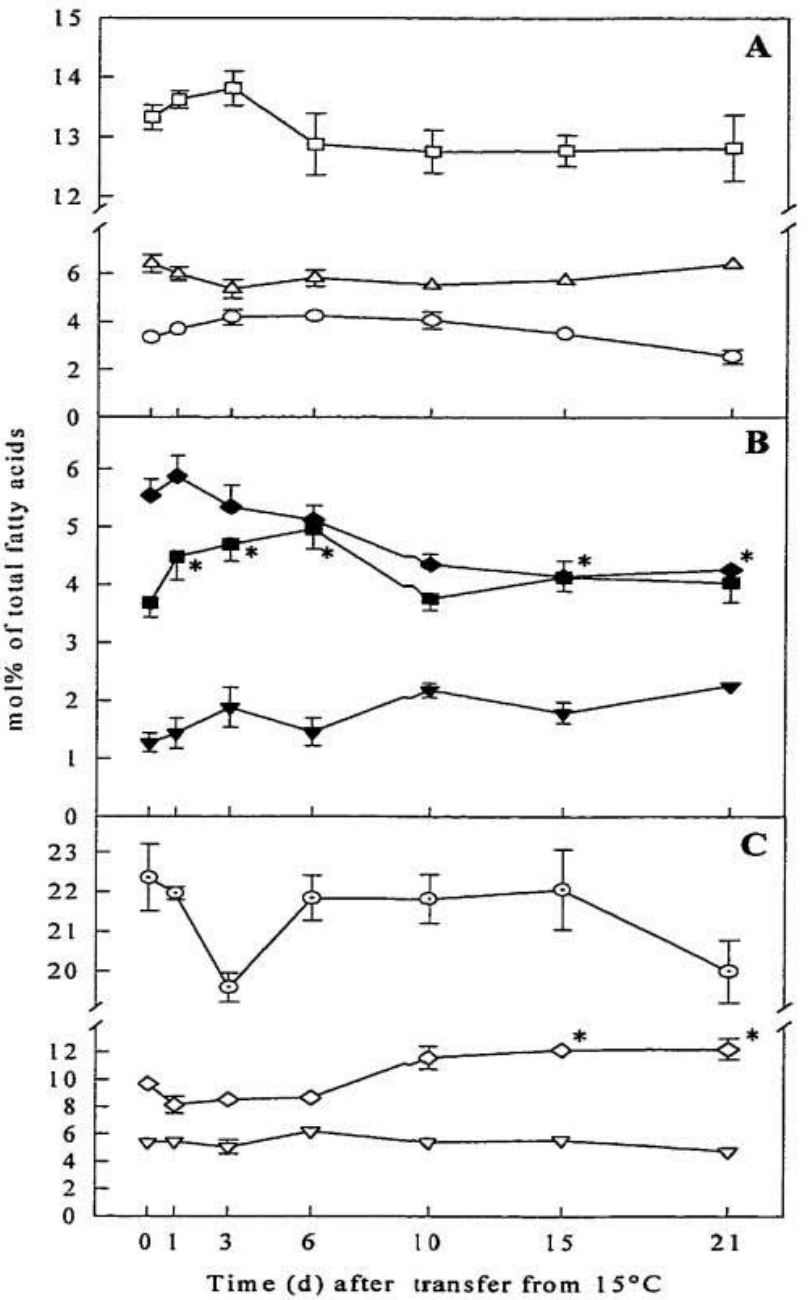


Fig. 3.11 Relative changes in the unsaturated-to-saturated fatty acid ratio (UFA/SFA) and unsaturation index (UI) of scallop hemocytes following transfer from 15 to 5°C. Values are means ± SEM (n = 4). * Significantly different (ANOVA, $P < 0.05$) from time zero.

