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Acclimatory response to environmental temperature in two species of marine crab: the stenothermal cancer pagurus and the eurythermal carcinus maenas

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ACCLIMATORY RESPONSES TO ENVIRONMENTAL TEMPERATURE IN TWO SPECIES OF MARINE CRAB: THE STENOTHERMAL *CANCER PAGURUS* AND THE EURYTHERMAL *CARCINUS MAENAS*

ABSTRACT

Temperature acclimation effects on leg muscle plasma membrane composition and biophysical state, neuromuscular function and whole organism thermal tolerance, and their dependence on the background seasonal acclimatization were investigated in the eurythermal *Carcinus maenas* and the stenothermal *Cancer pagurus*. Correlations between the changes observed at each of these levels are discussed.

The average winter (5°C), annual (8°C), and summer (15°C) sea-water temperatures and a warmer summer temperature (22°C) were chosen as acclimation temperatures for their ecological significance.

Warm-acclimation (22°C) increased the thermal tolerance (CTMax) of both species and induced partial compensation of the neuromuscular function (assessed from leg nerve axonal conduction velocity and the amplitudes of excitatory junction potentials in dactylopodite closer muscle fibres). The time-course of acclimation showed it was complete after two weeks, but a longer exposure to 22°C reduced neuromuscular performance, an effect that may not be acclimation related.

At plasma membrane level, the response to warm-acclimation depended on acclimation temperature. Acclimation to 15°C induced a significant increase in the S/U fatty acids ratio of PC and PE, but no increase in plasma membrane order. Acclimation to 22°C induced a significant increase in the Ch/PL molar ratio and in plasma membrane order, but no major changes in overall fatty acid composition of membrane phospholipids, compared to cold-acclimated crabs.

A seasonal background, determined by complex factors, underlied these responses to temperature-acclimation. At all acclimation temperatures, crabs acclimated in autumn and winter had lower S/U fatty acid ratios and Ch/PL molar ratios than in spring, and the temperature dependence of plasma membrane fluidity was reduced in summer, compared to spring and autumn. The response to 15°C-acclimation was greater in winter than in other seasons.

The CTMax and the magnitude or efficacy of the acclimation responses showed that the stenothermal species was more thermally sensitive than the eurythermal species.

ACCLIMATORY RESPONSES TO ENVIRONMENTAL TEMPERATURE IN TWO SPECIES OF MARINE CRAB: THE STENOTHERMAL *CANCER PAGURUS* AND THE EURYTHERMAL *CARCINUS MAENAS*

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Being a Thesis presented in candidature for
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DECLARATION

I hereby declare that the work presented in this document is based on research carried out by me, and that this document has not been presented anywhere else for a degree.

Part of Chapter 2 has been published in the journal Comparative Biochemistry and Physiology (Cuculescu and Bowler, 1993) and parts of Chapter 3 and Chapter 4 have been published in Journal of Thermal Biology (Cuculescu, Hyde and Bowler, 1995)

Mirela Cuculescu

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CHEMICALS

Unless otherwise specified, all chemicals used were of analytical purity grade, purchased from either Sigma (Poole, Dorset) or BDH (Poole, Dorset).

GLOSSARY

2-AS = 2-(9-anthroyloxy) stearic acid

5-DSA = 5-doxyl stearic acid

12-AS = 12-(9-anthroyloxy) stearic acid

16-AP = 16-(9-anthroyloxy) palmitic acid

16:0/22:6, etc = notation for phospholipid molecular species, giving the number of carbon atoms in the chain and the number of double bonds of the two fatty acyl chains in the molecule

$\omega 3$, $\omega 6$, $\omega 9$ = the desaturation-elongation series according to the number of carbon atoms from the last double bond to the methyl end of the fatty acyl chain

C16:0, etc = notation for fatty acids C(number of carbon atoms in chain):(number of double bonds)

ARR = acclimation response ratio

AT = acclimation temperature

ATP = adenosine 5'-triphosphate

BHT = butylated hydroxytoluene

Ch = cholesterol

Ch/PL = cholesterol to phospholipid (molar ratio)

Cm (in figure legends) = *Carcinus maenas*

Cp (in figure legends) = *Cancer pagurus*

CTMax = critical thermal maximum

CTMin = critical thermal minimum

CV = conduction velocity

DPH = 1,3-diphenyl-1,3,5 hexatriene

EDTA = ethylene-diamine-tetraacetic acid

EGTA = ethylene-glycol-bis(β-amino ethyl ether) N,N,N',N'-tetraacetic acid

EJP = excitatory junction potential

ESR = electron spin resonance

FAME = fatty acid methyl esters

GLC = gas-liquid chromatography

HE (%) = homeoviscous efficacy

HEPES = N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]

H_{II} = inverted hexagonal phase

HR (%) = homeoviscous response

LD_{50} = 50% lethal dose

LPC = lysophosphatidylcholine

LT_{50} = 50% lethal time

NADH = nicotinamide adenine dinucleotide, reduced form

NMR = nuclear magnetic resonance

PA = phosphatidic acid

PC = phosphatidylcholine

PE = phosphatidylethanolamine

PI = phosphatidylinositol

P_i = inorganic phosphate

PL = phospholipid

PMSF = phenylmethylsulfonyl fluoride

PS = phosphatidylserine

PVP = polyvinylpyrrolidone

Py(3)Py = 1,3-bis(1-pyrenyl) propane

S/U = saturated to unsaturated (fatty acid ratio)

S.D. = standard deviation

SDS = sodium dodecyl sulphate

SM = sphingomyelin

TLC = thin layer chromatography

TMA-DPH = 1-(4-trimethylammoniumphenyl) 6-phenyl-1,3,5-hexatriene

TPL = total phospholipid fraction

Tris = tris(hydroxymethyl) aminomethane

Chapter 1. General Introduction

Environmental temperature has a profound, all-pervasive effect on living organisms and their vital functions, in particular upon those organisms that conform in body temperature to the environment. Temperature is one of the most important environmental parameters that determine the ecological and geographical distribution of microorganisms, plants and animals. Although the range of temperatures on earth is considered to be greater than the range within which life is possible, with air temperatures ranging from -70°C in polar regions to +85°C in hot deserts, and surface waters ranging from -1.82°C in Arctic and Antarctic waters, to +30°C in tropical landlocked bodies of water (Prosser and Heath, 1991), living organisms have invaded virtually all thermal habitats on this planet (Cossins, 1981). Both terrestrial and aquatic habitats display a great diversity of thermal conditions, the most important aspects being the mean temperature, the absolute range of temperature experienced and the rate of change of temperature (Cossins and Bowler, 1987). Correlations of animal occurrence have been made with maximum and minimum temperature, mean temperature and constancy of temperature (Prosser and Heath, 1991).

One major trend in the evolution of animals has been the development of progressively complex homeostatic mechanisms. As these mechanisms have evolved, organisms have become increasingly independent of fluctuations in their external environment (Vernberg and Vernberg, 1970). From the point of view of thermoregulatory abilities animals have been classified as 'homeothermic', those that maintain a well-regulated, relatively constant body temperature and 'poikilothermic', those that have a variable body temperature. Another classification groups animals in 'endothermic' or 'ectothermic'. Endothermic animals produce heat within their own tissues to thermoregulate, whilst ectothermic animals rely on gained heat from the environment for thermoregulation (Cowles, 1962). These two classifications are formally independent, meaning that in a habitat with constant temperature, like deep oceans and polar seas where temperature is 2°C to 4°C all the time, any ectotherm

can be homeothermic (Louw, 1993). Fluctuations in environmental temperature raise particular problems for the ectothermic organisms, which generally lack mechanisms for physiological temperature regulation of their body temperature which is therefore determined by ambient temperature and the physical characteristics of the environment (Precht, 1973).

A great variety of adaptive strategies have evolved in ectothermic animals, in order to cope with changes in ambient temperature or to avoid unfavourable temperatures. The intensity of stress arising from a change in temperature depends on the magnitude of the temperature change and the rate at which the change occurs (Somero and Hochachka, 1976).

Two main types of responses can be distinguished: behavioural and physiological (metabolic) (Louw, 1993, Prosser and Heath, 1991, Cossins and Bowler, 1987). Behavioural responses are rapidly developed and include migration to a niche with preferred temperature and the use of position and posture for temperature regulation. These can be used in response to short-term fluctuations in temperature by mobile organisms, whereas sessile organisms will have to develop various physiological responses, which include metabolic changes, cardiovascular adjustment and evaporation, in order to survive rapid changes in temperature. Two types of physiological adaptations can be distinguished: capacity adaptations, which allow the organisms to maintain certain functional parameters at a relatively constant, optimal level, even when ambient temperature fluctuates, and resistance adaptations, which enable organisms to survive extreme temperatures. Capacity adaptations are mostly phenotypic, whereas resistance adaptations may involve genotypic adaptations (Precht, 1958). In some cases, too rapid temperature changes may prevent even mobile ectotherms from developing the simplest behavioural responses, such as rapid movement to a more favourable temperature, and may have lethal effects if other capacity or resistance adaptations are absent. Both sessile and mobile ectotherms develop physiological adaptations in response to longer term changes in temperature, such as seasonal variations. Some species display also behavioural responses to

seasonal variations of temperature, such as migration or dormancy (Prosser and Heath, 1991).

Marine ectotherms can be confronted with three types of temperature variation, each with its own time course. These include rapid changes associated with the tidal cycle and sudden changes in weather, which in intertidal and estuarine habitats may exceed 15-20°C. There are also slower seasonal temperature changes. Long-term changes in temperature, such as those experienced by populations colonising a new habitat with a different thermal regime from that experienced by the species during its evolutionary history, also occur (Somero and Hochachka, 1976).

Responses to longer term seasonal changes in temperature, or adaptation to a new or an altered habitat (such as those where a source of pollution appears) will involve compensatory changes in cellular physiology and biochemistry (Cossins and Bowler, 1987) which require longer periods of time, and the longer the exposure time, the more thoroughly can the organism rebuild its cellular chemistry to facilitate survival and enhance success in its new thermal regime (Somero and Hochachka, 1976). In each case, the animals develop a suite of physiological responses to cope with alterations in the thermal regime of their habitat. Those that are not able to adjust to the new conditions will either migrate to a different area or will be eliminated. The elimination can be either rapid, by thermal death, or gradual, due to the cessation of more complex functions, such as development, growth or reproduction. These functions are successfully performed over ranges of temperature narrower than those of whole animal survival (Cossins and Bowler, 1987). Survival and perpetuation of animals with greater adaptive flexibility or better fit for the new conditions will gradually change the genotypic background of a population following chronic alterations in the environmental parameters of its habitat or following colonisation of a new habitat, thus allowing a more fundamental response to an environmental change (Somero and Hochachka, 1976).

Evolutionary adaptation to a new thermal habitat may involve not only a tolerance for a new average temperature, but also an ability to cope with a different

range of temperatures, which means that a species which colonises an environment with widely fluctuating temperature must become eurythermal, while a species which colonises a highly thermally stable environment can afford to lose some of its ability to withstand temperature fluctuations, as evidenced by the stenothermality of Antarctic fishes (Somero and Hochachka, 1976).

Several patterns of temperature acclimation of rate processes have been observed, and can be classified according to schemes devised either by Precht (1958), Precht, Laudien and Havsteen (1973), or Prosser (1973). These are described in detail and discussed by several authors, for example Cossins and Bowler (1987), or Cossins and Sinensky (1984).

Changes in the kinetic energy of the environment usually are rapidly and quantitatively transferred to the whole body of ectotherms and affect the physiology of organisms via the cellular chemistry in two ways (Hochachka and Somero, 1984, Cossins and Bowler, 1987): firstly, the rate of biochemical reactions is changed, because of the changes in the percentage of molecules in a given population that possess sufficient energy to react, and secondly, the equilibria between the formation and the disruption of non-covalent interactions will be altered. These interactions are involved in stabilizing the higher levels of protein structure and the structure of biological membranes. Given the aqueous environment of cells and tissues, all these alterations are also related to the changes induced by temperature in the solvent properties of water, which affect the weak-bond systems (Franks, 1985). These problems must be overcome by ectotherms adapting to seasonal changes in temperature or to extreme thermal environments, and by non-mobile ectotherms which can not develop rapid behavioural responses (Hazel, 1989).

In spite of the critical role played by biomembranes in many vital cell functions, only weak, mostly hydrophobic, non-covalent interactions between constituent lipid and protein molecules maintain the structural organization and integrity of biological membranes. These allow great regulatory flexibility, but also

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make them particularly sensitive to perturbation by relatively small changes in temperature (Williams and Hazel, 1994a). The phase state, molecular order and rates of molecular motion of the membrane components will all be significantly perturbed by changes in environmental temperature (Lee and Chapman, 1987). Structural flexibility is an obligate requirement for both the catalytic efficiency of enzymes and the functional integrity of "fluid mosaic" membranes (Singer and Nicholson, 1972), but the range of temperature which maintains adequate structural flexibility is rather narrow, and temperatures below or above its limits will alter both enzyme and membrane function (Williams and Hazel, 1994a).

Cell membranes have several important functions: (1) constrain the free diffusion of solutes and catalyze specific exchange reactions, maintaining in these ways the unique chemical compositions of both the cellular and subcellular components; (2) store energy in the form of transmembrane ion and solute gradients and regulate the rate of energy dissipation; (3) provide an organizing matrix for the assembly of multicomponent metabolic pathways; and (4) govern the transfer of information between the compartments they separate by possessing ligand-specific receptors (Hazel and Williams, 1990). Membrane lipids mediate these functions by acting as physical barriers to electrolyte diffusion, solvents for a variety of membrane constituents, and as anchors, activators and conformational stabilizers of membrane proteins (Hazel and Williams, 1990).

For unicellular organisms, cell membranes are the primary site of interaction between the organism and its environment. The same statement is valid for higher organisms, including human beings, referring to membranes of more and more specialized epithelial and excitable cells (Lagerspetz, 1987). Poikilotherms, in particular, may experience large changes in their membrane fluidity as a result of fluctuations in environmental temperature, with disruptive effects upon all the membrane-dependent functions (Cossins, Bowler and Prosser, 1981).

The ability to modulate membrane fluidity to compensate partially for the direct effects of temperature has been demonstrated in many species of poikilotherms

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(Cossins, 1983). Eurythermal animals, which live in a thermally fluctuating environment, have greater adaptive flexibility than stenothermal animals, which live in habitats with narrower ranges of temperature fluctuations (Prosser and Heath, 1991). Although strong evidence exists to demonstrate that one of the aspects that determines the increased potential for capacity adaptations in eurytherms, therefore the greater ability to cope with large fluctuations in temperature without losing vital function, is the greater thermal independence of enzyme activity and Km, the exact molecular basis of this difference is not known (Somero, 1995) and other physiological differences between steno- and eurythermic animals are poorly understood (Cossins and Bowler, 1987).

The necessity of further investigation of the mechanisms of temperature adaptation of eurythermal and stenothermal marine species is widely recognized, not only by thermal and environmental biologists, in the current context of predicted global warming and increased awareness of thermal pollution of coastal marine environment from power stations (Logue, Tiku and Cossins, 1995), which may alter the thermal regimes of various marine habitats (Bamber, 1990) and perturb the ecological balance of whole communities, with short and long term effects which are not entirely predictable.

As stated above, the structural and functional integrity of biological membranes are particularly sensitive to changes in environmental temperature. Early work (Heilbrunn, 1924), carried out before the achievement of the current understanding of the structure and composition of membranes, and later detailed studies on a variety of cellular membranes (Bowler, Gladwell and Duncan, 1973) suggested that membranes and membrane adaptations play an important role in determining the thermal sensitivity of cells and that the stability of membrane lipoprotein complexes was the critical target in heat injury (Bowler and Manning, 1994). Also early investigations of the effects of cold temperatures showed that exposure of microorganisms, plants and animals to low temperature induced an

increase in the unsaturation of their storage lipids or membrane phospholipids (Cossins, 1983, Hazel, 1984). Chapman, Byrne and Shipley (1966) proposed that 'the particular distribution of fatty acyl residues present in a membrane provides the appropriate membrane fluidity at a particular environmental temperature to match the diffusion rate or rate of metabolic processes required for the tissue'.

This hypothesis was proved only after the development of biophysical techniques for measuring the physical condition of membranes in the early 1970s, when Sinensky (1974) demonstrated that the bacteria *Escherichia coli* grown at several temperatures displayed similar membrane fluidities and phase states at each of the growth temperatures. He termed this phenomenon 'homeoviscous adaptation', which emphasized the importance of compensatory changes in membrane fluidity for the maintenance of membrane function in conditions of altered temperature. The term fluidity usually described the physical state and dynamics of the lipid domains of the membranes (Pringle and Chapman, 1981).

Over the last two decades, the techniques available for measuring the membrane biophysical state have developed and more information has been gathered about the ways in which the physical state of membrane lipids may influence the function of membrane proteins. This has improved the understanding of the complex interactions between membrane lipids and proteins in biomembranes and refined the concept of membrane 'fluidity' (Cossins, 1994). The lipid bilayer is a complicated anisotropic microenvironment, where microdomains of different compositions or in different phase states coexist (Hazel, 1989), the structure and dynamics of which can not be accurately described by any of the existing measurement techniques (Gennis, 1989). Both the range and the rate of motion contribute to the anisotropy measured by the widely used DPH fluorescence polarization technique, with the range of motion, defined by the static ordering of the hydrocarbon chains, being the predominant term. The two contributions can be separated only by the time-resolved analysis of anisotropy (Cossins, 1994) and it has been suggested that the adaptive

regulation of membrane order is more important for evolutionary adaptation than that of the rate of motion, or fluidity (Behan-Martin *et al.*, 1993).

The existence of a directly causal relationship between membrane lipid composition and membrane biophysical state is still a controversial matter (Cossins, 1994). It is generally assumed, based on existing reports and on studies on synthetic lipids, that regulation of membrane fluidity is achieved via changes in the membrane lipid composition (Cossins and Raynard, 1987), which include alterations in the proportions of saturated and unsaturated fatty acids, branched-chain fatty acids (in some microorganisms), PE relative to PC, plasmalogens compared to diacyl phospholipids (in the nervous tissue), certain molecular species of PC or PE, or of cholesterol relative to phospholipids (Thompson, 1983, Hazel and Williams, 1990). Of the relatively few papers which investigated changes in both fluidity and lipid composition on the same preparations, some found consistent effects of temperature on both aspects, while others reported sometimes large changes in one of these two, with very little or no changes in the other (Cossins, 1994, Hazel, 1995).

It is now recognized that membrane acclimation or adaptation to temperature may involve more subtle restructurations than changes in overall lipid composition and/or in bulk fluidity (Cossins, 1994). For example, Farkas *et al.* (1994) suggested that the changes in the molecular species of membrane phospholipids, via reshuffling of the fatty acyl chains, without new synthetic requirements, play a more important role in membrane acclimation to a new temperature than the changes in overall fatty acid composition. Fluidity adjustment is probably only one of the possible ways in which cells manage to maintain appropriate control on the membrane-dependent processes (Cossins and Prosser, 1982).

Recently, the homeoviscous adaptation theory has been critically reviewed by several authors (Hazel and Williams, 1990, Cossins, 1994, Hazel, 1995) who concluded that this theory cannot explain all the diversity of patterns of responses observed in biomembranes of different species, in various conditions. Another problem is that despite the large amount of experimental evidence accumulated, the

adaptive significance of these compensations in membrane fluidity and/or lipid composition has not been unequivocally demonstrated (Cossins, 1994).

Behan Martin *et al.* (1993) and Cossins and Prosser (1978) showed that different vertebrate species, ranging from the Antarctic fish *Notothenia* to rat and pigeon, which are evolutionary adapted to different thermal regimes, have similar degrees of membrane order when measured at their respective body temperature, or typical habitat temperature, suggesting that the conservation of this feature plays an important role in temperature adaptation. Similar trends have been observed for invertebrate species (Farkas, Storebakken and Bhosle, 1988, Dahlhoff and Somero, 1993, Lahdes, Kivivuori and Lehti-Koivunen, 1993).

Hazel (1972) provided one of the first clear demonstrations of the adaptive benefits of the homeoviscous response. He showed that succinate dehydrogenase reconstituted with mitochondrial phospholipids of cold-acclimated goldfish had enhanced enzymatic activity compared to the same enzyme when reconstituted with the corresponding phospholipids of warm-acclimated goldfish, suggesting that SDH activity was dependent on the hydrophobic environment created by the surrounding phospholipids and the modification of the latter during temperature acclimation can modulate the enzyme's activity. Other delipidation and reconstitution studies have demonstrated that phospholipids are required for the enzymatic activity of a large number of membrane bound proteins and that the magnitude of the reactivation was dependent mainly on the unsaturation of the phospholipid acyl chains (Cossins, 1983). The thickness of the phospholipid bilayer, determined by the chain length and conformation of the phospholipid fatty acids, is also important for the activity and molecular dynamics of membrane enzymes (Cornea and Thomas, 1994).

Periodical reviews, such as those of Cossins (1983, 1994), or Cossins and Bowler (1987) have briefly presented and discussed several examples of studies which found correlations between the homeoviscous adaptation of various types of membranes, in particular of excitable membranes, such as fish brain and synaptic membranes, and compensatory changes in enzyme function, membrane permeability,

thermostability of the activity of membrane enzymes, whole organism thermotolerance or shifts in the break points of the Arrhenius plots of the rates of membrane-related processes. Some of the evidence coming from studies which used other membrane fluidizing agents (local anaesthetics, alcohols) showed that these had similar effects upon membrane function with the increase in temperature, and in some cases by increasing membrane fluidity these agents reduced the thermal tolerance of the exposed cells (Bowler and Manning, 1994).

The reviews of Macdonald (1988, 1990, 1994) raised a question about the direct involvement of homeoviscous adaptation in the control of the electrical properties of excitable membranes and of neuro-neuronal and neuromuscular transmission, in conditions of altered environmental temperature. Also examples of thermal compensation of membrane function without a significant extent of homeoviscous adaptation are available, such as the compensation of kidney function in the eurythermal roach *Rutilus rutilus* (Schwarzbaum, Wieser and Cossins, 1992).

Another theory proposed to explain the observed changes at membrane level is the homeophasic adaptation. This theory originated from the observation that structural rearrangements and subsequent functional perturbations are much greater when the phase state of a membrane is changed, during cooling, than when only a decrease in fluidity takes place (Hazel, 1989). The term has been proposed by McElhaney (1984) who showed that in *E. coli* growth was impaired at temperatures below which the percentage of lipid present in the gel phase exceeded 50%, although considerable variations in fluidity were tolerated without deleterious effects at growth temperatures above this threshold. The homeophasic adaptation theory assumes that the adaptive role of the changes associated with homeoviscous adaptation is primarily to ensure the preservation of a particular phase state, rather than to fine tune the membrane fluidity. The validity of this theory seemed to be restricted to simpler organisms, such as bacteria, since most studies on multicellular organisms have not detected phase transitions at physiological temperatures (Hazel, 1989). The membrane lipid composition of the species of fish in which homeoviscous adaptation has been

demonstrated suggests that, due to the high content of polyunsaturated fatty acids, the temperature of phase transition of these membranes is well below the physiological range of the species (Cossins and Raynard, 1987) and therefore a phase transition does not appear to constitute a serious challenge.

Phase transitions have been observed in reconstituted vesicles of phospholipids extracted from planktonic crustaceans in winter and in summer, with a compensatory shift of the temperatures of onset and completion of the phase transition towards lower temperatures in winter (Farkas, Nemecz and Csengeri, 1984), but the extrapolation of these observations to the phase behaviour of intact membranes from the same animals is speculative. However, the significance of homeophasic adaptation for multicellular organisms may be related to the microdomain heterogeneity existent in these membranes, the exact structure and role of which are still poorly understood.

The membranes of eukaryotic cells contain a whole diversity of phospholipids, which differ by the nature of their headgroup and their fatty acyl chains. Phospholipids are polymorphic and can assume different phases, depending upon the temperature, pressure, degree of hydration and the chemical structure of the phospholipid (Hazel, 1989). Three phases are relevant for the organization of biological membranes: the lamellar gel phase, the fluid lamellar (liquid crystalline) phase and an inverted hexagonal phase (H_{II}). The H_{II} phase consists of hexagonally packed water cylinders, delimited by the headgroups of phospholipid molecules, which have their acyl chains oriented radially (Figure 1.1). Some phospholipids, in particular PE and cardiolipin (in the presence of calcium) which have conically-shaped configurations, undergo a transition to this phase at temperatures above the gel/fluid transition, as this phase can readily accommodate their configuration and an increased level of conformational disorder (Hazel, 1989). Although the H_{II} transition is normally observed at temperatures above 45°C (for PE, Ellens, Bentz and Szoka, 1986), low water concentrations lower this temperature to the physiological range, suggesting a role for it in membrane fusion (Verkleij, 1984).

Figure 1.1. The phase states of phospholipids in biological membranes: the lamellar gel phase, the lamellar fluid or liquid crystalline phase and the inverted hexagonal (H_{II} phase); the H_{II} is presumed to be involved in membrane fusion (the arrows indicate changes in temperature; the physiological temperature refers to the temperature at which an organism is either adapted or acclimated) (from Hazel, 1995 and Hazel, 1989)

Gel Phase

Region of Phase Separation

Fluid, or Liquid-Crystalline Phase

Inverted Hexagonal Phase

High Temperature

Low Temperature

Physiological Temperature

Membrane Fusion
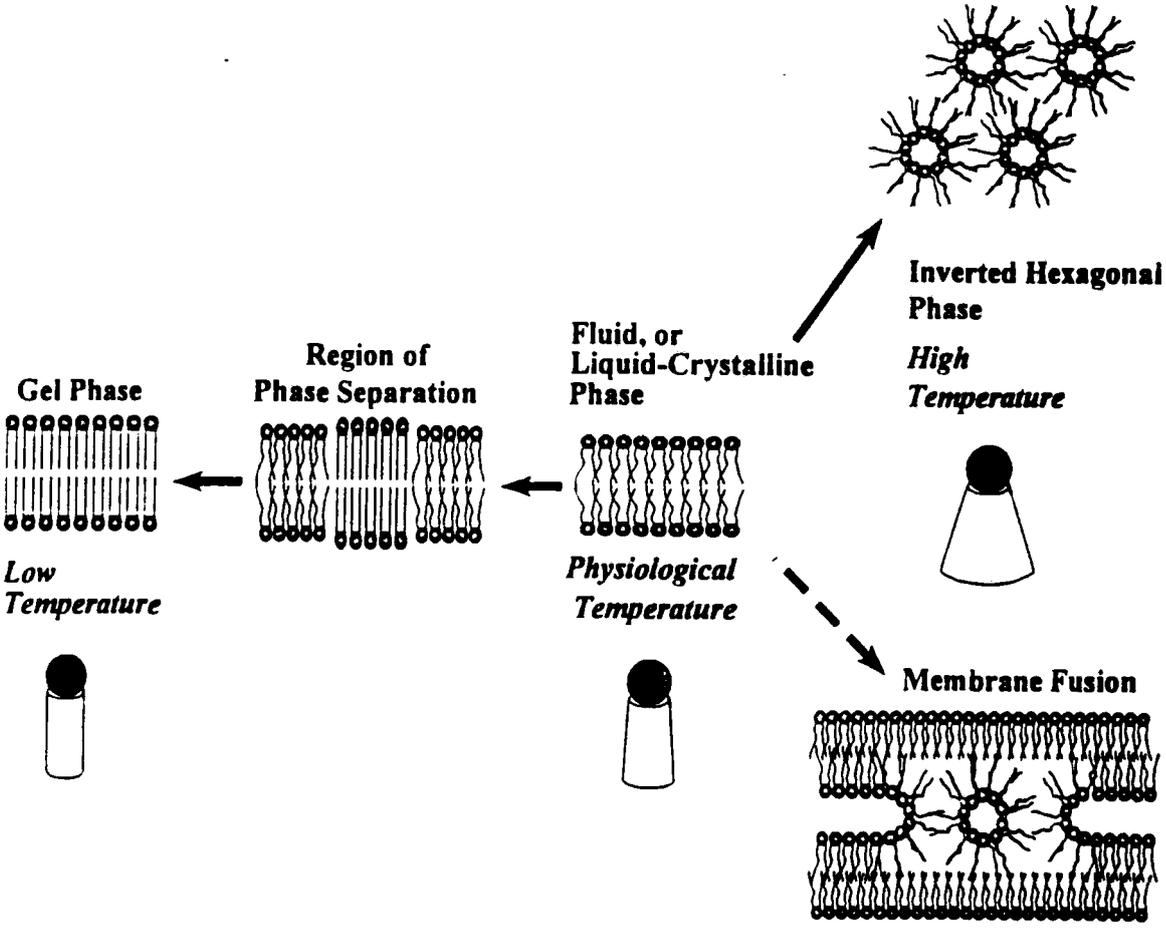
Hazel (1995) describes a dynamic phase model of membrane adaptation, developed on the basis of the homeophasic adaptation concept. According to this model, organisms alter their membrane lipid composition in order to conserve the relationship between the temperature at which they grow or function and the temperatures of the gel/fluid and H_{II} phase transitions. In a steady state, the temperature at which the organism lives is situated on the temperature scale above the temperature of the gel/fluid transition of its membrane lipids, and below the temperature of the fluid lamellar/H_{II} transition. The concept of dynamic phase behaviour predicts that at physiological temperatures, a membrane must be positioned close enough to the H_{II} transition, i.e. being sufficiently unstable, to permit the fusion events associated with normal membrane traffic, yet be stable enough to prevent these processes from occurring in an unregulated fashion (Lindblom and Rilfors, 1989). If the body temperature is changed, it will get too close to one of the phase transition temperatures and the equilibrium between the stability and unstability of its membranes will be disturbed, therefore the composition of membranes must be changed in order to shift the phase transition temperatures and re-establish this equilibrium.

This model seems to explain some of the observed membrane restructurings which were not consistent with the homeoviscous adaptation theory, such as the positive correlation between growth or acclimation temperature and the PC/PE ratio in animal cell membranes (Hazel and Landrey, 1988a), or the accumulation of polyunsaturated fatty acids rather than monounsaturated fatty acids in membranes of cold adapted or acclimated animals (Baezinger, Jarrell and Smith, 1992). Monounsaturated fatty acids induce a more marked decrease in membrane order, but they cannot pack regularly to form a tightly sealed bilayer that restricts cation permeability. The multiple double bonds of the polyunsaturated fatty acids will also increase the extent of acyl chain motion, and yet order the membrane in their immediate vicinity (Brenner, 1984), maintaining simultaneously an appropriate dynamic state of the bilayer and permeability. The dynamic phase behaviour model

may also explain why different types of membranes display different magnitudes of compensatory changes in fluidity or lipid composition, ranging from perfect compensation to none (Cossins, 1994). Each membrane type has its own specific composition and fluidity and consequently a particular interval between its gel/fluid and H_{II} phase transition. If for some membrane types this interval is wide enough to allow large variations in body temperature without bringing this too close to one of the phase transitions, then the adaptive response induced will be of little extent (Hazel, 1995).

Temperature acclimation and adaptation have been associated with other modifications at cellular level, such as variations in the concentrations of membrane-bound enzymes and in the cellular distribution and arrangement of membranes and proliferation of mitochondrial and sarcoplasmic reticulum membranes following cold acclimation (Penney and Goldspink, 1980, Sidell, 1983, Egginton and Sidell, 1989).

The understanding of the adaptive significance of the modulations in membrane composition is linked to the understanding of the mechanisms of control of membrane fluidity or phase state, that trigger and achieve these modulations, and of the ways in which the biophysical state of the lipid bilayer influences the function of membrane proteins (Cossins, 1994). In ectothermic animals, the changes in the lipid composition of their cell membranes are graded in response to the variation in ambient temperature (Hazel, 1988) and rapid compensatory changes in membrane fluidity occur (Wodtke and Cossins, 1991).

A tempting scheme is that of a closed feedback control system, described by Cossins (1983) which assumes that the direct effect temperature has on membrane physical state is detected by a 'membrane sensor' which activates the enzymes involved in the restructuring of lipid composition, then this is carried out and the process stops at a new steady state when the membrane sensor detects the appropriate biophysical state (Figure 1.2). Since then, this model has gained support from studies using immunological and genetic probes for the analysis of the temperature-induced

Figure 1.2. Schematic representation of the closed feed-back control system proposed for the control and regulation of membrane function via membrane fluidity and membrane composition (from Cossins, 1983)

Temperature disturbance

| Membrane biosynthesis | Membrane composition | Membrane fluidity | Membrane function |

FEEDBACK LOOP
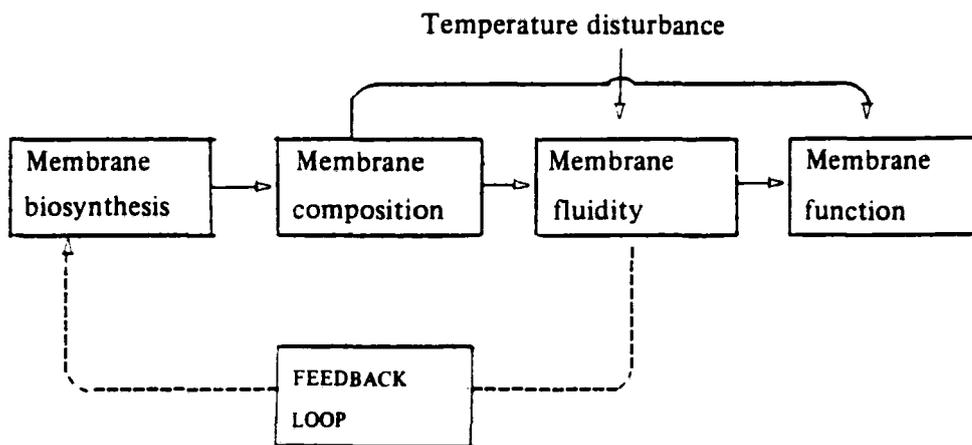
changes in expression of lipid biosynthetic enzymes at both the transcriptional and translational levels, reviewed by Macartney, Maresca and Cossins (1994) and from studies using catalytic hydrogenation of plasma membrane fatty acids as a method to induce specific alterations only in membrane fluidity, unlike temperature which affects all cellular processes (Vigh *et al.*, 1993, and related papers, discussed by Maresca and Cossins, 1993). These studies demonstrated that, in general, either an alteration in membrane composition (via hydrogenation of phospholipid fatty acids, dietary modulation or addition of cholesterol) which increases membrane order, or a direct increase in membrane order due to lowering of temperature rapidly activate the acyl desaturases, whereas alterations in membrane composition which decrease membrane order, such as addition of fluidizing agents, decreased the desaturase activity (Macartney, Maresca and Cossins, 1994).

The work presented in this thesis was planned as a comparative investigation of the mechanisms of temperature acclimation at cellular, functional and whole organism level in two species of marine crustaceans with different thermal ecology: the relatively stenothermal edible crab, *Cancer pagurus*, and the eurythermal shore crab, *Carcinus maenas*. One of the aims was to assess the correlation between temperature acclimation-induced changes at each of these levels. Another aim was to demonstrate that individuals of the stenothermal species are more thermally sensitive than the eurythermal ones at whole organism level, and to search for evidence at a cellular and functional level. The influence of the background seasonal acclimatization on the response to laboratory acclimation was also sought.

Acclimation temperatures of ecological significance were chosen and after a period of acclimation at constant temperature and photoperiod, animals were used for the three types of experiments planned. In order to study changes at cellular level, plasma membranes were isolated from leg muscle homogenates of animals acclimated to different temperatures and were used in part for fluorescence polarization measurements, to determine the extent of homeoviscous adaptation. The remaining

plasma membrane preparation was used for obtaining a total lipid extract, which was analysed for total phospholipid and cholesterol levels, and further separated in individual phospholipid classes. The fatty acid composition of the major phospholipid classes, phosphatidylcholine and phosphatidylethanolamine was determined using gas-liquid chromatography. At functional level, the temperature dependences of certain parameters of neuromuscular transmission, and the influence of temperature acclimation upon these, was measured in axonal or neuromuscular preparations from cold and warm-acclimated crabs. The whole organism thermal tolerance was determined using the critical thermal maximum method (Cowles and Bogert, 1944).

The results are presented starting with those describing the changes at cellular level, and ending with those at whole organism level. Each chapter starts with a brief review of the literature considered relevant for the particular aspect dealt with in that chapter and ends with a discussion of the results. To reduce repetition to a minimum, the correlative interpretations of the results are discussed in the final chapter, of general discussion and conclusions.

Chapter 2. The Isolation of a Plasma-Membrane Rich Fraction from the Leg Muscle of Two Species of Marine Crab, *Carcinus maenas* and *Cancer pagurus*

2.1. Introduction

The cell membrane is a complex heterogeneous system of lipids and proteins, whose role in the processes of thermal adaptation has been suggested, then demonstrated by numerous studies (Cossins, 1983, Pringle and Chapman, 1981). In ectothermic organisms, the most common response to alterations in environmental temperature is a change in the degree of saturation of the fatty acyl chains of membrane phospholipids, in order to compensate for the changes in membrane fluidity induced by temperature (Cossins, 1981). The observed compensatory changes, first described by Sinensky (1974) in bacteria and termed homeoviscous adaptation, suggested that membrane lipids play an important role in controlling membrane function. The attempts to understand how adjustments in membrane lipids regulate the membrane function during thermal adaptation must be based on studies of the direct effects of temperature on membrane structure and membrane-dependent functions (Cossins, 1983). Due to structural and functional differences between plasma membranes and intracellular membranes, these type of studies are more useful when carried out on isolated membranes of defined type and known level of contamination with other types (Thompson, 1983). Another reason is that compositional adjustments were frequently found to be membrane-specific (Hazel and Zerba, 1986, Lee and Cossins, 1990) and subtle but important differences in the nature of the homeoviscous response in different membrane fractions may be obscured by analyses of total tissue lipids (Hazel, 1989).

As the primary site of interaction between a cell and its external environment (Gennis, 1989) the plasma membrane is assumed to be involved in thermal sensitivity and is considered to be the primary site of lesion in thermal damage (Bowler, 1987).

For the purposes of the present study (see Chapter 1. Introduction), the plasma membrane of crab leg muscle fibres was chosen to be isolated and purified for subsequent analysis of fluidity and lipid composition. A relatively simple and rapid method was required, which would have allowed the routine preparation of membranes from a large number of animals, in a short period of time, at the end of each acclimation period. Marine ectotherms generally have high levels of polyunsaturated fatty acids in their membrane lipids (Newell, 1979), fatty acids which can be easily oxydized and degraded (Christie, 1982), which was the reason why a rapid method was sought.

Although a variety of methods of purification of different types of membranes are described in the literature (Findlay and Evans, 1987), methods specific for the isolation of plasma membrane from crustacean muscle are few (Sevillano, Calvo and Cabezas, 1980, Altamirano, Hamilton and Russell, 1988, Syvertsen and Fonnum, 1989, Krizanova, Novotova and Zachar 1990, Gray *et al.*, 1991). These methods are either novel, developed to suit the purpose of that particular study (Altamirano, Hamilton and Russell, 1988, Gray *et al.*, 1991), or are adaptations of methods successfully developed for vertebrate skeletal muscle (Boegman, Manery and Pinteric, 1970, Rosemblatt *et al.*, 1981). The isolation of plasma membrane from muscle tissue in general (Kidwai, 1974) is complicated by the presence of large amounts of contractile proteins. Most methods quoted above for crustacean muscle were based on lengthy and complex procedures, including centrifugations on sucrose gradient, and the quality of the final fractions was not always evident because the yields were not clearly expressed as percentage recovery of the total activity of a marker enzyme in the crude homogenate. In the one case reported for the preparation of sarcolemmal membranes from muscle of *Carcinus maenas* (Sevillano, Calvo and Cabezas, 1980) the yield was low (<1%). For our subsequent membrane fluidity study and lipid composition analysis, a method with high yields and good levels of purification was needed.

More recent reviews of the methods available for membrane isolation and purification (Gennis, 1989) have pointed out that the use of sucrose gradients has serious drawbacks. The densities required to separate the membrane fractions involve the use of high concentrations of sucrose which are both very viscous and hypertonic, the latter often resulting in lysis and damage of the subcellular organelles. To overcome these drawbacks, other density gradient media have been developed and are increasingly used, like Ficoll, metrizamide, and Percoll (Gennis, 1989).

Percoll had been successfully used in our laboratory for the purification of plasma membranes from other sources (Manning *et al.*, 1989). The product consists of a colloidal suspension of PVP-coated silica, which has the major advantages that it does not penetrate biological membranes and the solutions have low viscosity and low osmolarity. The large particle size allows centrifugation at moderate speeds to result rapidly in self-generated Percoll density gradients (typically 30,000xg for 30 minutes), which have very high resolution of membrane fractions based on buoyant density (Gennis, 1989).

Two parallel membrane purification methods were selected for preliminary studies: an adaptation of the method used by Manning *et al.* (1989), based on centrifugation on Percoll self creating gradient, and an adaptation of another rapid method described by Maeda, Balakrishnan and Mehdi (1983), based on a short centrifugation on a 41% sucrose cushion, which was reported to give high yields and levels of purification for various types of tissues (Maeda, Balakrishnan and Mehdi, 1983, Hazel and Landrey, 1988a). A number of marker enzymes were selected to assess the levels of purification of the plasma membrane fraction and of contamination with membranes from other cellular organelles (Evans, 1982).

One important step in the purification of plasma membranes from muscle cells with good yields is the separation of the contractile proteins from the membrane fragments. Filtration through a series of filters with different mesh sizes is recommended (Kidwai, 1974) before density gradient centrifugation. Alternatively, treatments with KCl solutions (0.6-0.8 M) or with chaotropic agents like LiBr (0.4-0.5

M), NaI (2M) or NaSCN (1M) are utilized (Hansen and Clausen, 1988). Sevillano, Calvo and Cabezas (1980) used both filtration through terylene mail and extraction with 0.2 LiBr for purification of plasma membranes from *Carcinus maenas* muscle. The extraction with NaI 2M was reported to reduce nonspecific ATPase activity and facilitate measurement of the activity of the ouabain-sensitive (Na^+-K^+)ATPase (Nakao *et al.*, 1965).

To avoid the filtration procedure, which was believed to reduce yields and also lengthen the duration of the preparation method, the extraction with 2M NaI was chosen before the centrifugation on Percoll gradient or 41% sucrose cushion. The centrifugation on 41% sucrose cushion, used in parallel with the centrifugation on Percoll on the same crude homogenate of crab muscle always gave poorer results in terms of both purification and yield recovery. The following sections will refer in detail only to the method based on centrifugation on Percoll.

2.2. Materials and Methods

2.2.1. Animals

Crabs (intermolt stage) were caught from the North Sea and kept in tanks with aerated filtered sea water at 7-8°C up to eight weeks prior to use for experiments. The crabs were fed weekly with frozen fish and the water was changed the day after.

2.2.2. Membrane Isolation Procedure

All the following steps were performed at 0-4°C in an ice bath or in a cold room. For each experiment, 4-7 g (fresh weight) of leg and claw muscle were rapidly dissected and transferred to a volume of ice-cold hypoosmotic homogenising buffer (0.25 M mannitol, 5 mM EDTA, 30 mM histidine, 0.005 mM PMSF, pH 6.8 with HCl). The volume was then brought to 22 ml and the muscle was homogenised in an all-glass manual Dounce homogenizer, with 9-10 strokes of a loose-fitting pestle. Two millilitres were sampled for the protein and enzyme-activity assays and 20 ml of the

crude homogenate were mixed with 20 ml of a freshly prepared mixture of solution A (8 m NaI) and solution B (10 mM $MgCl_2$, 6 mM ATP, 20 mM EDTA, pH 8.0 with Tris), 1:1 (v/v) resulting in a 2M NaI extraction medium (Nakao *et al.*, 1965). The muscle homogenate was extracted for 30 minutes, with continuous stirring.

After 30 minutes the extraction mixture was diluted to 100 ml with ice-cold distilled water (pH 7.2 with Tris) in order to reduce the concentration of NaI (0.8 M after dilution) and centrifuged at 1000xg for 10 minutes (MSE Europa 24M). Solid sucrose was added to 90 ml of the supernatant to give a 0.25 M sucrose solution, then 11.5 ml of Percoll, 1.3 ml of 0.1 M $CaCl_2$ and 1.6 ml of 2 M sucrose were added and thoroughly mixed (Manning *et al.*, 1989). This mixture (final concentrations 11% Percoll, 0.7 M NaI, 0.25 M sucrose, 1 mM $CaCl_2$) was centrifuged at 45,000xg for 30 minutes (MSE Prepspin 50, swing-out rotor), at 4°C. Compact, white-pinkish membrane bands formed at the top of the Percoll gradient and were collected, diluted to 100 ml with neutralized distilled water (pH 7.2 with Tris) and centrifuged for 1 hour at 100,000xg (MSE Prepspin 50, angle rotor) at 4°C to pellet the washed plasma membranes. The pellets were removed from the Percoll-sucrose cushion formed on the bottom of the tubes either by vortexing in a small volume of neutralized distilled water, or by gentle scraping with a smooth glass rod, and resuspended in 5-10 ml neutralized distilled water using 4-5 strokes of a teflon-glass homogenizer, power setting 3 (3600 rpm). Aliquots were taken for the protein and enzyme activity assays and the bulk of plasma membranes suspension was stored at -80°C.

All the dilutions of the crude homogenate or membrane suspension required for the protein and enzyme activity assays were prepared by re-homogenization in the appropriate volume of neutralized distilled water, using a teflon-glass homogenizer.

2.2.3. Enzymatic Assays

The purity and the yields of the plasma membrane preparations were assessed by measuring the activities of marker-enzymes (Evans, 1982) in both crude homogenate and final plasma membrane preparation (all assays were run in triplicate):

- (Na$^+$-K$^+$)ATPase (E.C.3.6.1.3.) (Atkinson, Gatenby and Lowe, 1973) and Alkaline Phosphodiesterase I (E.C.3.1.4.1.) (Beaufay *et al.*, 1974) for the plasma membrane;

- (Ca^{2+}-Mg^{2+})ATPase (E.C.3.6.1.3.) (Atkinson, Gatenby and Lowe, 1973) for the sarcoplasmic reticulum;

- Succinate Dehydrogenase (E.C.1.3.99.1) (Tsai, Chen and Canellakis, 1975) for the mitochondria;

- Lactate Dehydrogenase (E.C.1.1.1.27.) (Bernstein and Everse, 1975) for cytosol;

- and Thiamine Pyrophosphatase (E.C.2.5.1.3.) (Morré, 1971) for the Golgi apparatus.

Protein determinations were carried out using the Coomassie Brilliant Blue binding method (Bradford, 1976), with bovine serum albumin fraction V as a standard.

Assay for (Na$^+$-K$^+$)ATPase: The solutions used were a) reaction medium without ouabain (20 mM KCl, 6 mM MgCl$_2$, 200 mM NaCl, 40 mM imidazole, pH 7.2); b) reaction medium with ouabain (2 mM ouabain in solution a), pH 7.2); c) 12 mM Tris-ATP, pH 7.2 (the required amount of ATP disodium salt was dissolved in a small volume of distilled water, passed through Dowex resin layered on filter paper on a funnel for filtration under vacuum, in order to change it to the acid form, and collected in a volumetric flask; the resin was rinsed repeatedly with distilled water until the filtrate was brought close to the final volume; the filtrate was brought to pH 7.2 with 1M Tris and the volume adjusted to the final value); d) acid molybdate solution (10g ammonium molybdate, and 50 ml concentrated sulphuric acid brought to 1 litre with distilled water); and e) 1% Lubrol (Cirrasol) solution.

For the assay 1 ml of reaction medium with or without ouabain and 0.5 ml Tris-ATP were preincubated 5 minutes at the assay temperature (25°C, water bath) then 0.5 ml of an appropriate dilution of the membrane suspension or homogenate were added and tubes were incubated for 20 minutes. The reaction was stopped with 4 ml acid molybdate-Lubrol (1:1, vol), allowed 10 minutes for the colour development, then transferred on ice and the optical density at 390 nm read within 30 minutes. Blanks where the acid molybdate-Lubrol mixture was added before the enzyme

solution were prepared for each sample. The activity of the ouabain-sensitive (Na^+-K^+)ATPase was calculated from the difference in O.D. between the tubes without and with ouabain, and expressed as nmoles P_i/minute/mg protein. The optical densities were converted in nmoles inorganic phosphate (P_i)/ml reaction medium using a calibration curve prepared with dilutions of KH_2PO_4 (from 0 to 600 nmoles P_i/ml).