monoenes and polyunsaturates. No significant temporal pattern was observed in the major saturated fatty acid species (Fig. 3.12A). In contrast, monoene levels showed considerable variability (Fig. 3.12B). For example, while proportions of 16:1n-7 did not change significantly over the 21 days of cold acclimation, proportions of 18:1n-9 increased significantly ($P < 0.05$) on days 1 through 6 before returning after 10 days of cold exposure to levels characteristic of warm-acclimated scallops. Furthermore, the proportion of 20:1n-11 declined significantly ($P < 0.05$) after 15 days of cold exposure and remained significantly depressed for the duration of the acclimation period. Temporal changes in PUFA levels were confined to a few fatty acid species (Fig. 3.12C). For example, the proportion of 20:5n-3 showed an apparent decrease in levels over the first 6 days of cold exposure, ranging from 9.66 to 8.12%, after which levels gradually increased and were significantly ($P < 0.05$) higher than in 15°C-acclimated scallops after 15 days of cold acclimation. In contrast, levels of 20:4n-6 and 22:6n-3 showed no significant changes during acclimation.

Fig. 3.12 Temporal changes in the major A) saturated, B) monounsaturated and C) polyunsaturated fatty acids in hemocytes of the scallop following transfer from 15 to 5°C. Values are means ± SEM (n = 4). * Significantly different (ANOVA, $P < 0.05$) from time zero. Symbol index: (○) 14:0, (□) 16:0, (△) 18:0, (▼) 16:1n-7, (■) 18:1n-9, (◆) 20:1n-11 + 20:1n-9, (▽) 20:4n-6, (◇) 20:5n-3, (⊙) 22:6n-3.


A
B
C
mol% of total fatty acids
Time (d) after transfer from 15°C
0 1 3 6 10 15 21

4. Discussion

4.1. Temperature effect on gill membrane order and PL fatty acid composition

Electron spin resonance (ESR) spectroscopy has been extensively used to estimate conformational and dynamic changes in the physical state of biological membranes (Alonso et al., 1995; Dey et al., 1993; Kamada and Otsuji, 1983; Sinensky, 1974). The order parameter values of the commonly used ESR spin labels, 5-, 12 and 16-doxyl stearic acid, indicate the degree of hindrance to the rotation of the spin label associated with a given membrane structure. Each spin label exhibits its freedom of anisotropic motion in relation to the position of the nitroxide ring that is located at different positions along the acyl fatty acid chain of the stearic acid molecule. This anisotropic motion reflects the molecular motion of the lipid bilayer and thus is a measure of the fluidity of the membrane. The probe used in this study, 5-doxyl stearic acid (5-SASL), mainly provides average information concerning the ordering state of the fatty acids within the outer region of the membrane; at the level of the fifth carbon atom in the bilayer.

In the present study, the total phospholipids of the entire gill were considered representative of those of an average membrane, although individual membranes may differ with respect to their lipid compositions and fluidity, as has been shown for fish liver and brain (Roy et al., 1997; Dey and Farkas, 1992). In addition, since these vesicles were formed from isolated phospholipids, the differences in order reflect differences in membrane phospholipid (PL) composition between 15 and 5°C-acclimated animals. However, it should be noted that the observed differences in order may vary from those of an intact membrane due to the

presence of other membrane constituents, such as sterols and proteins, that may further affect the physical properties of the lipid bilayer.

The results of the membrane fluidity measurements using 5-SASL showed that the gill phospholipids from cold-acclimated scallops were less ordered than those from warm-acclimated scallops after three weeks of laboratory acclimation (Fig. 3.1). When 5-SASL was used as a probe, temperature acclimation was found to shift the order parameter values by about 1-2°C along the temperature axis toward lower assay temperatures. This small shift in the order parameter values signifies a partial compensation in the homeoviscous response of gill membranes to a change in temperature. In other words, the sea scallop, *Placopecten magellanicus*, is capable of homeoviscous adaptation of its gill membranes, although to a small degree. Partial adjustments in membrane order following short term exposure to reduced temperatures have been reported for muscle plasma membranes of two marine crabs, *Carcinus maenas* and *Cancer pagurus* (Cuculescu et al., 1995), and neuronal microsomal membranes of a freshwater crayfish, *Astacus astacus* (Lehti-Koivunen and Kivivuori, 1998).