Assay for Alkaline Phosphodiesterase I: The reaction medium used was 1.875 mM p-nitrophenyl-thymidine-5'-phosphate, 0.125M $MgCl_2$ and 2.5 mM Zn acetate in a 0.125 M glycine-NaOH buffer, pH 9.6. A volume of 0.4 ml reaction medium was mixed with 0.1 ml of pretreated membrane suspension or homogenate (0.05 ml membrane suspension or homogenate incubated 10 minutes with 0.05 ml of 0.2% (w/v) Triton X-100 at 25°C). The assay was run for 10-20 minutes at 25°C, then the reaction was stopped with 1 ml of 0.1M NaOH and the tubes were placed on ice, then centrifuged at 900 x g for 15 minutes, at 4°C and the supernatants read at 400 nm against a blank of distilled water. The enzymatic activities were calculated on the basis of the molar extinction coefficient of p-nitrophenol, which is 18,300 at 400 nm (Beaufay *et al.*, 1974).

Assay for (Ca^{2+}-Mg^{2+})ATPase: The solutions used were: a) reaction medium without calcium (6 mM $MgCl_2$, 1 mM EGTA, 200 mM KCl, 1.5 mM sucrose, 50 mM imidazole, pH 7.1 with HCl); b) reaction medium with calcium (1 mM $CaCl_2$ in solution a), pH 7.1); c) 12 mM Tris-ATP prepared as for the (Na^+-K^+)ATPase assay; d) acid molybdate/ Lubrol (1:1, vol.) as for (Na^+-K^+)ATPase assay. A volume of 1 ml reaction medium, with or without calcium, and 0.5 ml Tris-ATP were incubated at 25°C, then the reaction was started with 0.5 ml of membrane suspension or homogenate. After 10-30 minutes the reaction was stopped with 4 ml acid molybdate/Lubrol and the P_i produced by the Ca^{2+}-activated ATPase was calculated as decsribed in the assay for the (Na^+-K^+)ATPase.

Assay for Succinate Dehydrogenase: The solutions used were a) 20 mM sodium phosphate buffer, pH 7.0 with 1% bovine serum albumin; b) 1% cytochrome c solution; c) 10 mM KCN; d) 10 mM KCN and 50 mM succinate. For the assay 1.2 ml

phosphate buffer, 0.1 ml cytochrome c and 0.1 ml membrane suspension or homogenate were added to each of two spectrophotometre cuvettes and the cuvettes were placed in the spectrophotometre, one as sample, one as reference, and equilibrated at 25°C. The reading was adjusted to zero, at 550 nm, and 0.4 ml of 10 mM KCN were added to the reference cuvette and 0.4 ml of 10 mM KCN with 50 mM succinate to the sample cuvette and mixed well. The increase in absorbance at 550 nm was recorded on the chart paper. The calculation of the enzymatic activity was based on the molar extinction coefficient of cytochrome c, which is 29,705 at 550 nm (Tsai, Chen and Canellakis, 1975).

Assay for Lactate Dehydrogenase: The solutions used were: a) 0.1 M potassium phosphate buffer, pH 7.5; b) 13 mM NADH in solution a), pH 7.5; c) 0.1 M pyruvate in solution a), pH 7.5, prepared at least 24 hours before use. To a quartz cuvette 2.9 ml of potassium phosphate buffer, 0.03 ml NADH and 0.02 ml pyruvate were added and equilibrated at 25°C, then the reaction was started with 50 µl membrane suspension or homogenate. The change in absorbance at 340 nm was recorded on chart paper. The calculation of the enzymatic activity was based on the molar extinction coefficient of NADH, which is 6220 at 340 nm (Bernstein and Everse, 1975).

Assay for Thyamine Pyrophosphatase: A volume of 0.1 ml membrane suspension or homogenate was mixed with 2.9 ml substrate solution (33 mM sodium barbital, 15 mM $CaCl_2$, 3.3 mM thyamine-pyrophosphate, pH 8.0) and incubated 20-30 minutes at 25°C. The reaction was stopped with 1 ml of 10% trichloroacetic acid, the tubes were centrifuged at 900 x g and 2 ml of the supernatant were used for the determination of inorganic phosphate, as described for the ATPase enzymes.

2.3. Results

The method we have developed combined the sodium iodide extraction (Nakao *et al.*, 1965) which facilitates the separation of the plasma membranes from

the contractile proteins and reduces non-specific ATPase activity, with the centrifugation on Percoll self-creating gradient (Manning *et al.*, 1989, Loten and Redshaw-Loten, 1986) which gives an efficient and rapid separation of the plasma membrane fragments from other intracellular components.

Table 2.1. shows the results obtained for the two species of marine crabs studied. The values shown are means ± standard deviations of 4 individual experiments for each species. For each individual experiment, the protein and the enzymatic assays were run in triplicate. The assays on the crude homogenate were often troublesome because of non-homogeneity and viscosity, as reported by others (review in Hansen and Clausen, 1988) and where necessary the diluted samples were re-homogenised and the assays repeated immediately.

The plasma membrane preparations from *Cancer pagurus* showed a 14.3±1.7 - fold enrichment, with 17.9±2.8% recovery of the total activity in the homogenate when (Na^+-K^+)ATPase activity was determined and a 12.6±1.5 - fold enrichment, with 16.1±3.5% recovery when Alkaline Phosphodiesterase I activity was determined. Close values were calculated for plasma membrane preparations from the other species of crab studied, *Carcinus maenas*, namely 13.0±1.2 - fold enrichment, with 18.6± 2.3% recovery and 12.5±2.3 - fold enrichment, with 17.7±2.7% recovery, respectively (Table 2.1.). Contamination with other types of intracellular membranes was low or undetectable.

2.4. Discussion

Few comparable studies have been carried out on crustacean muscle. Of the recent studies that have been made, some prepared plasma membrane fractions for different purposes (Gray *et al.*, 1991, Krizanova, Novotova and Zachar, 1990, Syvertsen and Fonnum, 1989), whilst others used different methods to characterize the membranes obtained (Altamirano, Hamilton and Russell, 1988); these differences in approach and technique made direct comparison with the present study difficult. The

		Cancer pagurus			Carcinus maenas		
		Yield (mg/g fw)	% Recovery*		Yield (mg/g fw)	% Recovery*	
Protein	H	185.5±23.9	1.3±0.2		200.2±22.8	1.4±0.1	
	M	2.3±0.4			2.8±0.3		

Enzyme studied		Specific ** activity	Fold-purification	Yield (% tot.act.)	Specific ** activity	Fold-purification	Yield (% tot.act.)
(Na^+-K^+) ATPase	H	$3.1±0.9^a$	14.3±1.7	17.9±2.8	$2.5±0.3^a$	13.0±1.2	18.6±2.3
	M	42.6±9.3			32.9±2.2		
Alkaline Phosphodiesterase I	H	$0.447±0.109^b$	12.6±1.5	16.1±3.5	$0.108±0.023^b$	12.5±2.3	17.7±2.7
	M	5.670±1.71			1.318±0.197		
$(Ca^{2+}-Mg^{2+})$ ATPase	H	$76.8±22.7^c$	-	-	$59.8±10.3^c$	-	-
	M	n.d.			n.d.		
Succinate Dehydrogenase	H	$2.9±0.6^d$	-	-	$2.6±0.4^d$	-	-
	M	n.d.			n.d.		
Lactate Dehydrogenase	H	$268.3±18.9^e$	-	-	$257.4±71.3^e$	-	-
	M	n.d.			n.d.		
Thyamine pyrophosphatase	H	$14.8±4.3^f$	1.9±0.3	2.4±0.5	$14.1±2.1^f$	1.8±0.3	2.6±0.5
	M	28.8±11.8			26.3±7.7		

H = crude homogenate; M = plasma membrane preparation; * = percentage of total protein (mg) in crude homogenate recovered in membrane fraction; ** = letters a-f define the unit for the specific activity for each enzyme; a = nmoles P_i/min/mg protein; b = nmoles p-nitrophenol/min/mg protein; c = nmoles P_i/min/mg protein; d = nmoles cyt c/min/mg protein; e = nmoles NAD/min/mg protein; f = nmoles P_i/min/mg protein; n.d. = not detected; - = not possible to calculate.

plasma membrane preparations from barnacle muscle (Altamirano, Hamilton and Russell, 1988) were 10-fold enriched as determined by saturable ouabain binding, but the method involved an overnight centrifugation on continuous sucrose gradient and the yield, in terms of mg membrane protein/g original tissue, was low. The method used by Sevillano, Calvo and Cabezas (1980) was based on a complex method developed by Boegman, Manery and Pinteric (1970) which was found at that time to give the best results for the isolation of plasma membranes from skeletal muscle from various sources (Cabezas et al., 1982).

The purpose of the present study was to develop a rapid method, with high yields, as this would be essential for the further use of these plasma membrane preparations. The use of Percoll for the separation of plasma membranes from other intracellular membranes provided an improved method for the production of plasma membrane preparations with high yield and purity as it avoided the complex preparation of the sucrose gradients and the long centrifugations required for a good separation of membranes. Data are available from a variety of cell and tissue sources and the values reported ranged from 6 to 30-fold purification and 7 to 30% recovery of the total activities in the crude homogenate, depending on the types of cells or tissues used and on the marker enzymes assayed (Inui et al., 1981, Loten and Redshaw-Loten, 1986, Payastre et al., 1988, Manning et al., 1989, Horne, Reed and Said, 1992).

The level of purification and the yields obtained with the method described here were comparable with those reported for methods based on Percoll and the degree of contamination of the plasma membrane preparations with sarcoplasmic reticulum, mitochondria or cytosol was below the limit of detection of activity for the specific marker-enzymes assayed. The Golgi marker used (TPPase) revealed a 1.8-1.9-fold enrichment for both species studied (Table 2.1) which could indicate a minor contamination with Golgi membranes or could be due to non-specific distribution of the TPPase (Morré et al., 1979). Similar (Paul, Hurtubise and LeBel, 1992) or higher (Persson and Jergil, 1992) levels of Golgi-enrichment were found in plasma membrane

preparations with higher fold-enrichments in the plasma membrane markers, following purification by affinity partitioning with wheat germ agglutinin as the specific ligand.

During preliminary studies on crude homogenate and plasma membrane preparations from crab muscle other enzymes were investigated as potential markers, but no significant activity was detected for 5'-nucleotidase (E.C.3.1.3.5., Atkinson, Gatenby and Lowe, 1973) as a plasma membrane marker, or for monoamine oxidase (E.C.1.4.3.4., McEwen, 1971) as an outer mitochondrial membrane marker. N-acetyl β-glucosaminidase (E.C.3.2.1.30., Sellinger *et al.*, 1960) was considered as a lysosomal marker, however no activity was detected in the plasma membrane preparations and the activities calculated for the crude homogenate were low and inconsistent (data not shown).

An investigation of the effect of SDS treatment on the (Na^+-K^+)ATPase activity in the crude homogenate and in the plasma membrane preparation obtained with the method described here revealed the existence of some latent activity (approximately 20%) in our preparations, probably due to the resealing of the isolated plasma membrane fragments. This would suggest that the actual yields obtained were better than those calculated. However, routine use of SDS or other detergents is not appropriate when the membrane fraction is to be used subsequently for lipid analysis.

We considered that the method described here, based on NaI extraction and centrifugation on Percoll self-creating gradient, had the advantages of being rapid, minimising the risk of degradation of the membrane lipids, which were the subject of further studies on these preparations and it gave high yields and good levels of purification of the plasma membrane fraction, with low or not detectable contamination with other intracellular membranes. It did not involve the use of very expensive chemicals, and could be used as a routine procedure, and also the NaI extraction avoided complex and time-consuming procedures required for muscular tissue in order to separate the fibrillar proteins from the plasma membrane fragments.

Chapter 3: A Fluorescence Polarization Study of the Fluidity of Plasma Membranes of Cold and Warm-Acclimated Crabs

3.1. Introduction

Membrane fluidity is the general term used to describe the biophysical state of biological membranes, resulted from the complex dynamic interactions between membrane lipid and protein molecules. The dynamic, or 'liquid-like' character results from molecular motions ranging from a rapid flexing of the fatty acyl hydrocarbon chain by rotations about carbon-carbon bonds to rotational diffusion and translational (lateral) diffusion of membrane lipids and proteins in the plane of the membrane and along an axis vertical to the plane of the membrane (Shinitzky, 1984).

The phase state, molecular order and rates of molecular motion of the lipids, hence fluidity, in biological membranes are all significantly perturbed by changes in environmental temperature (Cossins and Raynard, 1987). This perturbation will also have functional consequences, as most membrane-dependent biochemical processes are chemical or physico-chemical reactions which are influenced by the immediate microenvironment, and changes in temperature would affect them directly via thermodynamic effects and indirectly via changes in the fluidity of the lipid bilayer (Pringle and Chapman, 1981). The idea that membrane fluidity is under physiological control and organisms develop compensatory alterations in their membranes to offset the direct effects of environmental temperature on membrane physical state and to maintain their vital functions was based on the accumulated experimental evidence that organisms modulate their lipid composition following changes in growth or acclimation temperatures (Shinitzky, 1984). The concept of homeoviscous adaptation to temperature, formulated by Sinensky (1974), implies that the biophysical state of biomembranes can be properly quantified and that the effects of the changes in lipid composition and membrane physical condition upon the function of membrane proteins and of membranes as a whole are well-understood (Cossins, 1994).

Different techniques are available for the measurement of the physical state of biomembranes, such as ESR, NMR, fluorescence spectroscopy techniques and calorimetric techniques. Calorimetric techniques are used to determine the phase state of membrane lipids and temperatures of phase-transition (Hazel, 1989). NMR provides detailed information on the motion of specific segments of hydrocarbon chains, while ESR and fluorescence spectroscopy provide various quantitative parameters of membrane fluidity describing the motion of molecular probes in the plane of the membrane. Examples of such parameters are the rotational correlation time and the order parameter derived from spin probes or fluorescent probes, and the steady-state anisotropy, or polarization, of fluorescent probes (Gennis, 1989). The absolute interpretation of spectral data is based on complex models and is controversial (Van der Meer, 1984). Each of the various spectroscopic and calorimetric techniques is sensitive to rather different combinations of the complex molecular motion of membrane lipids and in some cases membrane properties defined by one technique do not agree either quantitatively or qualitatively with the properties defined by another (Cossins, 1994). Most techniques do not distinguish between the effects of the range of motion (order) and the rate of motion (viscosity) on the spectral parameters of the probes used (Hazel, 1989). A widely used technique is the steady-state fluorescence polarization, which requires a relatively cheap and easy to operate instrumentation and small amounts of membrane samples, although there is no consensus on the orientation and nature of the motions of the most used probe, DPH, in the bilayer (Van der Meer, 1984) and the measurement is considered to be biased toward membrane order (Cossins, 1983). Only time-resolved fluorescence polarization measurements were able to provide separate estimates of membrane order and rates of probe motion (Cossins and Lee, 1985, Behan-Martin *et al.*, 1993).

Despite the drawbacks of the techniques available for estimating the membrane fluidity and order, they have made possible comparisons between the properties of membranes from organisms that have undergone different thermal treatments, or that are evolutionarily adapted to different temperatures, providing

information that is more significant for environmental physiology than an absolute value of membrane fluidity or order (Cossins, 1994).

Numerous studies, using DPH steady-state fluorescence polarization or E.S.R., have clearly demonstrated that homeoviscous adaptation occurs in various species, i.e. membranes from cold-acclimated organisms are more disordered than corresponding membranes from warm-acclimated ones (Cossins, 1983, Cossins, 1994). The extent of fluidity compensation, calculated as homeoviscous efficacy (%HE) (Cossins, 1983) or homeoviscous response (%HR) (Wodtke and Cossins, 1991) varies widely with the species investigated and the type of tissue or organelle (Hazel, 1989) and with the range of acclimation temperatures (Cossins, Kent and Prosser, 1980). Metabolically active membranes, characterized by low cholesterol/phospholipid ratios, of which a typical example are the mitochondrial membranes, exhibit the greatest fluidity compensation, with inert membranes, such as myelin, at the other extreme (Cossins and Prosser, 1982). Sarcoplasmic reticulum membranes are an exception to this trend as no differences in their fluidity were found between cold and warm-acclimated animals (Cossins, Christiansen and Prosser, 1978). In other cases, differences in the homeoviscous response were found between whole cells (no response) and plasma membranes isolated from those cells (45-50% compensation), suggesting that homeoviscous adaptation is developed only in specialized parts of the cell membrane, which are preferentially enriched in the plasma membrane preparation (Williams and Hazel, 1994b). Lee and Cossins (1990) found a differential homeoviscous response in brush-border (none) and basolateral (%HE=75%) membranes of carp intestinal mucosa, supporting the theory that the biochemical composition and physico-chemical structure of the different cellular membrane systems are under a sophisticated cellular control, and only necessary adaptive responses will be elicited.

Evolutionary homeoviscous adaptation has also been demonstrated (Cossins and Prosser, 1978, Behan-Martin *et al.*, 1993). Behan-Martin *et al.* (1993) compared brain membranes of fish from diverse thermal environments (Antarctic, temperate and

tropical), rat and pigeon and showed that the fluidity of the membranes varied inversely with the body temperature, but the values of the fluorescence anisotropy measured at their respective body temperatures were similar. A more detailed analysis of synaptic membranes from Antarctic *Notothenia*, trout and rat using time-resolved analysis of DPH and *trans*-parinaric acid fluorescence anisotropy decay (Behan-Martin *et al.*, 1993) indicated that the interspecific differences observed under steady illumination were due to different degrees of ordering of the probe's microenvironment rather than to different rates of probe-wobbling motion (fluidity). This suggests that the static ordering properties of membranes are under adaptive regulation, rather than motional rates, and the time-resolved methods of analysis, although technically much more difficult, would give more accurate measures of responsiveness (Cossins, 1994).

The lipid bilayers have a complex anisotropic structure. Phospholipids, their main component, are polymorphic, assuming a variety of different phases depending upon the temperature, pressure, degree of hydration and the chemical structure of the phospholipid (Chapman, 1975, Mattai, Sripada and Shipley, 1987). The phases that are relevant for the organization of biological membranes are the lamellar gel phase, the fluid lamellar or liquid-crystalline phase and an inverted hexagonal phase (Hazel, 1989). Transitions between these phases are driven by temperature changes, and the particular phase or combination of phases coexistent at a given temperature will depend on the chemical composition of the lipid. Evidence from X-ray diffraction measurements on *Tetrahymena* membranes indicate that microsomal membranes have both fluid and ordered states of lipids and that the proportion changes with temperature, the percentage in the fluid state being greater at high temperatures (Connoly *et al.*, 1985a, 1985c). Membranes of higher organisms appear to exist predominantly in the liquid-crystalline state at physiological temperatures (Quinn, 1981) but the existence of a certain structural heterogeneity has been demonstrated in the plane of some membrane types, due to fluid-phase immiscibility of different types of lipids (Wu and McConnell, 1975) or to the presence of a 'boundary' layer of lipids

around protein molecules (Bennet, McGill and Warren, 1980). Because of their complex composition (phospholipids, cholesterol, proteins), the phase transitions from gel to liquid-crystalline state occur over broad ranges of temperature (10-30°C) in biological membranes, compared to the narrow and well-defined range of phase-transition temperature in pure phospholipid bilayers (2-5°C, Melchior and Steim, 1976). At temperatures within the phase transition, microdomains with different degrees of order will coexist in the plane of membrane (Shimshick and McConnell, 1973, Karnovsky *et al.*, 1982) which means that gradual cooling of membranes would increase the lateral asymmetry. From a practical point of view, these aspects complicate the interpretation of the membrane fluidity measurements, because the more common probe techniques, such as DPH fluorescence polarization or nitroxy-labelled fatty acids ESR provide information on average fluidity or order, and are not able to differentiate between these different microdomains (Cossins and Raynard, 1987). The distribution of the probes within these microdomains with different physical properties may be restricted only to some of them, or may change with the measurement temperature, which means that the exact source of change in average membrane fluidity will be difficult to establish.

Seelig and Seelig (1980) have observed (NMR) distinct structural zones parallel to the membrane surface, that are created by the increasing mobility of the fatty acyl chains with depth into the bilayer and by the presence of unsaturated carbon-carbon bonds. Thus, a gradient of fluidity exists across the membrane bilayer, with the middle part being more fluid and the regions closer to the headgroup area more ordered.

There is also evidence for a compositional asymmetry between the two hemilayers of the membrane bilayer (Rothman and Lenard, 1977) and recent papers confirm that this is correlated with a temperature-dependent difference in fluidity between the two hemilayers, which results from different temperature sensitivities of the fluidity of each hemilayer (Williams and Hazel, 1994b). They found the inner

hemilayer of trout hepatocytes to be more fluid and its fluidity less temperature-sensitive than that of the outer hemilayer.

It is still uncertain if the adaptive significance of the observed changes in lipid composition and membrane fluidity, i.e. homeoviscous adaptation is to preserve the membrane phase structure or to fine-tune membrane fluidity (Cossins and Raynard, 1987). The uncertainty results from the fact that the role of the lipid bilayer in controlling membrane function has not been unambiguously established and the need for lipid diversity in eukaryote membranes remains unresolved (Cossins, 1994). Homeoviscous adaptation was correlated with the maintenance of the liquid-crystalline state, and with changes in permeability properties, membrane-transport processes, activity of membrane-bound enzymes, enzyme thermostability, parameters of neural function, and whole animal sublethal and lethal temperatures (Cossins and Bowler, 1987), based on various types of experiments, including delipidation and reconstitution of membrane-bound proteins with different lipid mixtures, use of other fluidizing agents (pressure, local anaesthetics, alcohol, other chemicals) and of dietary modulations (Cossins and Sinensky, 1984, Hazel, 1989, Bowler and Manning, 1994, Cossins, 1994). The mechanisms and the time-course of the processes involved have been recently reviewed and discussed by Williams and Hazel (1994a) and Macartney, Maresca and Cossins (1994).

Most studies discussing the adaptive role of homeoviscous adaptation refer to the effects of cold-adaptation. Although cold-induced phase transitions and separations have been widely reported among membranes of homeotherms, phase transitions based on unambiguous physical evidence have not been widely reported in multicellular ectotherms (Hazel, 1989). This is related to the lower temperatures to which these organisms are adapted and the presence of lipids with lower melting points than in the membranes of homeotherms. At the warm extreme, a shift of 6-7°C in the polarization vs temperature curves of goldfish synaptic membranes for a change in acclimation temperature of 20°C, was associated with an increase of 6-7°C in the heat coma temperatures of the warm-acclimated goldfish, compared to the cold-

acclimated ones (Cossins, Friedlander and Prosser, 1977). This suggests that for temperate marine ectotherms the homeoviscous adaptation is more important in coping with high temperatures, than with cold ones (Logue, Tiku and Cossins, 1995), which may be of particular importance under the current threat of predicted global warming. Bowler (1987) has reviewed the evidence that in ectotherms the thermostability of membrane-bound enzymes increases with acclimation temperature.

Invertebrates have been little studied with respect to homeoviscous adaptation, compared to the extensive work on fish (Pruitt, 1990). The purpose of the work presented in this chapter was to investigate the effects of temperature acclimation on the muscle plasma membrane fluidity of two species of marine crab, a stenothermal (*Cancer pagurus*) and a eurythermal one (*Carcinus maenas*). The questions posed were if the two species are able to adjust their membrane fluidity when acclimated to warm temperatures, if the eurythermal species develops a better response than the stenothermal one, and if the seasonal acclimatization has any influence upon the response to warm acclimation. Correlations with changes in lipid composition, neuromuscular function and whole organism thermal sensitivity will be discussed in Chapter 7 (General Discussion).

3.2. Materials and Methods

3.2.1. Animals

Crabs (adult, intermolt) caught in spring and autumn 1993 were acclimated for three weeks at 5°C, 8°C, 15°C and 22°C, respectively. The 3 weeks of acclimation at 22°C were preceded by a transition period of a week at 15°C. Crabs caught in summer 1992 were acclimated only to 5°C and 15°C. The animals were kept in filtered and aerated sea water, in 10 litres transparent plastic tanks. They were fed on fish (commercial source) just before transfer to the acclimation temperatures and after the first week (also after the second week, for the 22°C acclimated crabs), then

starved for the last two weeks prior to the plasma membrane preparation. The photoperiod was 12 hours light - 12 hours dark.

3.2.2. Plasma Membranes

Plasma membranes of crab leg muscle fibres were prepared as described by Cuculescu and Bowler (1993), then resuspended in a small volume of low ionic strength buffer (10 mM imidazole, 1 mM EDTA, pH 7.2 /HCl, at 20°C) and stored at -80°C until needed for the membrane fluidity measurements (see also section 2.2).

3.2.3. Fluorescent Probes

Two fluorescent probes were used: 1,6-diphenyl 1,3,5 hexatriene (DPH) (Aldrich Chemical Co.) and 1-[4-(trimethylammonium) phenyl]-6-phenylhexa-1,3,5 triene (TMA-DPH) (Molecular Probes Inc., Cambridge Bioscience). The first probe, DPH, has been extensively used to study the fluidity of liposomes and biological membranes. The DPH molecule is hydrophobic and partitions preferentially into the hydrocarbon region in lipid bilayers. Its rotational characteristics are dependent upon the molecular motion of the neighbouring lipid molecules and, therefore, provide an indirect index of the molecular motion of the lipids and a convenient technique for following changes in the fluidity of the membrane interior. The model used and the calculation of polarization of DPH fluorescence are described by Shinitzky and Barenholtz (1974). A decreased value of polarization indicates an increased rate of rotation of the probe and therefore a less viscous membrane environment. The second probe, TMA-DPH, was synthesized by Prendergast, Haugland and Callahan (1981) as a derivative of DPH with a cationic moiety affixed to the para position of one of the phenyl rings, which would be rapidly anchored to the lipid-water interface and yet intercalated into the lipid milieu. It has been used as a specific plasma membrane fluidity probe, for the flow cytometry studies. While DPH is rapidly internalized by whole cells and labels the intracellular membranes as well as the plasma membrane, TMA-DPH remains confined to the plasma membrane for at least 30 minutes

(Dynlacht and Fox, 1992). The patterns of motion and the region of the bilayer reported on by TMA-DPH are quite different from those of DPH, while the photophysical properties remain similar (Prendergast, Haugland and Callahan, 1981). TMA-DPH should be probing essentially the glycerol backbone region and the fatty acyl chain regions probably as far down as C_8-C_{10} possibly only in the outer hemilayer of the membrane (Prendergast, Haugland and Callahan, 1981). Engel and Prendergast (1981) discussed the model used for the interperetation of the motion parameters of DPH and TMA-DPH in terms of membrane structure and mobility and the limitations imposed on the angular displacements of TMA-DPH compared to DPH.

3.2.4. Experimental Set-Up

The polarization of DPH and TMA-DPH fluorescence under steady illumination was measured using an analogue T-format fluorimeter (Applied Photophysics Ltd.). The fluorescent probes were excited at 357 nm with an intense beam of vertically polarized light and the emitted light was simultaneously detected by two photomultipliers, each with two cross-polarized channels mounted at right angles to the incident light. All the signals detected by the photomultipliers were sent to a BBC microcomputer (Acorn Computers Ltd., Cambridge) connected to an Acorn 12-bit analogue-to-digital converter, which averaged the values at intervals of 0.7 min, calculated the polarization ratio and produced an output file with all the measurements.

The temperature of the sample cuvette contents was controlled by circulating water from a temperature-programmable Julabo (Seelbach, Germany) water bath, provided with refrigerating and heating units, controlled by the same computer that integrated the measurements. A linear thermistor inserted in the cuvette monitored the temperature with an accuracy of $\pm 0.1°C$ and transmitted the information back to the computer. The sample cuvette contents were continuously stirred with a mini-magnetic stirrer driven by an electromagnet positioned beneath the cuvette. The light

scattering artifact was estimated for a few preparations using an identical sample, but without added fluorescent probe (Cossins, 1977). The values found were very low (<0.5%) and corrections were not made.

The steady-state polarization of the fluorescent light was defined as

$$p = \frac{I_{II} - I_{\perp}}{I_{II} + I_{\perp}} \quad ,$$

where I_{II} and I_{I} are the intensities of fluorescent light detected through polarizers oriented parallel and perpendicular, respectively, to the direction of polarization of the excitation beam. In practice, the ratio $I = I_{II}/I_{\perp}$ was determined in each of the two photomultipliers, a vertical one ($I_{v} = I_{II}v/I_{\perp}v$) and a horizontal one ($I_{h} = I_{II}h/I_{\perp}h$), and the ratio of I_{v} and I_{h} was used by the computer to calculate the value of polarization using the equation:

$$p = \frac{(I_{v} / I_{h}) - 1}{(I_{v} / I_{h}) + 1}$$

3.2.5. Experimental Procedure

Thawed plasma membrane suspensions were sonicated (2x10 seconds), on ice, then diluted with 10 mM sodium phosphate buffer, pH 7.6 to an optical density of 0.1±0.01 at 500 nm. Approximately 2.4 ml of diluted plasma membrane suspension were thoroughly mixed with 2 µl of the fluorescent probe solution (2 mM DPH in tetrahydrofuran or 2 mM TMA-DPH in dimethylformamide), and incubated for 10 minutes (DPH) or 5 minutes (TMA-DPH) at room temperature to allow the probe to equilibrate between the membrane and the aqueous medium. A sample cuvette was then placed in the fluorescence spectrometer, cooled down to 5°C and the steady-state fluorescence polarization was measured over a temperature range from 5 to 35°C, with a rate of increase of 1°C / minute.

3.2.6. Homeoviscous Efficacy

The efficacy of the acclimation process was estimated using the method described by Wodtke and Cossins (1991) for log(pol) vs temperature plots that do not have similar slopes, therefore the distance between the curves for cold and warm acclimated individuals along the temperature axis varies with temperature, and the formula given by Cossins (1983) for homeoviscous efficacy can not be used. Wodtke and Cossins (1991) defined the homeoviscous response (%HR) as:

$$\%HR = 100 \cdot \Delta(\log P) \cdot (\Delta T)^{-1} \cdot [-\Delta(\log P)/C°]^{-1}$$

where $\Delta(\log P)$ is the difference in log polarization measured at 22°C for the 22°C- and 8°C-acclimated membranes, ΔT is the difference in acclimation temperature in °C, and $\Delta(\log P)/C°$ is the slope of the linear regression of logP against temperature for the 22°C-acclimated membranes. The natural logarithm (lnP) was calculated.

A modification of this formula was also used to calculate the %HR from the polarization vs. temperature curves. If the slope value was replaced with $\Delta'(\log P)/\Delta T$, where $\Delta'(\log P)$ is the difference in log polarization at 22°C and at 8°C for the 22°C-acclimated membranes, and ΔT has the same significance, then the formula becomes:

$$\%HR = -100 \cdot \Delta(\log P)/\Delta'(\log P)$$

and values of polarization can be used instead of logP. The graphic interpretation of the formula for %HR is detailed in a review by Cossins (1994).

Wodtke and Cossins (1991) used the value of %HR referring specifically to the adaptive change in membrane order which occurs during cold-acclimation of warm-acclimated fish. In our case, we used the equivalent formula for warm-acclimation, as the average sea-water temperature at the time of the catch was below 22°C (8-10°C in spring, 10-15°C in summer and 5-8°C in autumn) and the crabs were usually maintained at 8°C for a few days before the set-up of the acclimation experiments.

3.2.7. Statistical Analysis

Two approaches were used for the statistical analysis of the fluorescence polarization data. The first approach treated the values of polarization measured at a given experimental temperature for each combination of acclimation temperature, species, season, and fluorescent probe, as independent groups (from those measured at other temperatures) and the oneway analysis of variance, combined with the 'least significant difference' range test was carried out to test the influence of acclimation temperature, species or season, and repeated for each of 5°C, 8°C, 10°C, 15°C, 20°C, 22°C, 25°C, 30°C and 35°C temperatures. The second approach considered each set of data defining one polarization vs temperature curve separately. The regression analysis of ln polarization against experimental temperature yielded the equation for each individual curve. The values of the intercepts and slopes were then grouped for each combination of acclimation temperature, species, season and fluorescent probe and the oneway analysis of variance combined with the 'least significant difference' range test was carried out to test for the influence of each variable. The level of significance was $p < 0.05$.

3.3. Results

The steady state fluorescence polarization of DPH and TMA-DPH incorporated into plasma membrane preparations from leg muscle of crabs acclimated to different temperatures was measured over the temperature range 5 to 35°C.

The experiment was repeated in three different seasons: summer 1992 (July), spring (April) 1993 and autumn 1993 (end of September).

Figures 3.1, 3.2 and 3.3 show the polarization versus temperature curves for DPH and TMA-DPH in membranes of 5°, 8°, 15° and 22°C acclimated *Cancer pagurus* caught in spring, summer (only 5° and 15°C acclimation groups) and autumn, respectively. Figures 3.4, 3.5 and 3.6 show the curves for DPH and TMA-DPH in

Figure 3.1. The temperature dependence of fluorescence polarization of **DPH** and **TMA-DPH** probes in plasma membrane preparations of *Cancer pagurus* acclimated in **spring**. The curves are averages of n plots for individual preparations $\pm$ S.D. (5°C AT n=4, 8°C AT n=4, 15°C AT n=4, 22°C AT n=6, **AT** = acclimation temperature)
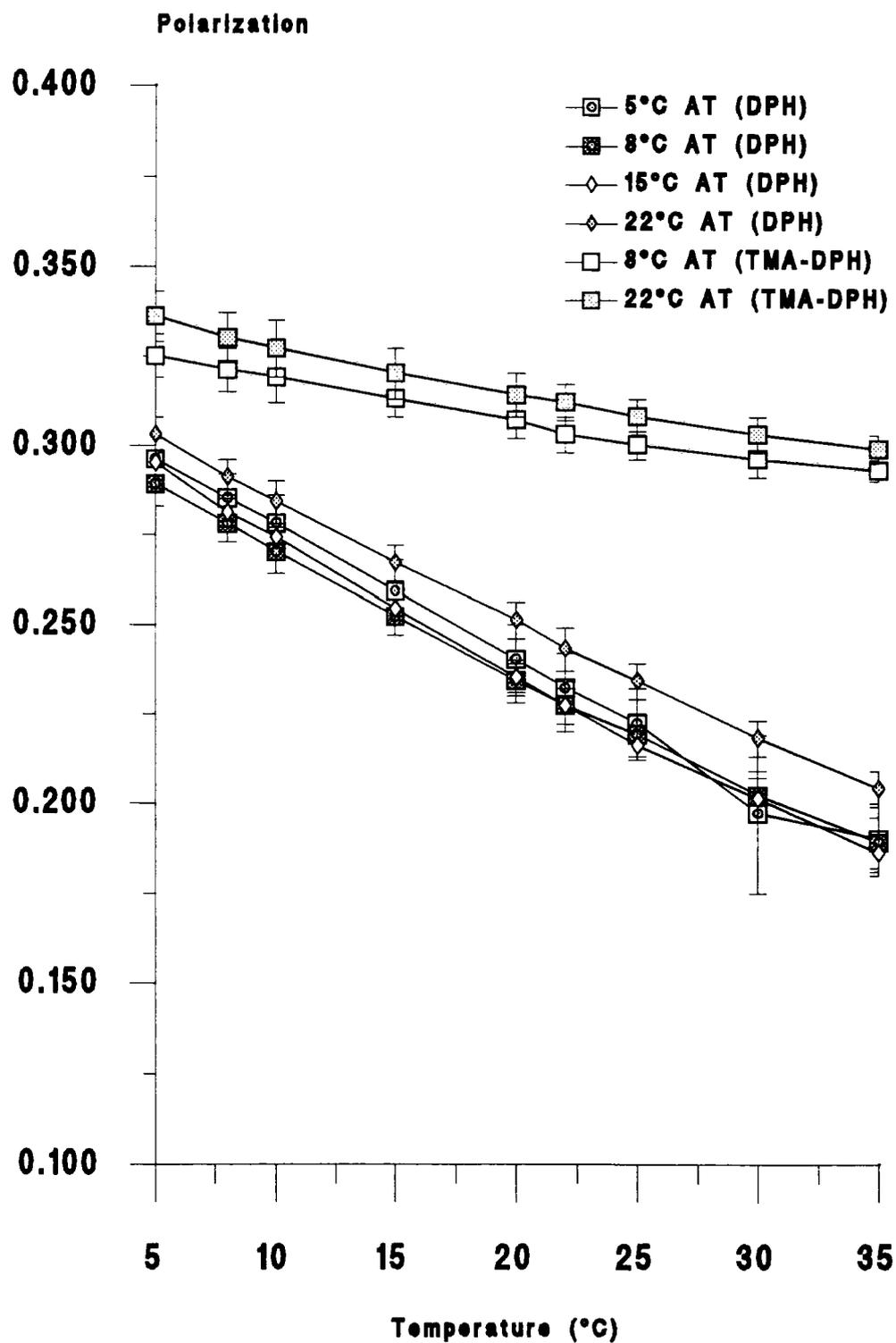
Figure 3.2. The temperature dependence of fluorescence polarization of **DPH** probe in plasma membrane preparations of *Cancer pagurus* acclimated in **summer**. The curves are averages of n plots for individual preparations ± S.D. (5°C AT $n=4$, 15°C AT $n=4$, AT = acclimation temperature)
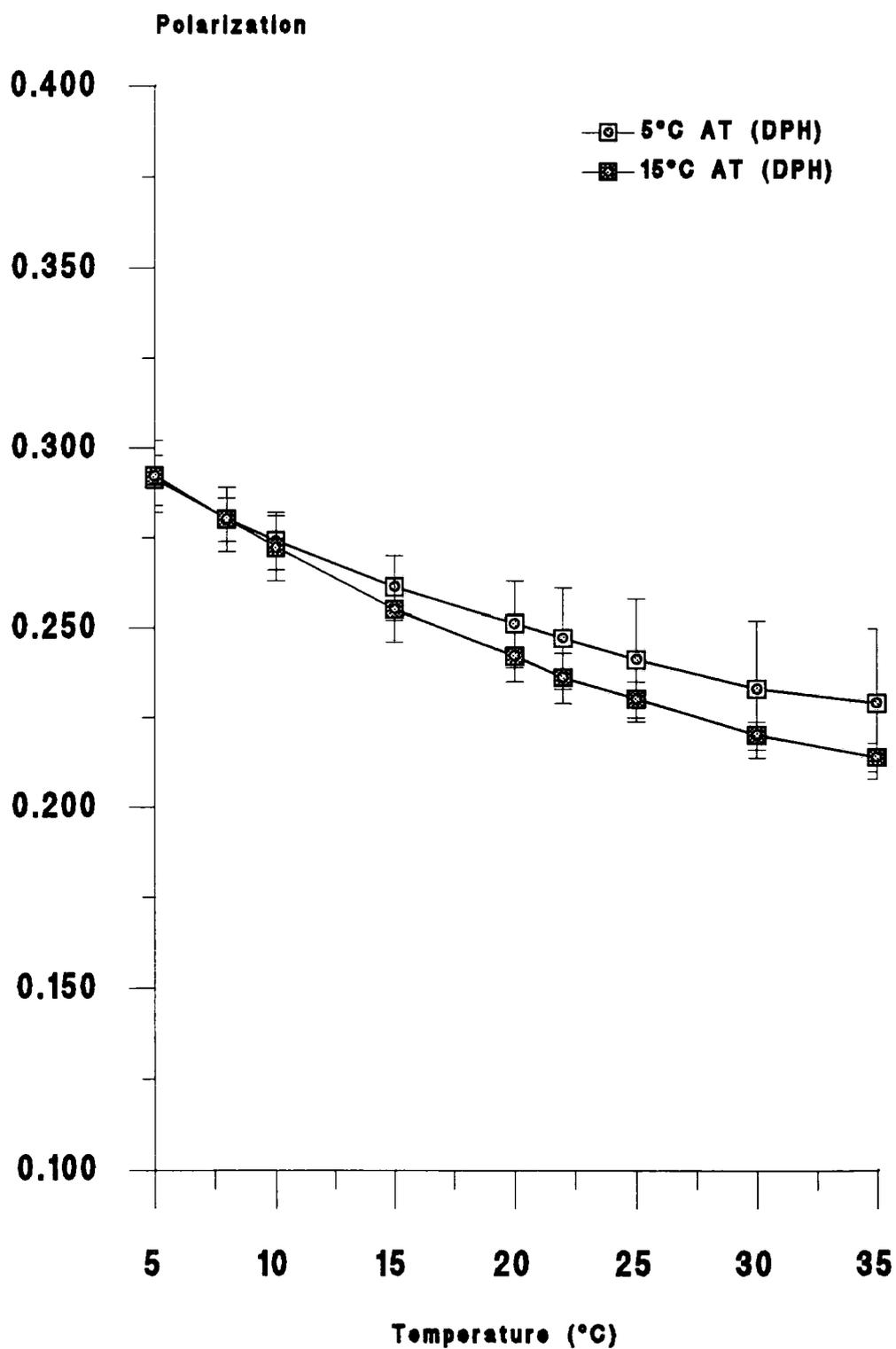
Figure 3.3. The temperature dependence of fluorescence polarization of **DPH** and **TMA-DPH** probes in plasma membrane preparations of *Cancer pagurus* acclimated in **autumn**. The curves are averages of n plots for individual preparations ± S.D. (5°C AT $n=2$, 8°C AT $n=4$, 15°C AT $n=2$, 22°C AT $n=4$, AT = acclimation temperature)
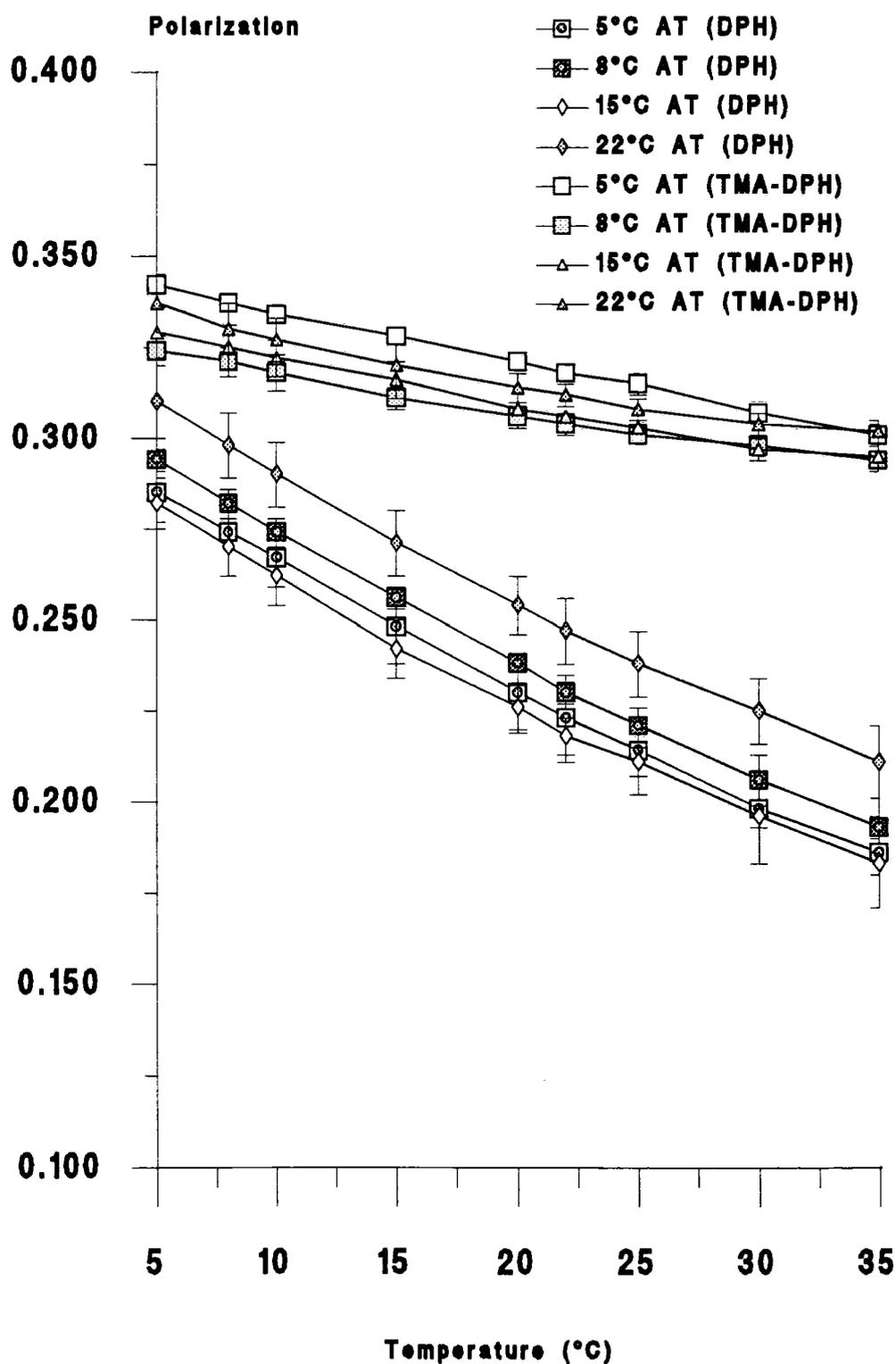
Figure 3.4. The temperature dependence of fluorescence polarization of **DPH** and **TMA-DPH** probes in plasma membrane preparations of *Carcinus maenas* acclimated in **spring**. The curves are averages of n plots for individual preparations ± S.D. (5°C AT n=4, 8°C AT n=4, 15°C AT n=4, 22°C AT n=2, AT = acclimation temperature)
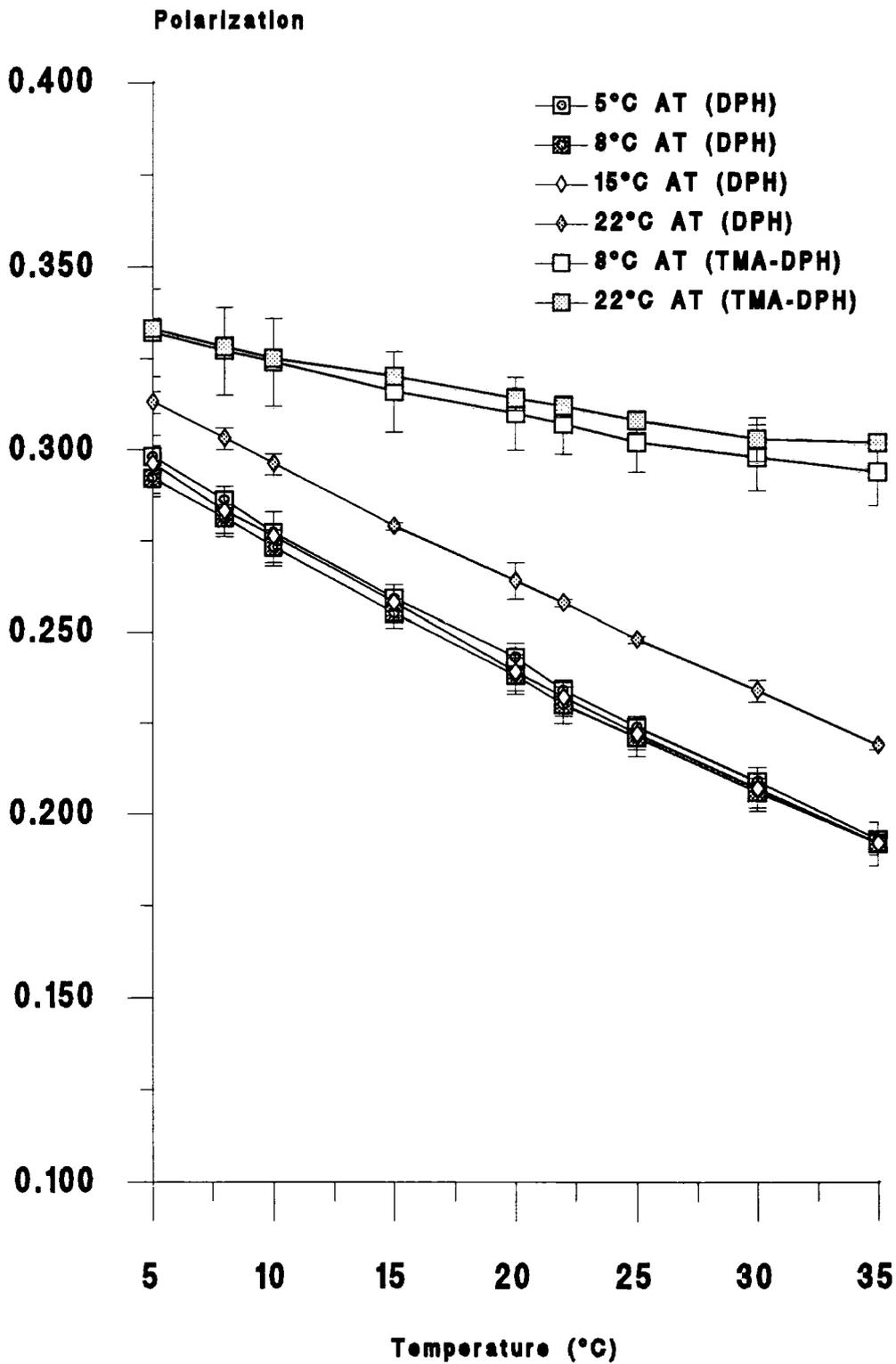
Figure 3.5. The temperature dependence of fluorescence polarization of **DPH** probe in plasma membrane preparations of *Carcinus maenas* acclimated in summer. The curves are averages of *n* plots for individual preparations ± S.D. (5°C AT *n*=4, 15°C AT *n*=4, **AT** = acclimation temperature)
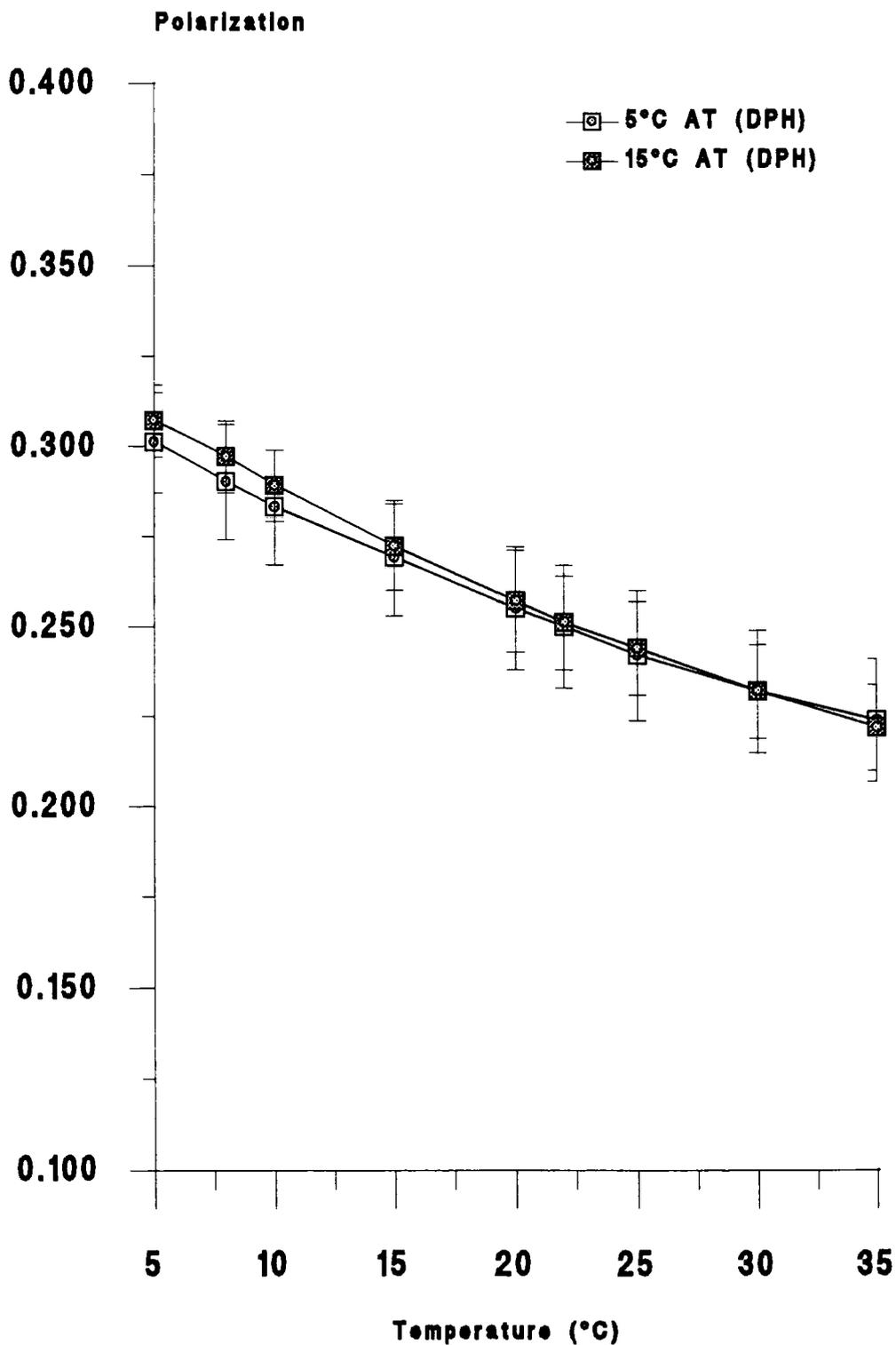
Figure 3.6. The temperature dependence of fluorescence polarization of **DPH** and **TMA-DPH** probes in plasma membrane preparations of *Carcinus maenas* acclimated in **autumn**. The curves are averages of n plots for individual preparations ± S.D. (5°C AT n=2, 8°C AT n=4, 15°C AT n=2, 22°C AT n=4, AT = acclimation temperature)
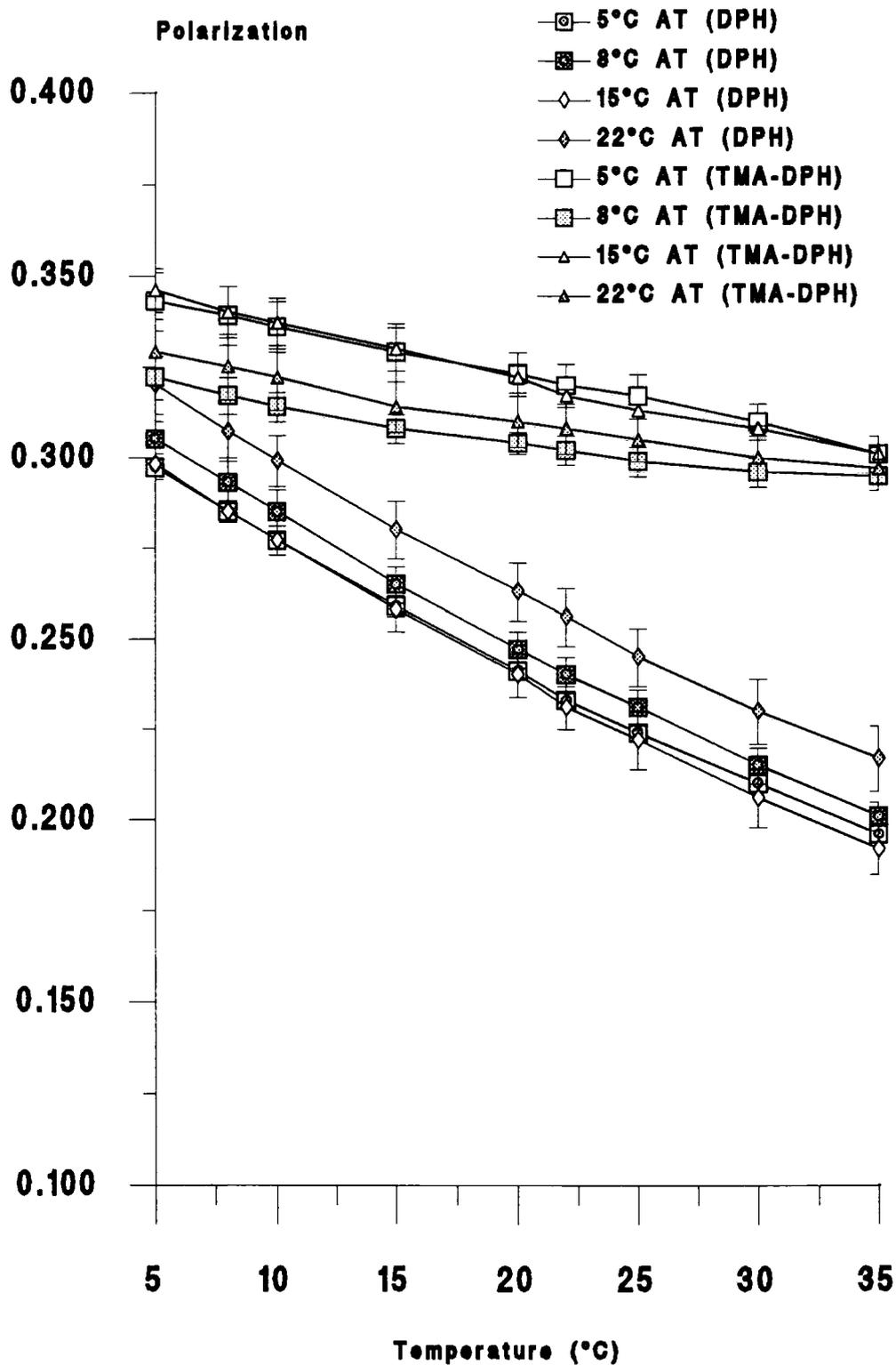
membranes of *Carcinus maenas* caught in spring, summer (only 5° and 15°C acclimation groups) and autumn, respectively.