Although gill membranes may display an incomplete homeoviscous response to changes in temperature of 10°C, this does not necessarily imply a partial compensation of membrane-associated functions, which would be otherwise affected by the physical state of the lipid bilayer (Cossins and Prosser, 1978). For example, Gabbianelli et al. (1996) observed little change in the fluidity of erythrocyte plasma membranes isolated from trout (*Salmo irideus*) adapted to seasonal changes in environmental temperature, but Na^+, K^+-ATPase activity of these cells were significantly enhanced, suggesting that even small changes in

membrane fluidity may enhance the functional state of a membrane. However, until more is known about the functionality (i.e. activity of membrane-bound proteins) of scallop gill membranes during acclimation to different temperatures, it is not possible to ascertain the effectiveness of such changes in the order of scallop gill membranes.

Changes in the fluidity of scallop gill membranes seem to be regulated in part through changes in the composition of their PL fatty acids (Table 3.2). The differences in lipid profiles of 15 and 5°C-acclimated scallops are consistent with the known inverse relationship between temperature and the amount of unsaturated fatty acids in tissue lipids of fish and invertebrates (Hazel and Williams, 1990; Farkas et al., 1988; Farkas et al., 1980). This increase in unsaturation is reflected in a significant increase in the UI of gill PL fatty acids during cold acclimation (Table 3.2). The increase in the UI is primarily attributable to a significant rise in the relative proportion of PUFA, in particular 20:5n-3. However, no change in the UFA/SFA ratio was observed between scallops acclimated for 21 days at 15 and 5°C because the PUFA was replaced with C_{20} monoenoic fatty acids rather than SFA.

4.2. Changes in hemocyte fatty acid composition and fluidity

The adaptive response of *P. magellanicus* to a prolonged period of reduced temperature was determined by measuring the membrane order and fatty acid composition of hemocytes isolated from 15 and 5°C-acclimated scallops. Membrane fluidity measurements revealed that hemocytes underwent an adaptive response to a sudden decrease in acclimation temperature by decreasing the order of their membranes (Fig. 3.8). This adaptation in

membrane order seems to be mediated in part by changes in the fatty acid composition of the hemocytes (Table 3.5). The differences in the fatty acid profiles of total lipid extracts from 15 and 5°C-acclimated scallops are consistent with prior descriptions of cold acclimation effects on other ectotherms (Hazel, 1988), and include an increase in unsaturated fatty acids at low acclimation temperatures and an increase in SFA at higher temperatures. These changes are reflected in a 15% increase in the UFA/SFA ratio of cold-acclimated scallops. Furthermore, this ratio was negatively correlated with hemocyte membrane order (Fig. 3.9). A similar correlation was observed between the adjustment of fluidity and the ratio of UFA/SFA in the PL fatty acid composition of synaptosomal membranes of goldfish following acclimation from 25 to 5°C (Cossins et al., 1977). Thus, it appears that changes in the fluidity of hemocyte membranes are partially dependent upon overall changes in their saturated and unsaturated fatty acid content although, once again, PUFA seems to be replacing C_{20} monounsaturated fatty acids (Table 3.5).

4.3. Temporal changes in membrane order during cold acclimation from 15 to 5°C

An inverse relationship between temperature and the fluidity of thermally acclimated membranes in ectotherms is well established (Hazel and Williams, 1990; Williams and Somero, 1996), and is illustrated by the data for fully acclimated scallops in Fig. 3.1. However, the data in Fig. 3.2 clearly show that this inverse relationship fluctuates in scallop gill membranes during the process of cold acclimation, following a decrease in acclimation temperature from 15 to 5°C. A paradoxical increase in the membrane order of scallop gill

membranes was observed during the initial stages of the acclimation time course. Membranes were significantly more ordered than warm-acclimated membranes after 6 days of cold exposure. Gill membrane order was negatively correlated with the proportion of those unsaturated fatty acids that were significantly elevated following 21 days of cold acclimation, in particular 18:1n-7 and 20:5n-3 (Table 3.3).