The values of the DPH fluorescence polarization were always lower than those of TMA-DPH, suggesting that the plasma membranes were more fluid at the level probed by DPH than at that probed by TMA-DPH (Figures 3.1, 3.3, 3.4 and 3.6). The increase in fluidity with the experimental temperature was more rapid at DPH level (slopes in the range of -0.010 to -0.016 $\Delta\ln(\text{pol})/°C$) than at TMA-DPH level (slopes in the range of -0.003 to -0.005 $\Delta\ln(\text{pol})/°C$), thus the difference in fluidity between the middle of the bilayer and the glycerol backbone region increased with temperature (Tables 3.1 and 3.2).

The DPH fluorescence polarization measurements indicated that there was no difference in fluidity between the plasma membranes from 5°, 8° and 15°C acclimated crabs, in either species (Figures 3.1-3.6), but the plasma membranes from 22°C acclimated crabs were more ordered than those from all other acclimation groups (Figures 3.1, 3.3, 3.4 and 3.6).

The TMA-DPH fluorescence polarization measurements were carried out on plasma membranes from all four acclimation groups only in the autumn experiment. In spring only preparations from the 8° and 22°C acclimation groups were studied and no significant difference in fluidity at this level was found between them, in either species, suggesting that the adaptive changes occurred only at DPH level (Figures 3.1 and 3.4). In autumn, in *Cancer pagurus*, the fluidity of plasma membranes at TMA-DPH level increased in the order: (5° and 22°) < 15° < 8°C (Figure 3.3 and Table 3.1), and in *Carcinus maenas* in the order: (5° and 15°) < (8° and 22°C) (Figure 3.6 and Table 3.2).

Tables 3.1 and 3.2 show the equations of the temperature dependence of fluorescence polarization (lnP) for the two probes and a summary of the statistical analysis for *Cancer pagurus* and *Carcinus maenas*, respectively.

On the basis of the equations given in Tables 3.1 and 3.2, the efficacy of the homeoviscous adaptation observed at DPH or TMA-DPH level was calculated as the

Table 3.1. Summary of the fluorescence polarization study on muscle fibre plasma membranes of *Cancer pagurus* acclimated to different temperatures (equations fitted to the average curve for each AT; **AT** = acclimation temperature; **H.R.%** = homeoviscous response; **5-22, 8-22**, etc. = the acclimation temperatures corresponding to the two curves pol=f(t) or ln(pol)=f(t) that were used for calculating the H.R.%; letters in the statistical analysis column designate groups with significantly different means; **a ≤ ab≤b**, p≤0.05)

Probe	Season	AT (°C)	n	Equation	Stat. An.	H.R.% pol=f(t)	H.R.% ln(pol)=f(t)
DPH	spring	5	4	$\ln(pol) = -1.127 - 0.016\,t$	a	5-22: 18.3	5-22: 21.3
		8	4	$\ln(pol) = -1.165 - 0.014\,t$	a	8-22: 31.3	8-22: 36.3
		15	4	$\ln(pol) = -1.142 - 0.015\,t$	a	15-22: 66.7	15-22: 73.6
		22	6	$\ln(pol) = -1.127 - 0.013\,t$	b		
	summer	5	4	$\ln(pol) = -1.213 - 0.007\,t$			
		15	4	$\ln(pol) = -1.197 - 0.010\,t$			
	autumn	5	2	$\ln(pol) = -1.179 - 0.014\,t$	a	5-22: 42.8	5-22: 45.2
		8	4	$\ln(pol) = -1.153 - 0.014\,t$	a	8-22: 31.4	8-22: 38.5
		15	2	$\ln(pol) = -1.195 - 0.014\,t$	a	15-22: 116.7	15-22: 135.2
		22	4	$\ln(pol) = -1.111 - 0.013\,t$	b		
TMA--DPH	spring	8	4	$\ln(pol) = -1.108 - 0.004\,t$			
		22	6	$\ln(pol) = -1.077 - 0.004\,t$			
	autumn	5	2	$\ln(pol) = -1.053 - 0.004\,t$	b		
		8	4	$\ln(pol) = -1.113 - 0.003\,t$	a	8-22: 42.1	8-22: 48.2
		15	2	$\ln(pol) = -1.095 - 0.004\,t$	ab		
		22	4	$\ln(pol) = -1.080 - 0.004\,t$	b		

Table 3.2. Summary of the fluorescence polarization study on muscle fibre plasma membranes of *Carcinus maenas* acclimated to different temperatures (equations fitted to the average curve for each AT; AT = acclimation temperature; **H.R.%** = homeoviscous response; **5-22, 8-22**, etc. = the acclimation temperatures corresponding to the two curves pol=f(t) or ln(pol)=f(t) that were used for calculating the H.R.%; letters in the statistical analysis column designate groups with significantly different means; **a < b**, p≤0.05)

Probe	Season	AT (°C)	n	Equation	Stat. An.	H.R.% pol=f(t)	H.R.% ln(pol)=f(t)
DPH	spring	5	4	ln(pol) = -1.137 - 0.014 t	a	5-22: 42.9	5-22: 48.5
		8	4	ln(pol) = -1.158 - 0.014 t	a	8-22: 60.9	8-22: 69.0
		15	4	ln(pol) = -1.143 - 0.015 t	a	15-22: 123.8	15-22: 127.4
		22	2	ln(pol) = -1.098 - 0.012 t	b		
	summer	5	4	ln(pol) = -1.160 - 0.010 t			
		15	4	ln(pol) = -1.132 - 0.010 t			
	autumn	5	2	ln(pol) = -1.145 - 0.014 t	a	5-22: 34.4	5-22: 41.2
		8	4	ln(pol) = -1.117 - 0.014 t	a	8-22: 29.4	8-22: 33.5
		15	2	ln(pol) = -1.136 - 0.015 t	a	15-22: 100.0	15-22: 108.8
		22	4	ln(pol) = -1.077 - 0.014 t	b		
TMA--DPH	spring	8	4	ln(pol) = -1.087 - 0.004 t			
		22	2	ln(pol) = -1.088 - 0.004 t			
	autumn	5	2	ln(pol) = -1.047 - 0.004 t	b		
		8	4	ln(pol) = -1.126 - 0.003 t	a	8-15: 75.0	8-15: 60.7
		15	2	ln(pol) = -1.041 - 0.005 t	b		
		22	4	ln(pol) = -1.100 - 0.004 t	a		

'homeoviscous response' (HR%) using the method described by Wodtke and Cossins (1991) for curves that do not have the same slope (see section 3.2. Materials and Methods). Values are shown in Tables 3.1 and 3.2. Due to the variability of data between individual preparations, the standard deviations of the average slopes and intercepts were rather large. In order to check if these did not induce large errors in the calculation of the homeoviscous responses, a modification of the formula given by Wodtke and Cossins (1991) was used to calculate the homeoviscous response for pairs of curves 'polarization vs. temperature' instead of 'ln(polarization) vs. temperature'. The values obtained are also shown in Tables 3.1 and 3.2, and in most cases the two methods yielded close values.

When the cold acclimated groups (5° and 8°C) were compared to the 22°C acclimated group, the homeoviscous responses, at DPH level, were greater for *Carcinus maenas* than for *Cancer pagurus*. The HR% were in the range of 20-45% for *Cancer pagurus* and of 30-70% for *Carcinus maenas*.

When the 15° and 22°C acclimated groups were compared, an approximately 70% compensation was calculated for *Cancer pagurus* in spring and a superoptimal response (115-135%) in autumn. In *Carcinus maenas*, the HR% was superoptimal in both seasons (100-130%), (Tables 3.1 and 3.2).

The homeoviscous response at TMA-DPH level (autumn) was 40-50% in *Cancer pagurus*, when the 8°C and the 22°C acclimated groups were compared, and 60-75% for *Carcinus maenas* between the 8°C and 15°C acclimated groups.

A comparison between *Carcinus maenas* and *Cancer pagurus* acclimated at the same temperature showed that plasma membranes from *Cancer pagurus* were, on average, slightly more fluid, at DPH level, than those of *Carcinus maenas*. The difference was significant in a few cases, which are shown in Figure 3.7.

A general comparison of the efficacy of the homeoviscous adaptation (at DPH level) in the two species, showed better %HR for *Carcinus maenas* in spring, of 40-70% as compared to 20-35% in *Cancer pagurus*, and similar values in autumn, of 30-

Figure 3.7. Comparison between the fluidity of *Cancer pagurus* (Cp) and *Carcinus maenas* (Cm) plasma membranes: a) 22°C-acclimated crabs, spring, DPH probe (*n*=6, *n*=2); b) 8°C-acclimated crabs, autumn, DPH probe (*n*=4); and c) 15°C-acclimated crabs, autumn, TMA-DPH probe (*n*=2), (The curves are averages of *n* plots for individual preparations ± S.D., AT = acclimation temperature)
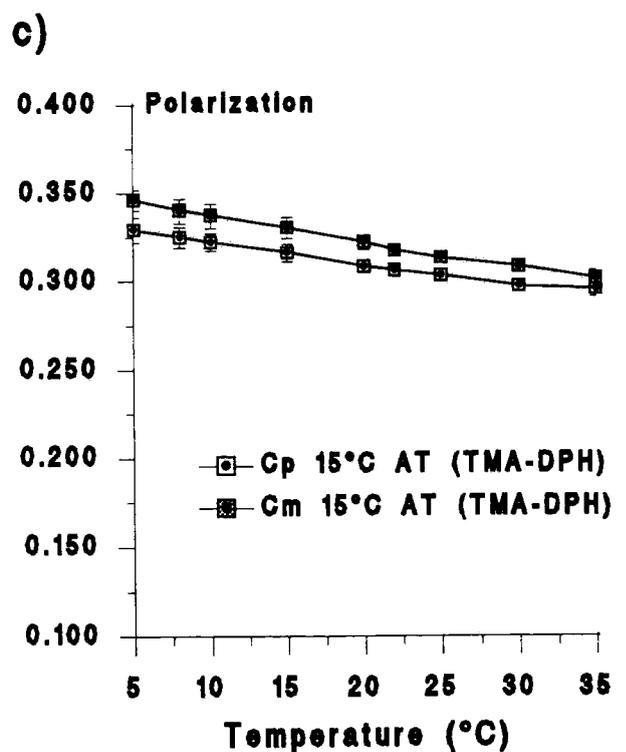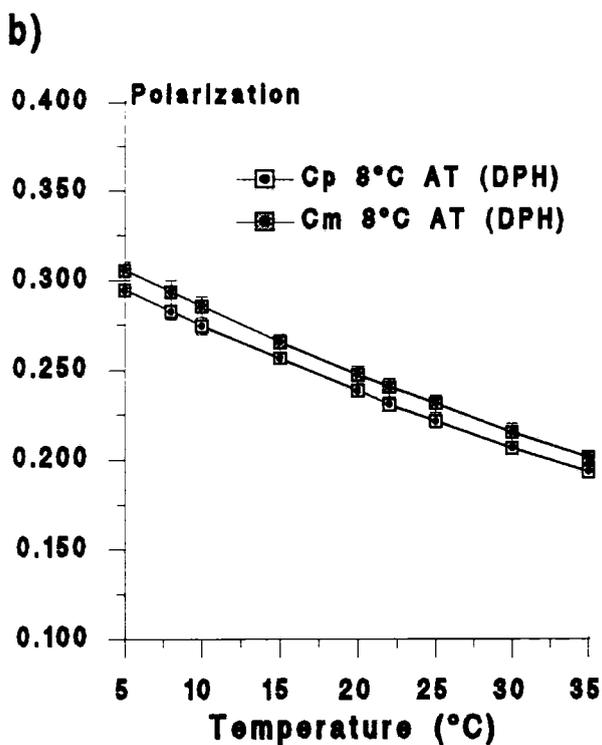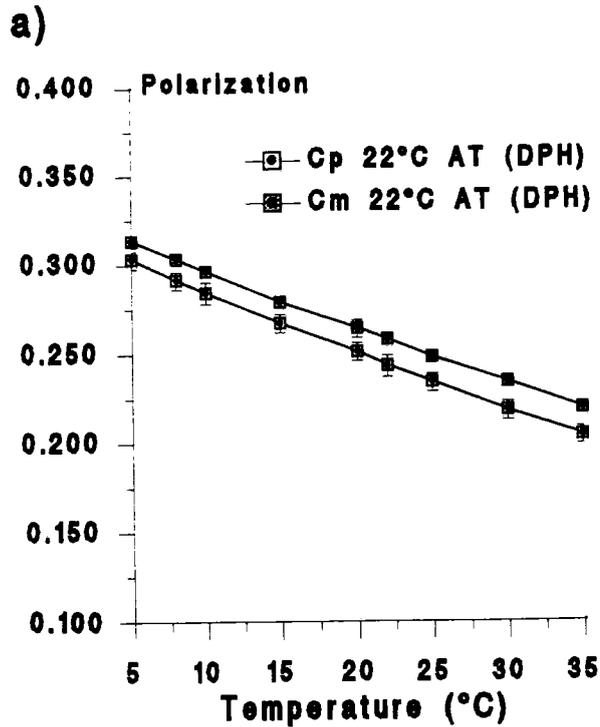
40% for both species, when the fluidity of membranes from 5°C and 8°C-acclimated crabs was compared with that of membranes from 22°C-acclimated crabs.

The comparison between seasons showed that the fluidity of the plasma membranes from crabs caught in summer was significantly less dependent on experimental temperature (slope = -0.007 to -0.010 Δln(pol)/°C) than that of plasma membranes from crabs caught in spring or autumn (slope = -0.014 to -0.016 Δ ln(pol)/°C). At experimental temperatures above 20°C for 5°C acclimated crabs, and above 25°C for 15°C acclimated crabs, the plasma membranes from the summer crabs were significantly more ordered, at DPH level, than those of spring or autumn crabs (Figures 3.8 and 3.9). In most cases, the differences between crabs caught in spring and autumn, and acclimated at the same temperature, were not significant. The 8°C-acclimated *Carcinus maenas* caught in spring had slightly more fluid plasma membranes than those caught in autumn (and acclimated at 8°C).

In *Cancer pagurus*, the %HR (DPH) calculated for 5°C and 22°C-acclimated membranes was lower in spring (18-21%) than in autumn (42-45%). The values of %HR (DPH) calculated for 8°C and 22°C-acclimated membranes were similar in spring and autumn (30-38%) (Table 3.1). In *Carcinus maenas* the %HR (DPH) calculated for 5°C and 22°C-acclimated membranes was similar in spring and autumn, and the %HR (DPH) calculated for 8°C and 22°C-acclimated membranes was reduced nearly 2-fold in autumn (29-33%) compared to spring (60-69%) (Table 3.2.).

3.4. Discussion

The DPH fluorescence polarization measurements showed that only warm-acclimation to 22°C induced a significant increase in the order of muscle plasma membranes of *Cancer pagurus* and *Carcinus maenas*. No difference was observed, at DPH level, between the cold-acclimated (5°C and 8°C) and the 15°C-acclimated crabs, independent of the season when the crabs were caught. These findings indicate

Figure 3.8. Seasonal comparison between the temperature dependences of **DPH** fluorescence polarization in plasma membranes of *Cancer pagurus* (Cp) acclimated to 5°C or 15°C in spring (**Sp.**, $n=4$), summer (**Summ.**, $n=4$) and autumn (**Au.**, $n=2$) (The curves are averages of n plots for individual preparations ± S.D., AT = acclimation temperature)
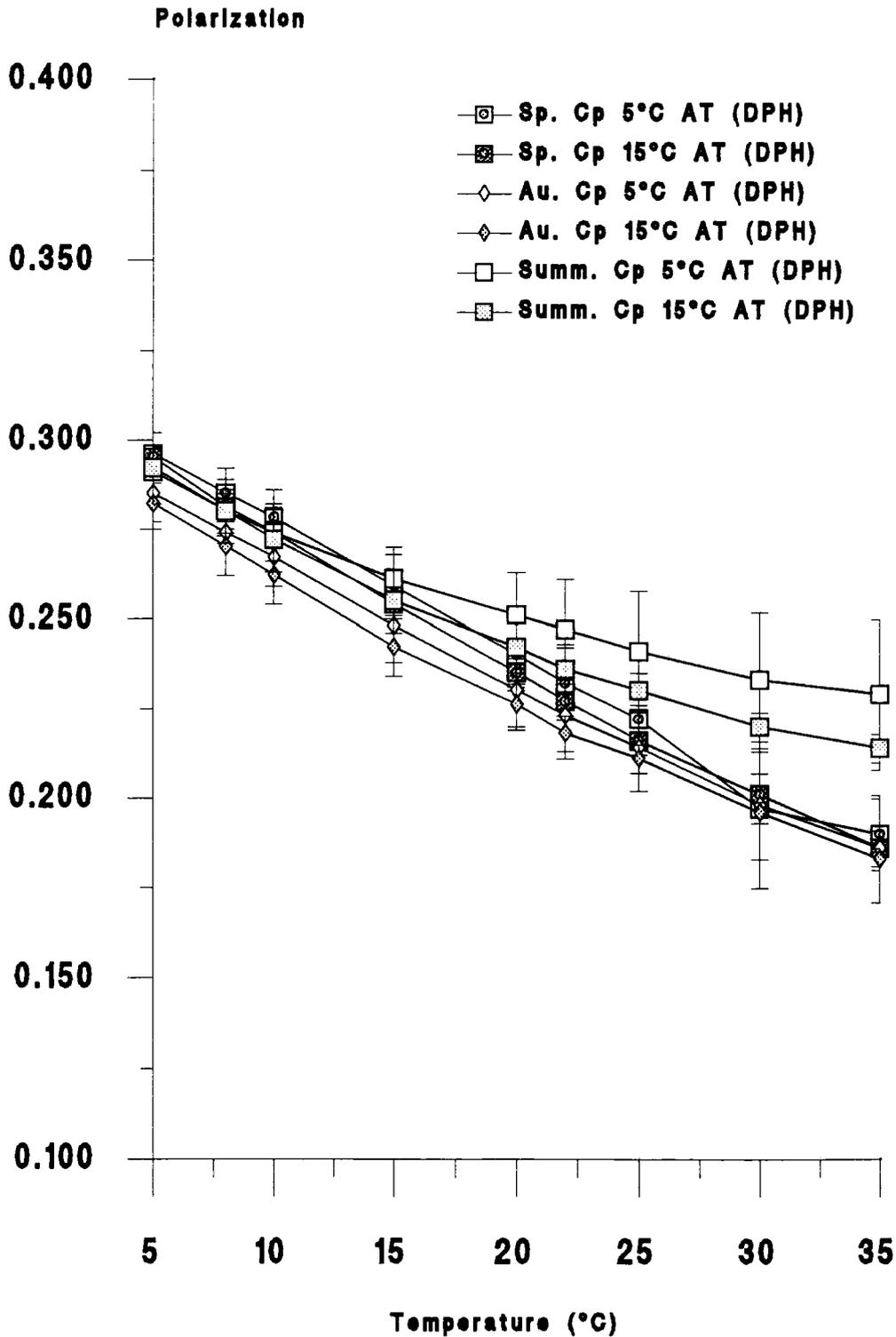
Figure 3.9. Seasonal comparison between the temperature dependences of **DPH** fluorescence polarization in plasma membranes of *Carcinus maenas* (Cm) acclimated to 5°C or 15°C in spring (**Sp.**, *n*=4), summer (**Summ.**, *n*=4) and autumn (**Au.**, *n*=2) (The curves are averages of *n* plots for individual preparations ± S.D., AT = acclimation temperature)
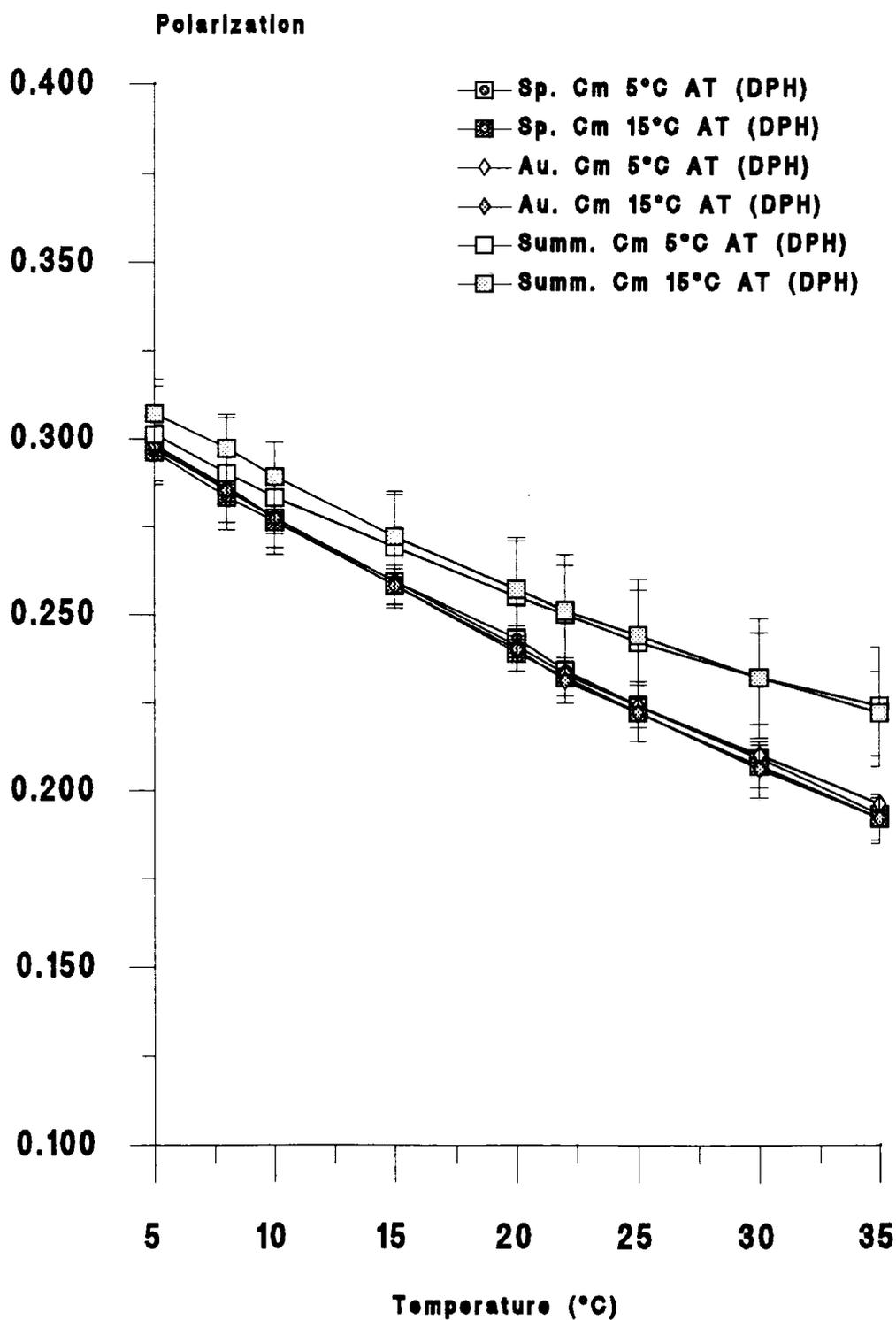
that both the stenothermal and the eurythermal species were able to develop an acclimatory response to a warm temperature, the extent of which was greater in the eurythermal species, *Carcinus maenas* (30-70%), than in the stenothermal species (20-45%), as expected. The fact that *Cancer pagurus* were able to acclimate to 22°C was remarkable, as this temperature is presumably warmer than the upper limit of their natural range of temperatures.

It was difficult to compare our results with other reported data, as there are only few papers describing the effects of temperature acclimation on the membrane fluidity of crustaceans (Farkas, Nemecz and Csengeri, 1984, Kivivuori, Laine and Lagerspetz, 1984), in comparison with the detailed studies carried out on fish (Cossins, 1994, Williams and Hazel, 1994a, Cossins, 1983). Most papers on crustaceans compare the fluidity of membranes from species living at different latitudes and/or in habitats with different thermal regimes (Farkas, Storebakken and Bhosle, 1988, Lahdes, Kivivuori and Lehti-Koivunen, 1993, Farkas *et al.*, 1994). There are a few papers which describe the effects of temperature acclimation on the membrane fluidity of other aquatic invertebrates, such as abalone (Dahlhoff and Somero, 1993) or the freshwater mussel *Anodonta cygnea* (Lagerspetz, Anneli-Korhonen and Tiiska, 1995).

Farkas, Nemecz and Csengeri (1984) compared the response to short-term cold-exposure (3 days) in two species of warm-adapted freshwater planktonic crustaceans, a winter-active (*Cyclops vicinus*) and a winter-dormant species (*Daphnia magna*). Initially, there was no difference in the order parameter of phospholipids isolated from membranes of the two species. Cold-exposure increased the order parameter (ESR, 5-doxyl stearic acid) of phospholipids from the winter-active species, *Cyclops vicinus*, but the temperatures of the onset and completion of the phase-separation were adaptively shifted to lower values than in phospholipids isolated from the warm-adapted animals, the compensation being nearly perfect. The same treatment had no influence on the order and the phase transition temperatures of the phospholipids from the winter-dormant species.

Kivivuori, Laine and Lagerspetz (1984) found that cold-acclimation increased the fluidity of crayfish neuronal membranes.

Species of abalone living at different habitat temperatures were acclimated for 7 weeks to temperatures spanning the extremes of the known habitat temperature range of each species (Dahlhoff and Somero, 1993) and temperature-compensatory changes were observed in the fluidity (DPH) of mitochondrial membranes in all species studied, eurythermal and stenothermal, with the exception of one eurythermal species.

Lagerspetz, Anneli-Korhonen and Tiiska (1995) did not find any change in the membrane fluidity (DPH) of isolated gills of 4°C-acclimated *Anodonta cygnea* (freshwater mussel) after they were kept at 24°C for a day.

The fact that crustaceans are able to adapt their membrane fluidity to environmental temperature has been clearly demonstrated by studies comparing species which are evolutionarily adapted to different temperatures (Farkas, Storebakken and Bhosle, 1988, Lahdes, Kivivuori and Lehti-Koivunen, 1993, Farkas *et al.*, 1994). Farkas *et al.* (1994) compared two species of shrimp living at different latitudes and average habitat temperatures, *Pandulus borealis* (Norway, 2°C) and *Parapandulus sp.* (Egypt, 27-30°C) and found that the fluorescence anisotropy of different anthroyloxy fatty acids (2-AS, 12-AS and 16-AP) was higher when embedded in phospholipid vesicles of the warm-adapted species, than in those of the cold-adapted species. The greater fluidity of membranes of the cold-adapted species was more obvious in the outermost segment of the bilayer, i.e. in the 2-AS segment. A proposed explanation of the fact that the fluidity of the deeper regions of the membranes remained relatively unaltered (Farkas *et al.*, 1994) was that the segments below the 12th carbon atom were fluid enough to enable the species to survive under the new thermal conditions. Lahdes, Kivivuori and Lehti-Koivunen (1993) found that the neuronal and gill membranes of an Antarctic amphipod, *Orchomene plebs* (Weddell sea, -1.6°C), were more fluid than those of a Baltic isopod, *Saduria entomon* (Baltic sea, 0.5-1°C). Farkas, Storebakken and Bhosle (1988) showed that

phospholipid vesicles obtained from tropical copepods (*Calanus ssp.*) were more ordered than those from copepods living around the West coast of Norway at colder temperatures (*Calanus finmarchicus*). All these papers refer to freshly collected animals, either maintained a few days at temperatures equal to the average environmental temperature at the time of collection or rapidly frozen until membrane preparation.

In fish, the most common response to laboratory acclimation was a compensatory shift in the polarization versus temperature curves towards lower temperatures for cold acclimated fish (Logue, Tiku and Cossins, 1995, Williams and Hazel, 1994a), in agreement with the homeoviscous adaptation theory (Sinensky, 1974).

Our results were in agreement with the homeoviscous adaptation theory when the DPH fluorescence polarization curves for membranes of 5°C-, 8°C- and 15°C-acclimated crabs were compared to those for 22°C-acclimated crabs, but they raised the question why the membranes from 15°C-acclimated crabs were not more ordered at DPH level than those of cold-acclimated crabs in either species, in any season.

Exceptions to the above mentioned rule of membranes of cold-acclimated fish being more disordered than those of warm-acclimated fish, or variations in the extent of the response, depending on the type of tissue or membrane investigated have been reported for fish (Cossins, 1994). Of particular interest, in relationship with our results, was the observation of Cossins, Kent and Prosser (1980) that microsomal membranes of liver from green sunfish had roughly identical fluidities in 5°C-,15°C- and 25°C-acclimated fish and an increased order, compared with lower acclimation temperatures, only in 34°C-acclimated fish. At the same time, liver mitochondrial membranes prepared from the same individuals showed a gradual decrease in fluidity with the increase in acclimation temperature, over the entire range of acclimation temperatures studied. The fluidity of fish muscle plasma membrane has not been studied but the sarcoplasmic reticulum membrane of goldfish is known as one of the typical examples of a metabolically active membrane that did not exhibit any

homeoviscous acclimation at DPH level when 5°C- and 25°C-acclimated fish were compared (Cossins, Christiansen and Prosser, 1978). However, Ushio and Watabe (1993) used a different fluorescent probe, Py(3)Py, and found that sarcoplasmic reticulum membranes from carp acclimated to 30°C for 5 weeks were less fluid than those from 10°C-acclimated carp.

A possible interpretation of our findings is that the muscle plasma membranes of the species investigated either did not alter their fluidity at the level probed by DPH at acclimation temperatures below a certain point between 15°C and 22°C, or the alterations had a very slow time-course and were not detectable after 3 weeks of warm-acclimation.

The fact that the 22°C-acclimated crabs spent, in total, four weeks at warm temperatures (1 week at 15°C and 3 weeks at 22°C), compared to 3 weeks for the 15°C-acclimated crabs, may be related to the assumption that the adaptive changes have a slow time course, but, on the other hand, the membranes of 15°C-acclimated crabs may undergo compensatory changes that are not detectable by DPH fluorescence polarization measurements. Time-course studies on fish have demonstrated that the compensation in membrane fluidity following transfer to cold temperatures was a rapid process (Wodtke and Cossins, 1991), but Williams and Hazel (1994a) pointed out that the rapid adjustments observed in membrane physical state and/or lipid composition short time after transfer to a lower or higher temperature than the acclimation or adaptation temperature, were not always the same with those found after longer periods at the new temperature.

The statement that probably no compensation in the membrane fluidity of 15°C-acclimated crabs occurred at DPH level brings the discussion to the TMA-DPH fluorescence polarization measurements. These also revealed some interesting differences between the fluidity of muscle plasma membranes from different acclimation groups, but the pattern was not as clear-cut as in the case of DPH probe. In the spring experiment, the membranes of 8°C- and 22°C-acclimated crabs had

similar fluidities at TMA-DPH level, in both species, suggesting that the compensatory changes occurred only at DPH level.

The comparison between all the four acclimation groups was possible only in the autumn experiment, when membranes of 15°C-acclimated *Cancer pagurus* were found to have an intermediary fluidity between the 8°C-acclimated membranes and the 5°C- and 22°C-acclimated membranes, with the last two groups being more ordered. The difference between the 8°C- and 22°C-acclimated membranes was significant and suggested that in *Cancer pagurus* caught in autumn, homeoviscous adaptation occurred at both levels of the bilayer, with a slightly higher %HR at TMA-DPH level compared to the DPH level. The intermediary fluidity of the 15°C-acclimated membranes fitted the series of decrease in fluidity with the increase in acclimation temperature, but the fact that 5°C-acclimated membranes were as ordered as the 22°C-acclimated membranes was confusing.

The results observed in *Carcinus maenas* caught in autumn were somewhat similar, in that the 5°C-acclimated membranes were more ordered than both the 8°C- and 22°C-acclimated membranes, and not different from the 15°C acclimated membranes. The similar fluidity of 8°C- and 22°C-acclimated membranes suggested that in *Carcinus maenas* in both seasons homeoviscous adaptation occurred mainly at DPH level, with a higher %HR in spring. However, in autumn the membranes from 15°C-acclimated *Carcinus maenas* displayed homeoviscous adaptation at TMA-DPH level with a %HR of 60-75% relative to the 8°C-acclimated membranes.

Therefore, in both species, the membranes from 5°C-acclimated crabs were as ordered (*Cancer*), or more ordered (*Carcinus*) at TMA-DPH level than those from crabs acclimated to warmer temperatures. The significance of these observations was not clear. Farkas, Nemecz and Csengeri (1984) reported a cold-induced increase in the order (at the level probed by 5-DSA, which in a simplistic manner can be considered close to that probed by TMA-DPH) of phospholipids isolated from a winter-active species of a planktonic crustacean, despite the perfect compensation of phase-transition temperatures, but the duration of cold-exposure in their experiments

was much shorter (3 days) than in our case (3 weeks), probably not sufficient for all the compensatory changes to be completed.

Further attempts to correlate these observations with the results of the membrane lipid composition analysis will be made in Chapter 7 (General Discussion). Another observation from our experiments was that both *Cancer pagurus* and *Carcinus maenas* seemed to become semi-dormant after a week or two at 5°C, in laboratory conditions (casual observations), with the photoperiod maintained at 12 hours light-12 hours dark. The reduction in activity may be related to the increased membrane order at TMA-DPH level.

The comparison between the stenothermal and the eurythermal species could be conducted on two lines: firstly, a direct comparison of the membrane fluidities between crabs acclimated at the same temperature and secondly, a comparison of the patterns of changes observed. The direct comparison showed that the *Cancer pagurus* plasma membranes were slightly more fluid than those of *Carcinus maenas* at all acclimation temperatures. By analogy with the differences observed between cold- and warm-adapted species (for example Farkas *et al.*, 1994), this observation could be correlated to the narrower range of temperature fluctuations experienced by *Cancer pagurus* in the natural habitat, which implies a lower upper-limit of this range. The patterns of the changes induced by temperature acclimation were similar at DPH level, with greater acclimation efficacy in *Carcinus maenas* than in *Cancer pagurus*, in particular in the spring experiment. Eurythermal ectotherms in general have greater abilities to develop adaptive responses to altered environmental conditions (Prosser and Heath, 1991). Other comparative studies have shown that planktonic crustaceans that remained active at low-temperatures had greater capacity to adjust their membrane fluidity following changes in thermal conditions than those which did not overwinter in an active state (Pruitt, 1990), and that both eurythermal and stenothermal species of abalone were able to acclimate the fluidity of their mitochondrial membranes (Dahlhoff and Somero, 1993).

The results of our summer experiment, when the rate of increase in fluidity with temperature was slower than in spring and autumn, in both species, suggested a seasonal acclimatisation effect, manifested even after three weeks of acclimation to 5°C or 15°C. The lower temperature sensitivity of membrane fluidity in summer has a potential protective role, as the temperatures experienced in summer are likely to be higher than in spring or autumn, especially for *Carcinus maenas*. It was interesting that this feature was conserved even after laboratory acclimation to a temperature as low as 5°C, and that no such effect was induced by warm-acclimation. This was probably related to the existence of factors other than temperature which influence the physiological status of animals under natural conditions (Cossins and Bowler, 1987), for example photoperiod, nutrition, reproductive and growth cycles. Farkas, Storebakken and Bhosle (1988) reported that phospholipid vesicles prepared from copepods collected in spring were more fluid and had lower phase transition temperatures than those from copepods collected in late autumn, and suggested that the spring animals still retained winter acclimatization, whilst the autumn ones still retained the summer acclimatization.

The significant difference in fluidity observed between the levels probed by DPH and by TMA-DPH was in agreement with the results obtained with other methods on a variety of membrane types (Shinitzky, 1984) that showed a gradient of fluidity between the middle of the bilayer and the surfaces. The temperature-dependence of the polarization of the two probes was also different, the DPH polarization decreasing approximately four times faster than the TMA-DPH polarization with temperature. Williams and Hazel (1994b) found large differences in the membrane fluidities sensed by DPH, TMA-DPH and PA-DPH (3-(p-(6-phenyl)-1,3,5-hexatrienyl)phenyl-propionic acid) in trout hepatocytes. According to the presumed probe location, the fluidity increased in the order of PA-DPH (outer hemilayer, least fluid) < TMA-DPH (inner hemilayer) < DPH (membrane interior,

most fluid) (Williams and Hazel, 1994b) showing that in their experiment the effect of probe location on the reported membrane fluidity was many times greater than the effect of temperature. However, the exact location of TMA-DPH in the cell membrane is controversial. It is accepted that it is inserted nearer to the surface of the bilayer, but Prendergast, Haugland and Callahan (1981), who originally synthesized this probe, reported that it stays in the outer hemilayer of the membrane, whilst other papers, including the above mentioned by Williams and Hazel (1994b) argue its preferential location in the inner hemilayer and relate it to the compositional asymmetry of the hemilayers.

The need for more studies of the temperature effects on membrane fluidity, in complex experimental designs and using different probes and more refined techniques is stated in the recent review by Cossins (1994).

Chapter 4: A Comparative Study of the Plasma Membrane Lipid Composition of Cold- and Warm-Acclimated Crabs

4.1. Introduction

Several early studies on the effects of temperature on the biochemical composition of ectothermic organisms showed that cold adaptation or acclimation involved an increase in the levels of unsaturated fatty acids in membrane phospholipids and warm adaptation or acclimation involved an increase in the levels of saturated fatty acids. After the development of the techniques for measuring the biophysical state of lipid bilayers, in the 1970s, such changes in the lipid composition were also correlated with alterations in the membrane fluidity (Cossins, 1994, Hazel, 1988, Prosser and Heath, 1991).

The role of membrane lipids in regulating the fluidity of cell membranes under conditions of altered environmental temperature has been originally proposed by Sinensky (1974) in his homeoviscous adaptation theory. Since then this theory has been supported by several studies, based on biochemical or biophysical methods, which showed that poikilothermic organisms adapt to changes in environmental temperature by modulating the lipid composition of their cell membranes, in order to maintain a relatively constant membrane fluidity (Cossins, 1983).

However, studies on synthetic phospholipids (Coolbear, Berde and Keough, 1983, Stubbs *et al.*, 1981) showed that there was no simple linear relationship between the degree of lipid unsaturation and membrane biophysical properties. The insertion of the first double bond in a fatty acyl chain decreases significantly the melting point of that fatty acid, and also the temperature of thermotropic phase transition of a phospholipid containing that fatty acyl, but the insertion of a second double bond has a smaller effect, and the third one can in some cases increase slightly the melting point (Stubbs and Smith, 1984, Coolbear, Berde and Keough, 1983).

Recent work on biological membranes, showed that in some cases temperature acclimation induced significant changes in overall lipid unsaturation, but bilayer order was not affected (Lee and Cossins, 1990, Cossins, Christiansen and Prosser, 1978). In other cases, the opposite was observed: the structural order of membranes changed in an adaptive manner with temperature acclimation, with very little changes in the overall fatty acid composition of membrane phospholipids (Buda *et al.*, 1994, Farkas *et al.*, 1994). Based on studies of the membrane phospholipid molecular species, Farkas *et al.* (1994) have proposed that the main mechanism of adjusting the biophysical properties of membranes to temperature challenge is a reshuffling of fatty acyl chains between phospholipid molecules, leading to the restructuring of phospholipid molecular species, which would also be a metabolically inexpensive process.