Although scallop gill membranes may undergo oscillations in membrane order during cold acclimation, when measurements are made at a common temperature (20°C) the slow changes in membrane order over time suggest that scallops may have a limited capacity to mobilize essential structural components required to offset the ordering effect of decreased temperature. This is in contrast to scallop hemocytes, which can adjust the order of their membranes rapidly following a decrease in temperature from 15 to 5°C (Fig. 3.8). However, both gill and hemocytes exhibited similar rates of adjustment in membrane fatty acid composition during the process of cold acclimation (see section 4.7). The fluidity of hemocyte membranes was assessed using intact cells, compared with the isolated phospholipids of gill, therefore the presence of membrane proteins in the former may account for the differences in membrane order between gill and hemocyte membranes (Fig. 3.2 and 3.8). Furthermore, the small difference observed in membrane fluidity between scallops fully acclimated to 5°C and those fully acclimated to 15°C may also imply that the cost of complete compensation in membrane order may be too high or the benefits too low to warrant large adjustments in membrane composition following a 10°C decrease in temperature.

4.4. Effect of temperature on the lipid composition of gill and hemocytes

No differences were recorded in the lipid composition of the gill tissue or hemocytes between scallops acclimated at 15°C and those acclimated for 21 days at 5°C (Table 3.1 and 3.4). The maintenance of sterol (ST) levels was unexpected, considering that a correlation between ST content and temperature is often observed in membranes of ectothermic organisms (Hazel and Williams, 1990). The main function of sterols is as a structural component of membranes; however, sterols may also aid in restricting the mobility of the fatty acyl chains within a membrane bilayer without conferring increased rigidity (Nes, 1974). Therefore, sterols should increase in importance in membranes acclimated to high temperatures, where increased thermal energy may lead to a highly disordered lipid bilayer. For example, Sørensen (1993) found high amounts of cholesterol in erythrocyte plasma membranes from the warm-acclimated flounder, compared with those from cold-acclimated fish.

Membranes of cold-acclimated ectotherms are generally associated with higher proportions of phosphatidylethanolamine (PE) and, less frequently, lower proportions of phosphatidylcholine (PC) than membranes formed at higher temperatures (Hazel, 1995). Cold acclimation is generally characterized by an increase in the relative proportion of 'conical' lipid molecules, having large hydrophobic volumes. In contrast, an increase in the concentration of 'cylindrical'-shaped lipids with small hydrophobic volumes is preferred in response to warm acclimation. Thus, a reduction in the acclimation temperature results in an increase in the proportion of conical lipids, in particular PE, in the membranes of cold-

acclimated ectotherms. Such alterations in PL class composition are believed to contribute to maintaining the stability and phase composition of the membrane in spite of the order-inducing effect of reduced temperature. Thus, the similarities between the ST and PL contents of scallops acclimated at 15 and 5°C suggest that such and adaptation does not occur in bivalve membranes, although this is not certain because changes in PL class composition were not resolved. However, the lack of change in scallop gill PL content in response to thermal challenge agrees with previous observations of gill lipids isolated from the mussels, *Mytilus edulis* (Zandee et al., 1980) and *Mytilus californianus* (Williams and Somero, 1996), acclimated at similar low temperatures.

4.5. Lipid composition of gills and hemocytes

The total lipid content of 5°C and 15°C-acclimated scallop gill ranged from 7.4 to 8.6 mg g^{-1} wet weight (Table 3.1). In both cases, PL was the highest contributor, ranging between 36-43% of the total a value about half that reported for wild *P. magellanicus* adapted to winter and summer temperatures (Napolitano and Ackman, 1992). The low level of PL reported in this study may be attributable to the high levels of free fatty acids (FFA) and ketones which are both normally minor components of bivalve lipids (Napolitano and Ackman, 1992).

Free fatty acids comprised 14-16% of the total lipids of scallop gill in this study, suggesting that lipid breakdown and hydrolysis may have been high during extraction and storage. After the gill was excised from the scallop, it was stored at -20°C, but without rapid

freezing with liquid nitrogen or storage in chloroform; both of which have been shown to reduce lipid degradation and to prolong the shelf-life of lipid samples (Sasaki and Capuzzo, 1984). Therefore, the hypothesis that the high FFA levels observed was attributable to handling and storage was evaluated by comparing the lipid class composition of scallop gill in this study to that of freshly extracted gill tissue. Table 3.6 shows that gill tissue generally contains low levels of FFA, as reported earlier by Napolitano and Ackman (1992). Thus, the high level of FFA in scallop gill in this study may not be of biological significance but rather an artifact of the sampling and storage procedure. Furthermore, if the PL, FFA, acetone-mobile polar lipid, diacylglycerol and triacylglycerol classes (i.e. total acyl lipid) of scallop gill are summed (Table 3.1), the proportion is similar to the total PL level reported for freshly extracted tissue (Table 3.6), thus accounting for the loss in phospholipids. In addition, although there may have been some lipid breakdown in the samples, it is reassuring that all samples experienced similar degrees of degradation, based on the small difference in the variance between samples.

Ketones formed another major component of the neutral lipid in scallop gill. The unexpectedly high levels of ketones may be attributable to the algal diet provided throughout this study. *Isochrysis* sp. has been known to accumulate high proportions of long-chain *trans*-unsaturated methyl (11%) and ethyl (4%) alkenones in response to changes in culture conditions (Dunstan et al., 1993; Volkman et al., 1989) and its use as food for the scallops may account for the elevated ketone levels (8-11%, Table 3.1) observed in the gills.

Table 3.6 Lipid class composition of freshly extracted scallop gill tissue expressed as percent of total lipid by weight.

Lipid class	mean ± SEM (n =2)	range (weight %)
Hydrocarbon	3.0 ± 0.80	2.1 - 3.7
Triacylglycerol	0.56 ± 0.56	0.0 - 1.1
Free fatty acid	6.1 ± 6.1	0.0 - 12
Sterol	16 ± 0.98	15 - 17
Acetone-mobile polar lipid	2.8 ± 0.58	2.2 - 3.4
Phospholipid	72 ± 0.58	63 - 81
Total polar lipid	75 ± 8.4	66 - 83

The lipid profile of the hemocytes (Table 3.4) recorded in this study was similar to that reported by Thompson (1977) for the same species. Hemocyte lipids were predominantly composed of the structural lipids PL and ST, which together accounted for 81-87% of the total lipid. In contrast to the gill, the remaining lipid classes of the hemocyte were minor and accounted for < 5% of the total lipid compared with the high levels (37-41% of total lipid) in the gill. Although the storage protocol for gill and hemocytes was the same, FFA was a minor component in hemocytes, suggesting that lipid breakdown was minimal compared with that in the gill.

4.6. Comparison of fatty acid composition between gill and hemocytes

An inverse relationship between acclimation temperature and the proportion of unsaturated fatty acids, especially PUFAs, is often shown when bivalves and other ectotherms acclimate to the cold (Hazel and Williams, 1990; Pazos et al., 1996). Such temperature-induced changes in PUFA were recorded for the gill tissue and hemocytes of cold-acclimated scallops in this experiment (Table 3.2 and 3.5). The composition of the PL fatty acids of scallop gills in this study is similar to that described for the same species collected from Georges Bank during summer (Napolitano and Ackman, 1993). However, the fatty acid composition of hemocytes has not been investigated previously in bivalves. Total lipid extracts of hemocytes were not separated into neutral and polar fractions to isolate the phospholipids, but it can be assumed that the total fatty acids primarily represent phospholipids, since the ratio of PL to total acyl lipid accounted for 78-84% of the total lipids

(Table 3.4). Although the fatty acid composition of hemocytes has not been previously described, the results agree with those reported for the phospholipids in several tissues of scallops and other bivalves acclimated at similar low temperatures (Napolitano and Ackman, 1993; Napolitano et al., 1992; Piretti et al., 1988).