Despite the complexity of the mechanisms presumed involved in the regulation of the biophysical state of cell membranes, there is a large number of papers which describe temperature-related changes in the lipid composition of various tissues or membranes, without any corresponding information on the physical properties of their membranes (Cossins, 1994). As already mentioned above, the most common response involved in thermal adaptation is a change in the degree of fatty acid unsaturation. Other types of responses involve changes in the proportion of branched-chain fatty acids (in bacteria), the fatty acid chain length, the proportion of phospholipid or glycolipid classes, in the sterol to phospholipid ratio or in the distribution of phospholipid molecular species (Thompson, 1983, Hazel, 1989).

Hazel (1989) has summarized the results of 12 studies on the fatty acid composition of PC and PE in relation to acclimation temperature and concluded that the unsaturation ratio decreases with cell or acclimation temperature for both phospholipids. The correlation coefficient was higher for selected membrane fractions than for total tissue. For PC the correlation was strongest for the mitochondrial fraction (-0.88) and weakest for synaptosomes (-0.34, but a lower number of studies

involved). For PE all membrane fractions exhibited a strong correlation, ranging from -0.84 to -0.89. In some cases, the increase in unsaturated fatty acids was due to an increase in monoene levels, in other cases the polyunsaturated fatty acids (PUFA) increased with cold-acclimation. Farkas (1979) and Farkas, Nemecz and Csengeri (1984) proposed that this difference is related to the level of activity maintained in the cold. In their experiments on planktonic crustaceans the PUFA increased only in the winter-active species. Consistent with this explanation, in fish the monoenes increased with cold-acclimation mostly in goldfish and related species that do not remain very active at cold temperatures (Hazel, 1988). It has been shown that in ectotherms that do accumulate PUFA during winter months, the capacity to metabolize unsaturated fatty acids varies and seems to determine whether the PUFA accumulated belong to the $\omega 3$ (Hazel, 1979) or the $\omega 6$ family (Chang and Roots, 1985, Schünke and Wodtke, 1983).

Hazel and Zerba (1986) showed that in rainbow trout liver mitochondria the molecular species composition of PC was significantly influenced by acclimation temperature. Fish acclimated to 5°C had nearly double amounts of 16:0/22:6 and triple amounts of 16:0/20:5 compared to 20°C-acclimated fish, but the levels of 16:1/18:1 were six-fold lower in the cold-acclimated fish. Farkas *et al.* (1994) found a 2-fold to 3-fold increase in 18:1/22:6 and 18:1/20:5 with cold-acclimation in species of shrimp and fish (liver), mainly in PE but also evident in PC, and an increase in 18:0/22:6 and 18:0/20:4 with warm-acclimation in both PE and PC of the same species. It has been suggested that the role played by some molecular species of PE (18:1/22:6 and 18:1/20:5) is more important than that of PC species in thermal adaptation of membranes (Dey *et al.*, 1993a).

Membranes of cold-adapted ectotherms generally contain higher proportions of PE and, less consistently, reduced proportions of PC and, as a consequence, the ratio of PC/PE tends to be lower in cold- than in warm-adapted animals (Hazel, 1989). The replacement of PC by PE at cold temperatures can be explained on the basis of molecular shape: the conical shape of PE, due to the smaller and less

hydrophilic headgroup and the bulkier hydrophobic domain (more unsaturated than PC), would have a disruptive influence upon membrane organization, since this shape does not pack efficiently in the lamellar phase (Hazel, 1989). In neural tissue, monosialogangliosides are also replaced by more polar, polysialized homologues in cold-acclimated and cold-adapted, compared to temperate water fish species (Rahmann and Hilbig, 1981, 1983).

Cholesterol has a great influence on the physical and functional properties of a membrane and is likely to play a role in the regulation of membrane biophysical properties during thermal adaptation (Shinitzky, 1984). However, in aquatic ectotherms membrane cholesterol levels do not vary in a consistent manner with acclimation temperature and the cholesterol content of some membranes, including synaptic membranes of goldfish brain (Cossins, 1977) and mitochondrial membranes of carp red muscle (Wodtke, 1981) was not influenced at all by acclimation temperature.

There are few studies which compare adaptive responses in eurythermal species of fish with those of stenothermal species (Schwarzbaum, Wieser and Niederstätter, 1991, Schwarzbaum, Wieser and Cossins, 1992) but only one for decapod crustaceans (Cuculescu, Hyde and Bowler, 1995). In general, eurythermal animals have greater ability to develop capacity adaptations than stenotherms (Prosser and Heath, 1991).

Winter-active and winter-passive species of crustaceans (Farkas, 1979, Farkas, Nemecz and Csengeri, 1984, Pruitt, 1988) and fish (Hazel, 1979) have been compared and it has been shown that the acclimation temperature-induced changes in membrane composition of poikilotherms were related to the over-wintering strategy of the animal.

The lipid composition of ectotherms varies to a considerable extent on a seasonal basis and although this seasonal variation may be related to factors other than temperature, such as the food availability, the reproductive and growth cycle or the photoperiod (Cossins and Bowler, 1987) it does appear as an important aspect for

thermal biology studies, as the response to the temperature stress imposed under laboratory conditions may depend on the preexistent state of acclimatization. The work of Farkas (1970) and Farkas and Herodek (1964) on freshwater planktonic crustaceans suggested a characteristic annual cycle in Crustacea, which consists of the disappearance of the long polyunsaturated fatty acids in spring and an accumulation of these during the decrease in temperature in autumn.

The effects of temperature acclimation or adaptation on lipid composition were studied in a few aquatic invertebrate species, including marine (Chapelle, 1978, Farkas and Nevenzel, 1981, Farkas *et al.*, 1994) and freshwater (Cossins, 1976, Farkas, 1979, Pruitt, 1988) crustaceans. However, the studies on marine crustaceans refer to whole animal or tissue, not to a specific membrane type. Since compositional adjustments are frequently membrane-specific (Hazel and Zerba, 1986) it has been suggested that analysis of whole-cell lipid compositions are of limited usefulness for studies trying to discover the physiological or functional consequences of membrane adaptation to thermal stress (Thompson, 1983, Hazel, 1989).

The work presented in this chapter concerns the effects of cold- and warm-acclimation on the total phospholipid and cholesterol content, the cholesterol/phospholipid molar ratio and the fatty acid composition of phospholipids of plasma membranes isolated from leg muscle of two species of marine crab: a stenothermal species, *Cancer pagurus*, and an eurythermal species, *Carcinus maenas*. The two species were compared in an attempt to identify differences in the type or intensity of response to thermal stress between stenothermal and eurythermal marine invertebrates. The seasonal influence upon the response to laboratory cold- or warm-acclimation was also investigated.

4.2. Materials and Methods

4.2.1. Animals

Crabs (intermoult stage) were caught from the North Sea (Hartlepool area) and maintained in laboratory conditions for 3 weeks at their respective acclimation temperatures (5, 8, 15 and 22°C) in aerated filtered sea water, in thermostated rooms (12 hrs light-12 hrs dark). Acclimation to 22°C also involved a week at 15°C, prior to transfer to 22°C for three weeks. The crabs were fed on frozen fish (commercial source) and the water changed the day after feeding. Table 4.1 shows the average fatty acid composition of the fish. All crabs were starved for the last two weeks of the acclimation period. Data from three experiments are presented: a winter experiment, with crabs caught in December 1992, a spring experiment with crabs caught in April 1993 and an autumn experiment with crabs caught in late September 1993.

4.2.2. Plasma Membranes

Plasma membranes from crab leg and claw muscle were prepared using the previously described method (Cuculescu and Bowler, 1993, and Chapter 2 of this thesis).

4.2.3. Total Lipid Extraction

Lipids were extracted from freshly prepared plasma membranes using the method of Bligh and Dyer (1959) as modified by Hajra, Seguin and Agranoff (1968). One volume of sample was vortexed with 3.75 volumes of chloroform/methanol (1:2, v/v) and 0.08 volumes of concentrated HCl, then allowed to stand for 5 minutes. Subsequent additions of 1.25 volumes of chloroform and 1.25 volumes of 0.2M KCl in H_3PO_4 2M were each followed by vortexing, then the mixture was centrifuged at 500 x g for 10 minutes to assist the phase separation. The bottom chloroform phase was collected, dried under N_2, then the lipid film resuspended in 2 ml of chloroform, sealed under N_2 in glass tubes and stored at -20°C. All solvents contained 0.05%

Table 4.1. The average fatty acid composition of the commercial frozen fish used as crab diet

FATTY ACID	Relative %
C14:0	3.2
C14:1 w9	1.8
C16:0	15.4
C16:1w9	4.5
C17:0	- 0.8
C17:1w9	0.4
C18:0	3.4
C18:1w9	13.3
C18:2w6	1.4
C18:3w6	0
C18:3w3	0.4
C20:0	1.4
C20:1w9	4.0
C20:2w6	0.3
C20:4w6	2.7
C20:5w3	15.1
C22:5w3	0.3
C22:6w3	30.5

BHT and all steps were performed at 4°C. These total lipid extracts were used for the total cholesterol and phospholipid assays and for thin layer chromatography.

4.2.4. Total Phospholipid Assay

Total phospholipid was determined with the rapid assay method of Stewart (1980), using a standard curve prepared with a mixture of PC/PE (2:1, w/w).

An ammonium ferrothiocyanate solution was prepared by dissolving 27.03g $FeCl_3$ ($6H_2O$) and 30.4g NH_4SCN in distilled water, final volume 1 litre. The solution, kept in a brown bottle, was stable for months at room temperature. For the assay, 2 ml of this solution were pipetted in siliconized glass tubes, then aliquots of samples (50-100µl) or standard PC/PE mixture (in chloroform), and pure chloroform to make the final volume of the chloroform phase 2 ml were added to these. The biphasic system was vortexed for 1 minute, then allowed to separate again. The lower chloroform phase, which contained the coloured complex phospholipid-ammonium ferrothiocyanate, was taken up in a Pasteur pipette and put in quartz spectrophotometer cuvettes and read immediately at 488 nm against a chloroform blank. The accurate timing of the vortexing and the rapid reading of the optical density (OD) of the volatile chloroform solution appeared to be important for the reproducibility of results. If necessary, the chloroform phase was clarified with a pinch of anhydrous sodium sulphate.

The standard curve ranged from 0 to 100µg phospholipid (in 2 ml assay volume). The use of a PC/PE mixture was recommended due to differences in the slopes of standard curves for choline containing phospholipids (PC, LPC, SM) and other phospholipids (PE, PS) (Stewart, 1980).

4.2.5. Total Cholesterol Assay

Total cholesterol was enzymatically assayed using a cholesterol assay kit (Sigma) and a standard curve prepared with the Sigma cholesterol calibrator. The assay was based on the method of Johnson (1979).

The reconstituted cholesterol reagent contained the following: cholesterol oxidase 300 U/l, cholesterol esterase >100 U/l, peroxidase 1000 U/l, 4-aminoantipyrine 0.3 mM, p-hydroxybenzenesulphonate 30 mM, buffer pH 6.5 and other nonreactive stabilizers and fillers (Sigma kit instruction booklet). For the assay, 50-100 µl of sample or the required amount of calibrator solution were dried under N_2, then redissolved in 20 µl of isopropanol. To the same tubes 1 ml of reagent was added, then the tubes were incubated 10 minutes at 25°C and the OD of the pink solution read at 500 nm.

The standard curve covered a range from 0 to 1 mg cholesterol in 1 ml assay volume.

4.2.6. Thin Layer Chromatography (TLC)

The TLC plates were made in the laboratory using glass plates (20x20 cm), a slurry of 45 g Silicagel H (Merck) in 105 ml distilled water and a hand-operated Shimadzu applicator, set for 0.5 mm thickness. Plates were air-dried overnight, then activated 2 hours at 110°C prior to use.

The total phospholipid fraction was separated from the other lipid classes present in the total lipid extract using uni-dimensional TLC with a solvent mixture of petroleum ether:diethylether:acetic acid (70:30:2), (Christie, 1982). The phospholipid fraction remained at the origin (Figure 4.1.a), the spots were scraped in Pyrex glass tubes, sealed under dry N_2 and stored at -20°C until required for the fatty acid derivatization.

The individual phospholipid classes were separated using two-dimensional TLC. The solvents used were (Christie, 1982):

I: chloroform : methanol : ammonium 28% (65:30:4) and

II (perpendicular direction): chloroform : methanol : acetic acid : water (135:65:18:3).

After separation in the first solvent system, the plates were well dried under N_2 for 30 minutes (in boxes with a nitrogen inlet pipe and an open side), in order to avoid interference with the components of the second solvent system.

Figure 4.1. (a) The separation pattern of lipid classes by unidimensional thin layer chromatography using petroleum ether:diethylether:acetic acid (70:30:2) (**PL** = phospholipids, **MG** = monoglycerides, **CH** = cholesterol, **DG** = diglycerides, **FFA** = free fatty acids, **FAE** = fatty acid esters, **TG** = triglycerides, **S.F.** = solvent front);
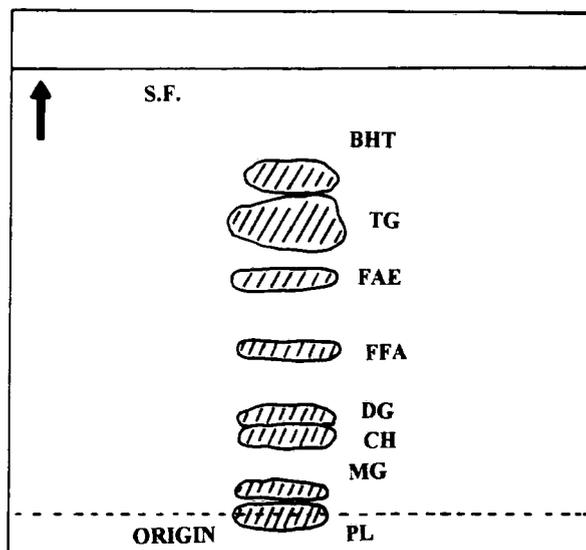
(b) The separation pattern of individual phospholipid classes by two-dimensional thin layer chromatography, using I: chloroform:methanol:ammonium 28% (65:30:4) and II (perpendicular direction): chloroform:methanol:acetic acid:water (135:65:18:3) (**LPC** = lysophosphatidylcholine, **NL** = neutral lipids, **PA** = phosphatidic acid, **PC** = phosphatidylcholine, **PE** = phosphatidylethanolamine, **PG** = phosphatidylglycerol, **PI** = phosphatidylinositol, **PS** = phosphatidylserine, **SM** = sphingomyelin)
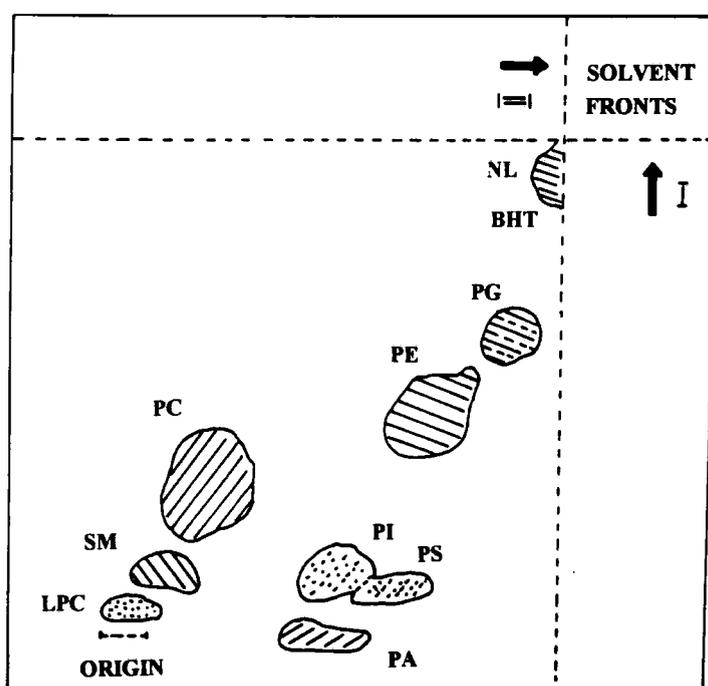
The spots were visualised by spraying with 0.01% Phloxine B in methanol 50% and examination under UV lamp (Figure 4.1.b), then scraped in Pyrex tubes, sealed under dry N_2 and stored at -20°C. The Phloxine B was removed by adding distilled water to the phase system used for the extraction of the fatty acid methyl esters (FAME) in hexane (see next paragraph).

4.2.7. Fatty Acid Composition Analysis

The fatty acid composition of the membrane phospholipids was determined using gas-liquid chromatography. The fatty acid methyl esters (FAME) were prepared using a modification of the method of Morrison and Smith (1964). The scraped spots were soaked in 0.5 ml boron trifluoride methanol solution, then the tubes were flushed with nitrogen, sealed and incubated for 15 minutes at 100°C. Cooled tubes were flushed again with N_2 to remove volatiles created during incubation, and 0.5 ml distilled water and 1 ml hexane were added to each tube and vortexed. The addition of distilled water to the phase system was an essential requirement as blanks of Silicagel from the sprayed plates for which water was not added to the phase system displayed small peaks in the position where C16:1 was normally eluted.

The FAME were extracted in the hexane phase and the tubes were centrifuged at 500xg for 10 minutes (MSE Coolspin, Fisons, swing-out rotor) in order to assist the separation of the hexane and aqueous phases and the sedimentation of the Silicagel. The top hexane phase was collected and concentrated to a small volume (10-20 µl) under N_2. Aliquots of these were injected onto the GLC column.

The separation of FAME was carried out using a Shimadzu GC-9A Series gas chromatograph (glass column 2.0 m long, 2.0 mm internal diameter and 6.0 mm external diameter, packed with cyano-silicone stationary phase, 105 Alltech CS-5, on a Chromasorb WAW 100-120 mesh support), connected to a Shimadzu C-R6A Chromatopac integrator. Nitrogen was used as the carrier gas and the resolved components were detected by a flame ionization detector (hydrogen/air). A temperature programme (25 minutes per sample) was run: initial temperature = 210°C

Figure 4.2. The GLC spectrum of the fatty acid methyl esters mixture derivatized from the PC fraction separated from the lipid extract of muscle plasma membranes of cold-acclimated *Cancer pagurus* (shown as an example of the spectra obtained for the plasma membrane samples). Values shown near peaks are the retention times, in minutes
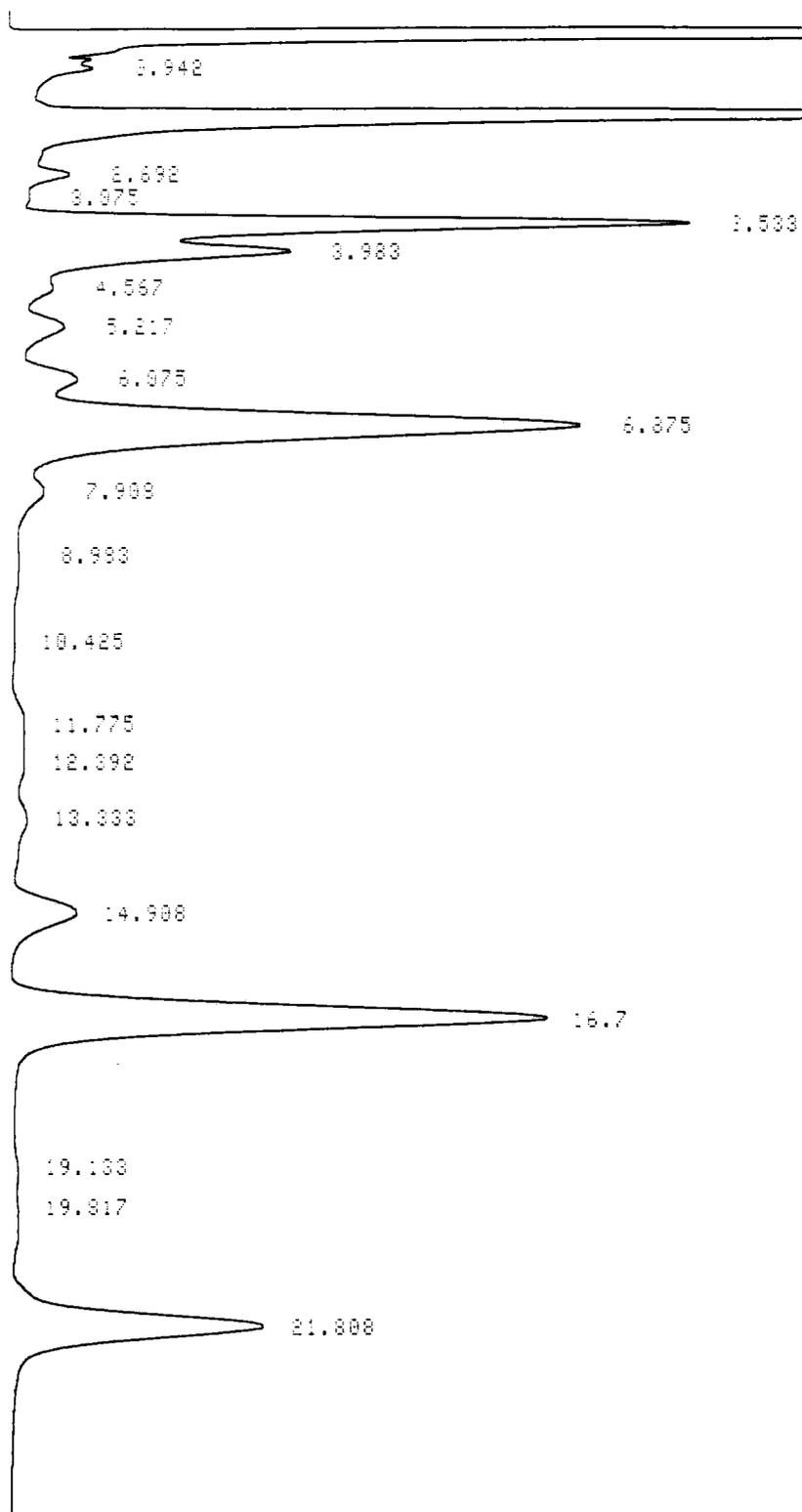
(10 minutes), rate of increase 4°C/minute (10 minutes), final temperature = 250°C (5 minutes), injection temperature = 260°C, detector temperature = 260°C, carrier gas flow rate 59 ml/minute. Peaks were identified by comparison of their relative retention times to C16:0 (RRT) with the RRTs of FAME standards (Ackman, 1969). Figure 4.2 shows a typical GLC spectrum for our samples. The attached integrator produced an output with the relative percentage of each peak and its corresponding RRT.

4.2.8. Statistical Analysis

The data were input in SPSS (Windows version) files and analysed using the SPSS software. Group means were compared using the one-way analysis of variance combined with the least significant difference range test (equivalent to an inter-group T-test). Where a significant difference was found, the groups were labelled with letters in alphabetic order, starting from the group with the lowest mean to those with higher means (a < b < c etc.). The level of significance was $p \leq 0.05$.

4.3 Results

4.3.1. Total Phospholipid, Cholesterol and Cholesterol/Phospholipid Molar Ratio

Tables 4.2 and 4.3 show the results of the total phospholipid and total cholesterol assays on lipid extracts from plasma membranes of cold- and warm-acclimated *Cancer pagurus* and *Carcinus maenas*, respectively, and the calculated cholesterol/phospholipid (Ch/PL) molar ratios.

In the winter experiment only two acclimation groups were compared: 5°C and 15°C. In *Cancer pagurus* no difference was observed between the 5°C and 15°C acclimated crabs. In *Carcinus maenas* the concentration of phospholipid was significantly lower in membranes of 15°C-acclimated crabs and the Ch/PL molar ratio slightly higher.