Significant differences in fatty acid composition were observed between gill and hemocytes in warm-acclimated scallops. Gill phospholipids contained lower proportions of saturates, higher proportions of PUFAs, smaller amounts of 18:0, 18:1n-7 and 20:4n-6 and larger amounts of 22:6n-3 than hemocytes (Table 3.2 and 3.5). Owing to these differences, gill phospholipids had a higher UFA/SFA and UI than hemocytes. These differences were still apparent following 21 days of cold acclimation.

4.7. Temporal changes in fatty acid composition of gill and hemocytes

The present study is consistent with the hypothesis that, to adapt to prolonged low temperatures, adult *P. magellanicus* adjust membrane fluidity by modifying the fatty acid composition of their membrane phospholipids (Fig. 3.6 and 3.12). In general, when exposed to a decrease in acclimation temperature from 15 to 5°C, gill and hemocyte phospholipids showed an increase in the PUFA content, in particular 20:5n-3. However, the adjustment to temperature change was relatively slow, requiring approximately 15-21 days to achieve significant alterations in the PL fatty acid composition. The time course of these changes in PL fatty acid composition is similar to that of the physiological response to temperature change exhibited by the mussel *Mytilus edulis*, which showed a complete acclimatory

response in oxygen consumption and filtration rates within 14 days following a reduction in temperature from 15 to 10°C (Widdows and Bayne, 1971). Similar slow adjustments in membrane fatty acid composition have also been reported in the adult oyster *Crassostrea virginica* acclimated for prolonged periods to similar low (5-7°C) acclimation temperatures (Chu and Greaves, 1991). This is in contrast to the fish *Cyprinus carpio*, which can adjust fatty acid unsaturation efficiently within hours and even minutes of a change in temperature (Buda et al., 1994; Farkas et al., 1984; Farkas et al., 1980). A long period of time necessary to achieve maximal changes in PL fatty acid composition in response to cold adaptation has also been reported for goldfish and rainbow trout (Sellner and Hazel, 1982; Cossins et al., 1977), which may reflect the reduced rate of lipid turnover and the lower rates of lipid synthesis at reduced acclimation temperatures (Hazel and Williams, 1990; Hazel, 1988). Furthermore, the apparent lack of differences in the PL fatty acid composition of scallop gill membranes may possibly be due to different rates of uptake of dietary fatty acids which has been known to occur when one changes the temperature at which ectotherms live (Clarke, 1983).

During cold acclimation of *P. magellanicus* the proportion of 18:1n-9 increased dramatically from days 1-6, although there was no significant difference between gill and hemocytes in the total monoene content over the entire acclimation time course (Fig. 3.4 and 3.10). The level of 18:1n-9 began to rise at a time when the proportion of 20:5n-3 fell below the value for warm-acclimated scallops. This inverse relationship suggests that the increase in 18:1n-9 may have been a response to the decline in levels of 20:5n-3. A similar relationship

between monoene and PUFA contents of microsomal membranes from livers of thermally-acclimated trout has been reported (Hagar and Hazel, 1985). Thus the fluctuations in monoene content (i.e 18:1n-9) during the initial stages of acclimation may be an acclimatory response by which scallops modulate membrane unsaturation in an effort to compensate for changes in PUFA. Furthermore, monoene content is strongly correlated with Δ^9-desaturase activity in trout (Hagar and Hazel, 1985), which may suggest that changes in 18:1n-9 in scallop membranes may be due largely to alterations in Δ^9-desaturase activity. Unfortunately, the presence and activity level of this enzyme in scallop gill membranes has not been clearly established.

The physiological importance of such changes in unsaturation is still unclear, although it has been suggested that this differential pattern of replacement may relate to the activity level of the organism at low temperatures (Hazel, 1988). The importance of PUFA in maintaining physiological functions at low temperatures has been shown by Farkas et al. (1981), who observed that a winter-active crustacean (*Cyclops vicinus*) shows increased PUFA levels compared with the elevated monoene levels of an inactive crustacean (*Daphnia magna*). Increased levels of monoenes are also observed in membranes of goldfish and related species that tend not to be very active at low temperatures (Schunke and Wodtke, 1983; Wodtke, 1978; Cossins, 1977), and therefore monoenes may most effectively aid in preserving membrane order in response to low environmental temperatures in these species. However, because of the asymmetrical shape of membrane proteins it is unlikely that a single lipid species consisting of a monoenoic fatty acid could allow optimal protein function, due

in part to the irregular alignment of the acyl chain with that of the protein molecule (Wieslander et al., 1980). Therefore, a balance between the proportion of specific MUFA may be essential for conserving membrane architecture and in determining the levels of protein function during the initial period of low acclimation in *P. magellanicus*.

Levels of PUFA, rather than monoenes, are generally higher in animals that are active during winter months and at low temperatures, although PUFA levels are generally slow to respond to changes in temperature (Hazel, 1995). For example, in the present study a lag time of 6 days was observed for changes in 20:5n-3 in membrane phospholipids of scallop gill and total lipid extracted from hemocytes following a decrease in temperature from 15 to 5°C (Fig. 3.6 and 3.12). Similarly, a lag time of 3-6 days has been recorded for changes in PUFA levels in microsomal membranes of rainbow trout gill and liver following a change in temperature from 20 to 5°C (Sellner and Hazel, 1982). Such temperature-induced changes in the fatty acid composition of the bivalve gill and hemocytes may therefore reflect conflicting requirements for particular fatty acid species imposed by changing thermal conditions. Changes in the molecular species of PC-containing long chain PUFA in plasma membranes of rainbow trout kidney show a comparable lag time of approximately 10 days (Hazel and Landrey, 1988b). The slow changes in PUFA levels in this study may also reflect acclimatory adjustments in the capacity for unsaturated fatty acid biosynthesis at reduced temperatures (Hazel and Williams, 1990; Hazel, 1988).

4.8. The role of PUFAs in the thermal adaptation of biological membranes

It is generally believed that PUFA, in particular docosahexaenoic acid (DHA, 22:6n-3), play an important role in maintaining the structural and functional integrity of cell membranes. This conclusion is primarily based on the frequently elevated levels of PUFA of winter-active ectotherms. For example, 22:6n-3 levels are nearly double those of the intestinal (Miller et al., 1976) and liver (Wodtke, 1978) lipids of cold-acclimated carp as compared with warm-acclimated fish, and three times higher in synaptosomal membranes of the Arctic sculpin than in the desert pupfish (Cossins and Prosser, 1978). The importance of PUFA in regulating membrane structure in response to changes in environmental temperature is also illustrated by the high levels of molecular species enriched in DHA. In particular, mitochondrial and plasma membranes of rainbow trout liver and kidney show elevated ratios of $C_{16:0}/C_{22:6}$-PC and $C_{18:0}/C_{22:6}$-PE in response to low temperatures (Williams and Hazel, 1994). DHA also plays an essential structural role in neural cell membranes of vertebrates. For example, 22:6n-3 accounts for approximately 40% of the total fatty acid in PE from fish brain, and about 15% of all the trout brain phospholipids consist of a single di-22:6n-3-PE molecular species (Bell and Dick, 1991). This evidence suggests that phospholipids, in particular PE, enriched in DHA probably represent an important structural component of neural cell membranes and are vital to normal development and functioning of the visual and neural systems of finfish.

Although there is compelling evidence that 22:6n-3 may be an essential fatty acid in regulating the physical properties of finfish membranes, this may not be the case in bivalve

molluscs. For example, the gill membrane order of thermally acclimated scallops showed a strong negative correlation with PL 20:5n-3 and 18:1n-7 levels (Fig. 3.7 and Table 3.3), whereas 22:6n-3 levels were not significantly correlated with membrane gill PL order. This contrasts with the membrane properties of the fish *Cyprinus carpio*, which were correlated with an increase in the level of 22:6n-3 and, to a lesser extent, 20:5n-3 (Behar et al., 1989; Farkas et al., 1980). Furthermore, it has been shown that 20:5n-3 has the lowest melting point of any PUFA (Bell et al., 1986), which may explain the greater importance of 20:5n-3 in gill membranes of thermally acclimated scallops, compared with 22:6n-3. These data, therefore, suggest that in scallop gills DHA probably has a function other than regulating membrane fluidity, in contrast to finfish, which seem to rely primarily on changes in DHA to regulate the fluidity of their membranes (Farkas et al., 1994; Fodor et al., 1994; Dey et al., 1993). This study also suggests that the modulation of membrane PL structure by 20:5n-3 may be an important mechanism in the thermal compensation of membrane function in bivalves.

4.9. Thermal stress and prostaglandin biosynthesis

Prostaglandins (PGs) and related eicosanoids are a unique class of biologically active metabolites of certain polyunsaturated fatty acids, specifically 20:3n-6, 20:4n-6 and 20:5n-3 (Stanley and Howard, 1998; Stanley-Samuelson, 1994). These polyunsaturates are known to be sequestered in the PL components of cell membranes and are made available for eicosanoid biosynthesis through the action of phospholipase A_2 (Stanley-Samuelson, 1994).

PGs have been found in a large range of animals including insects (Ogg and Stanley-Samulson, 1992), fish (Bell et al., 1994) and mammals (McKanna et al., 1998), but little is known of the action of PGs in bivalves. Invertebrate tissues are capable of synthesizing a wide range of eicosanoids including PGA_2, PGE_1, PGE_2 and $PGF_{2\alpha}$ (Stanley-Samuelson, 1987). Such PGs are produced by many tissues in response to specific stimuli and aid in a wide range of physiological functions, including control of fluid and electrolyte fluxes, behavioural thermoregulation, reproductive function and control of the cardiovascular and neuronal systems (reviewed by Stanley-Samuelson 1987).

Although PG production was not directly quantified in this study, it is possible that the decline in 20:5n-3 levels experienced during the initial stages of the acclimation time course (Fig. 3.6 and 3.12) may suggest the importance of this eicosanoid precursor in the production of 20:5n-3 series eicosanoids. In bivalves the production of PG has been little studied although PGE_2 has been detected in gills of the marine bivalve, *Modiolus demissus* (Freas and Grollman, 1980). This study showed that when gill filaments were incubated for 60 min in 25% seawater, there was a 10-fold increase in PGE_2 production, suggesting an increase in biosynthesis and release of this PG in response to a hypoosmotic stress. Thus changes in membrane 20:5n-3 levels in this study may indicate an adaptive response in bivalves following thermal stress, although additional work is needed to establish with certainty the functional significance of 20:5n-3, especially as it pertains to PG production.

4.10. Summary

Organisms must often cope with a variety of suboptimal conditions, in particular reduced temperature, imposed by changes in their environment. Such conditions are perceived as physiological stresses which an organism has to overcome to survive. Most temperate ectotherms possess survival mechanisms that enable them to cope with seasonal and short term cold conditions by avoiding or tolerating freezing. The ability to modulate the relative concentrations of various fatty acids has been widely recognized as one of the most fundamental mechanisms in stress resistance, in particular thermal stress. The results presented in this study demonstrate that the widely perceived and apparently straightforward relationship between lipid unsaturation and membrane fluidity is more complex than it may appear. In fact, adaptive changes in the content of a small number of lipid species, in particular the modulation of membrane PL structure by 18:1n-9, 20:1n-11 and 20:5n-3, rather than extensive changes in unsaturation levels, may be an important mechanism in the maintenance of membrane function in bivalves that experience fluctuating temperatures. This study, therefore shows that scallop gill and hemocyte membranes can undergo progressive changes in their membrane fluidity and fatty acid composition, an observation which is consistent with homeoviscous adaptation following a reduction in acclimation temperature.

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