Table 4.2. Total **phospholipid** and **cholesterol** concentrations (mg/g fresh weight) and the calculated **cholesterol/phospholipid molar ratios** in lipid extracts from plasma membranes of *Cancer pagurus* (means±S.D.): significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order **a<b**

SEASON	LIPID	ACCLIMATION TEMPERATURE				STAT.
		5°C (*n*=4)		15°C (*n*=4)		ANALYSIS
Winter	Total Phospholipid	0.75±0.19		0.69±0.17		
	Total Cholesterol	0.07±0.01		0.07±0.01		
	Molar Ratio Ch/ PL	0.19±0.02		0.21±0.06		
			8°C (*n*=4)		22°C (*n*=6)	
Spring	Total Phospholipid		0.94±0.14		0.67±0.13	- b - a
	Total Cholesterol		0.11±0.03		0.07±0.01	- b - a
	Molar Ratio Ch/ PL		0.34±0.09		0.29±0.04	
		5°C (*n*=4)	8°C (*n*=8)	15°C (*n*=4)	22°C (*n*=8)	
Autumn	Total Phospholipid	1.23±0.14	0.63±0.13	1.21±0.06	0.68±0.26	b a b a
	Total Cholesterol	0.11±0.01	0.08±0.02	0.11	0.11±0.01	b a b b
	Molar Ratio Ch/ PL	0.25±0.02	0.34±0.08	0.25±0.01	0.49±0.16	a a a b

Table 4.3. Total **phospholipid** and **cholesterol** concentrations (mg/g fresh weight) and the calculated **cholesterol/phospholipid molar ratios** in lipid extracts from plasma membranes of *Carcinus maenas* (means±S.D.): significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order
a≤ab≤b, b≤bc≤c

SEASON	LIPID	ACCLIMATION TEMPERATURE				STAT.
		5°C (*n*=4)		15°C (*n*=4)		ANALYSIS
Winter	Total Phospholipid	0.68±0.07		0.52±0.11		b - a -
	Total Cholesterol	0.11		0.10±0.01		
	Molar Ratio Ch/ PL	0.31±0.04		0.38±0.06		a - b -
			8°C (*n*=4)		22°C (*n*=4)	
Spring	Total Phospholipid		0.70±0.08		0.13	- b - a
	Total Cholesterol		0.15±0.06		0.06±0.02	- b - a
	Molar Ratio Ch/ PL		0.61±0.27		1.18±0.49	
		5°C (*n*=4)	8°C (*n*=8)	15°C (*n*=4)	22°C (*n*=8)	
Autumn	Total Phospholipid	0.66±0.07	0.43±0.14	0.81±0.14	0.54±0.14	bc a c ab
	Total Cholesterol	0.09±0.01	0.07±0.02	0.12±0.01	0.11±0.01	a a b b
	Molar Ratio Ch/ PL	0.39±0.04	0.50±0.18	0.42±0.07	0.61±0.17	a ab a b

In the spring experiment the total phospholipid and cholesterol assays were carried out only for the 8°C and 22°C acclimation groups. In both species, plasma membranes of 8°C-acclimated crabs had higher concentrations of total phospholipid and cholesterol than those of 22°C-acclimated crabs. The Ch/PL molar ratio was not influenced by the acclimation temperature in *Cancer pagurus* (Table 4.2). In *Carcinus maenas*, the average Ch/PL molar ratio was higher in warm-acclimated crabs, but the difference was not significant, due to large variability of individual data (Table 4.3).

In the autumn experiment the assays were carried out on all acclimation groups. The 8°C-acclimated crabs had the lowest concentrations of total phospholipid and cholesterol. In both species, the 5°C and 15°C-acclimated groups had the highest levels of total phospholipid (Tables 4.2 and 4.3). The concentration of total cholesterol varied very little between acclimation groups in *Cancer pagurus*, but in *Carcinus maenas* it increased with the acclimation temperature. The 15°C and 22°C-acclimated *Carcinus* had significantly higher levels of cholesterol than the 5°C and 8°C-acclimated ones. The Ch/PL molar ratios were higher in membranes of 22°C-acclimated crabs, in both species. For *Carcinus* the difference was significant only between the 22°C-acclimated crabs and the 5°C and 15°C-acclimated ones (Table 4.3).

The concentration of total phospholipid was higher in membranes of *Cancer pagurus* than in those of *Carcinus maenas* in both cold- (5°C or 8°C) and warm- (15°C or 22°C) acclimated groups, and in both winter and spring experiments. Therefore *Cancer pagurus* membranes had significantly lower Ch/PL ratios than those of *Carcinus maenas*. A similar situation was found in the autumn experiments (Tables 4.2 and 4.3, Figure 4.3). There were little differences in the concentration of cholesterol in membranes between the two species. No reason could be found for the similarity of the patterns of variation of the Ch/PL molar ratio with acclimation temperature in the two species, shown in Figure 4.3.

Figure 4.3. The cholesterol/phospholipid molar ratios (Ch/PL, means±S.D.) in plasma membranes isolated from crabs acclimated in **autumn**: comparison between *Cancer pagurus* (5°C AT $n=4$, 8°C AT $n=8$, 15°C AT $n=4$, 22°C AT $n=8$) and *Carcinus maenas* (5°C AT $n=4$, 8°C AT $n=8$, 15°C AT $n=4$, 22°C AT $n=8$)
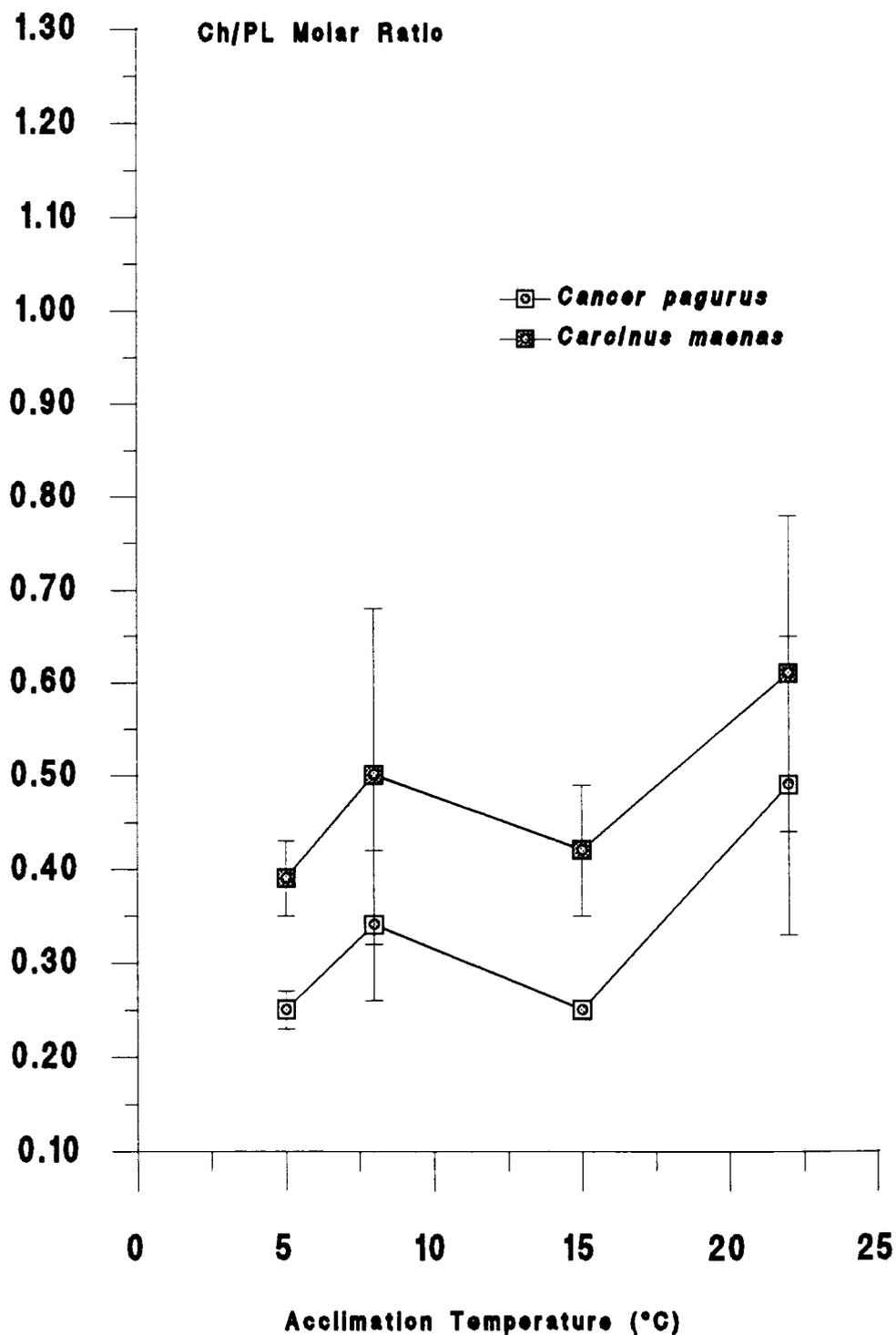
The difference observed in spring between the levels of total phospholipid and cholesterol in 8°C- and 22°C-acclimated crabs was greater in *Carcinus maenas* (Table 4.3) than in *Cancer pagurus* (Table 4.2).

A seasonal comparison between winter and autumn showed that both 5°C and 15°C-acclimated *Cancer pagurus* had lower levels of total phospholipid and cholesterol in winter than in autumn, and lower Ch/PL ratios (Figure 4.4.a). In *Carcinus maenas* only the 5°C-acclimated crabs had slightly lower Ch/PL molar ratios in winter compared to autumn (Figure 4.4.b).

Plasma membranes of 8°C-acclimated crabs contained more phospholipid and cholesterol in spring than in autumn, in both species, but with no seasonal difference in the Ch/PL ratio. A seasonal difference in the Ch/PL ratio was observed between the 22°C-acclimated crabs (Figure 4.4.a and b). Membranes of 22°C-acclimated *Cancer pagurus* contained more cholesterol in autumn, and consequently had higher Ch/PL ratios than in spring. Membranes of 22°-acclimated *Carcinus maenas* also had higher cholesterol levels in autumn, but the Ch/PL ratios were lower than in spring, due to significantly higher concentrations of phospholipid in autumn compared to spring.

4.3.2. Fatty Acid Composition

The major phospholipid classes in the total lipid extracts of crab muscle plasma membranes were phosphatidylcholine (PC) and phosphatidylethanolamine (PE). In both PC and PE, the fatty acids present in higher proportions were the polyunsaturated C20:5, C22:6 and C20:4, the monounsaturated C18:1 and C16:1 and the saturated C16:0 and C18:0.

The results shown in the following paragraphs are the relative percentages of individual fatty acids in PC, PE and in the total phospholipid fraction (TPL), together with the results of the sums of total saturated, monounsaturated and polyunsaturated fatty acids and the ratio total saturated/total unsaturated fatty acids (S/U). Other calculations shown are the distribution by chain length and the relative proportion of each of the desaturation-elongation fatty acid series: ω3 and ω6 (ω9 is identical with

Figure 4.4. The cholesterol/phospholipid molar ratios (Ch/PL, means±S.D.) in plasma membranes isolated from **(a)** *Cancer pagurus* and **(b)** *Carcinus maenas* acclimated in winter, spring and autumn: **seasonal** comparison

a) *Cancer pagurus*
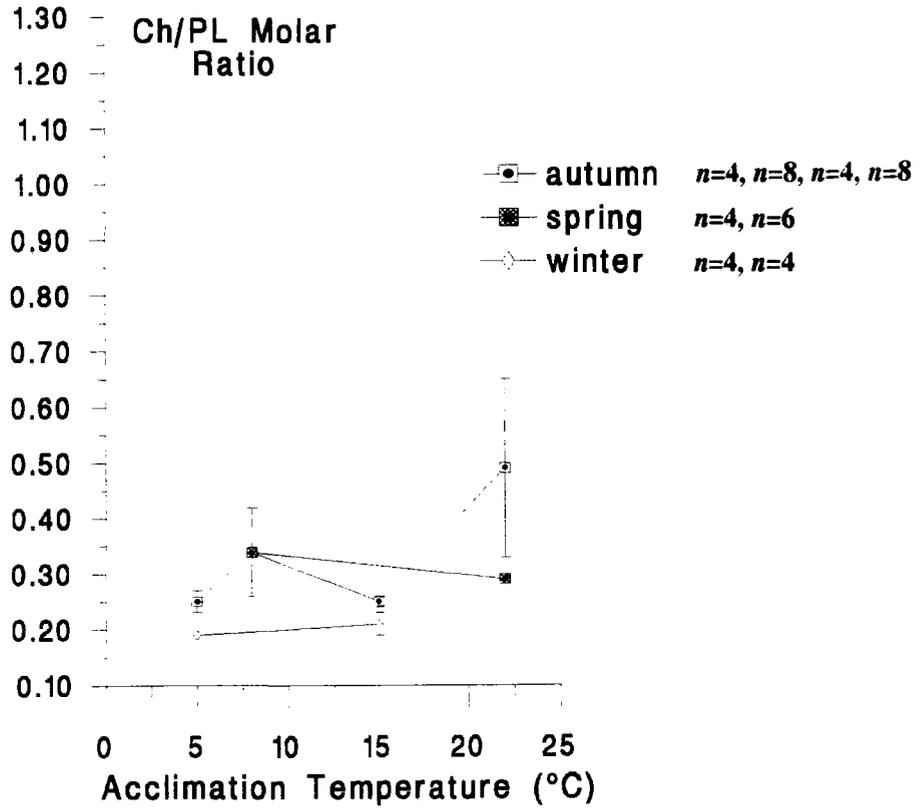
b) *Carcinus maenas*
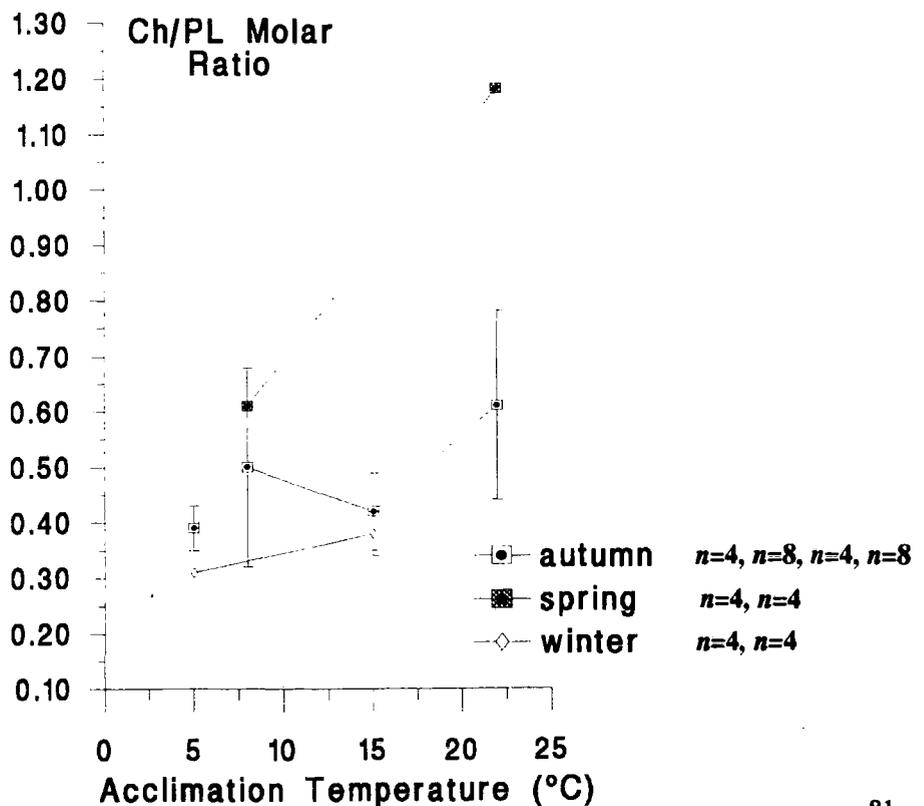
the total monounsaturated fatty acids, in our results). The data tables also include the results of the inter-acclimation groups t-tests.

As the acclimation-temperature-related variations in the relative proportions of individual fatty acids and also of chain-length groups and ω3 and ω6 series did not follow a clear and consistent pattern, the results description will refer mainly to the major categories of fatty acids (saturated, monounsaturated and polyunsaturated) and the S/U ratios.

4.3.2.1. Total Phospholipid Fraction (TPL)

Tables 4.4 and 4.5 show the summary of the fatty acid analysis on the total phospholipid fraction from plasma membranes of *Cancer pagurus* and *Carcinus maenas* acclimated in winter (5°C and 15°C acclimation temperature). Tables 4.6 and 4.7 show the corresponding results for crabs acclimated in autumn (5°C, 8°C, 15°C and 22°C acclimation temperature).

In the winter experiment, the 15°C-acclimated *Cancer pagurus* had significantly higher levels of saturated fatty acids, and slightly lower monounsaturated and polyunsaturated fatty acids than the 5°C-acclimated crabs (Table 4.4). Significant increases in C16:0 and C18:0, and decreases in C16:1 and C20:5 were observed. The ratio saturated/unsaturated fatty acids (S/U) was significantly higher for membranes of warm-acclimated *Cancer*. Warm-acclimation to 15°C induced less marked differences in the fatty acid composition of TPL fraction of *Carcinus maenas* membranes, and the S/U ratio was only slightly higher in membranes of 15°C-acclimated crabs, compared to the 5°C acclimated ones (Table 4.5).

In the autumn experiment no significant difference in the levels of total saturated, monounsaturated and polyunsaturated fatty acids was observed between the 5°C and 15°C-acclimated crabs, in either species (Tables 4.6 and 4.7). When all the four acclimation groups were compared, it was found that 22°C-acclimated *Cancer pagurus* had slightly higher levels of saturated fatty acids and lower levels of polyunsaturated fatty acids, leading to slightly higher S/U ratios than in the

Table 4.4. Fatty acid composition (relative percentages, means±S.D.) of the total phospholipid class (**TPL**) separated from lipid extracts of muscle plasma membranes of *Cancer pagurus* acclimated in **winter**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order **a<b**

FATTY ACID	ACCLIMATION TEMP.		STAT. ANALYSIS
	5°C (*n*=4)	15°C (*n*=4)	
C16:0	10.6±0.4	11.3±0.9	a b
C16:1w9	6.2±0.8	5.0±0.9	b a
C17:0	0.9±0.1	0.9±0.2	
C17:1w9	2.3±0.3	2.0±0.1	
C18:0	4.2±0.4	5.9±0.6	a b
C18:1w9	20.2±1.1	20.4±0.7	
C18:2w6	1.5±0.2	2.3±0.4	a b
C18:3w6	0.4±0.1	0.2	b a
C18:3w3	0.1±0.1	0.2±0.1	
C20:1w9	1.0±0.1	0.9±0.1	
C20:2w6	1.0±0.3	1.2±0.1	
C20:4w6	4.2±0.2	3.8±1.0	
C20:5w3	30.4±0.9	27.9±1.3	b a
C22:5w3	0.2±0.4	0.9±0.9	
C22:6w3	13.9±0.6	14.5±0.9	
Saturated	16.8±0.3	19.2±0.4	a b
Monounsat.	29.9±1.2	28.5±0.7	
Polyunsat.	52.6±0.9	51.7±0.9	
Ratio S/U	0.20±0.01	0.24±0.01	a b
C 16	16.8±0.6	16.3±0.6	
C 17	3.2±0.2	2.9±0.2	
C 18	26.5±0.6	29.0±0.6	a b
C 20	36.9±0.7	34.2±0.9	b a
C 22	15.2±0.5	16.0±0.7	
w3	45.1±0.8	43.6±0.7	b a
w6	7.9±0.3	8.3±0.4	

Table 4.5. Fatty acid composition (relative percentages, means±S.D.) of the total phospholipid class (**TPL**) separated from lipid extracts of muscle plasma membranes of *Carcinus maenas* acclimated in **winter**: no two groups are significantly different at p≤0.05

FATTY ACID	ACCLIMATION TEMP.		STAT. ANALYSIS
	5°C (*n*=4)	15°C (*n*=4)	
C16:0	10.3±0.7	10.3±0.6	
C16:1w9	5.6±0.8	5.2±0.8	
C17:0	1.2±0.2	1.2±0.1	
C17:1w9	2.1±0.7	2.1±0.6	
C18:0	5.3±0.4	6.1±0.7	
C18:1w9	20.4±1.8	20.2±2.4	
C18:2w6	1.3±0.2	1.0±0.3	
C18:3w6	0.2±0.1	0.2±0.1	
C18:3w3	0.4±0.2	0.2±0.1	
C20:1w9	1.4±0.4	0.9±0.2	
C20:2w6	1.4±0.2	1.3±0.1	
C20:4w6	5.2±1.2	4.6±0.4	
C20:5w3	27.8±3.4	29.3±3.1	
C22:5w3	0.2±0.2	0.2±0.2	
C22:6w3	14.1±2.1	13.6±0.5	
Saturated	18.1±0.6	18.7±0.4	
Monounsat.	29.9±1.1	28.6±0.9	
Polyunsat.	51.5±0.9	51.1±1.1	
Ratio S/U	0.22±0.02	0.23±0.03	
C 16	15.9±0.7	15.5±0.6	
C 17	3.3±0.4	3.2±0.3	
C 18	27.6±0.9	27.8±1.1	
C 20	36.0±1.4	36.2±1.6	
C 22	15.3±1.2	14.6±0.8	
w3	43.2±1.9	43.9±1.5	
w6	8.3±0.8	7.4±0.5	

Table 4.6. Fatty acid composition (relative percentages, means±S.D.) of the total phospholipid class (**TPL**) separated from lipid extracts of muscle plasma membranes of *Cancer pagurus* acclimated in **autumn**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order **a≤ab≤b, b≤bc≤c**)

FATTY ACID	ACCLIMATION TEMPERATURE				STAT. ANALYSIS
	5°C (*n*=2)	8°C (*n*=4)	15°C (*n*=2)	22°C (*n*=4)	
C16:0	10.7	10.9	10.7±0.2	10.8±0.1	
C16:1w9	7.1±0.7	6.5	5.5	4.5±0.2	c bc ab a
C17:0	1.2±0.4	0.9±0.2	0.8±0.3	0.9	
C17:1w9	1.3±0.1	1.1	1.0	0.8	c b b a
C18:0	4.3±0.1	4.9±0.1	4.6±0.2	5.8	a b b c
C18:1w9	19.7±0.1	21.2±0.2	20.9±0.5	23.4±0.3	a b b c
C18:2w6	3.2±0.1	2.4±0.1	0.4±0.1	2.4±0.1	c b a b
C18:3w6	0	0	0	0	
C18:3w3	0.1	0.1	0	0.1	b b a b
C20:1w9	0.7	0.8	1.1	0.7	a b c ab
C20:2w6	1.4±0.1	1.5	1.1	1.8	b b a c
C20:4w6	4.6±0.3	4.2	4.0	5.3±0.1	b ab a c
C20:5w3	28.8±2.5	30.3±0.1	32.6±0.2	28.0±0.1	a ab b a
C22:5w3	0.2	0.2±0.1	0.3	0.2±0.1	
C22:6w3	14.9±1.2	13.7±0.4	15.7±0.2	13.9±0.2	
Saturated	16.8±0.3	17.4±0.3	16.7±0.3	18.0	a ab a b
Monounsat.	28.9±0.9	29.5±0.2	28.5±0.5	29.4±0.7	
Polyunsat.	53.7±0.7	52.7±0.6	54.9±0.1	52.0±0.7	bc ab c a
Ratio S/U	0.20	0.21±0.01	0.20±0.01	0.22	ab bc a c
C 16	17.8±0.7	17.4	16.2±0.2	15.3±0.3	b b a a
C 17	2.5±0.3	2.0±0.2	1.8±0.3	1.7±0.1	b ab a a
C 18	27.2±0.3	28.6±0.5	26.0±0.2	31.7±0.7	ab b a c
C 20	35.5±2.1	36.8±0.1	38.9±0.2	35.9±0.3	a ab b a
C 22	15.7±1.3	14.2±0.8	16.7±0.2	14.4±0.6	ab a b ab
w3	44.5±1.2	44.6±0.7	49.4	42.5±0.8	a a b a
w6	9.1±0.5	8.1±0.1	5.5±0.1	9.5	c b a c

Table 4.7. Fatty acid composition (relative percentages, means±S.D.) of the total phospholipid class (**TPL**) separated from lipid extracts of muscle plasma membranes of *Carcinus maenas* acclimated in **autumn**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order a≤ab≤b, b≤ bc≤c

FATTY ACID	ACCLIMATION TEMPERATURE				STAT. ANALYSIS
	5°C (*n=2*)	8°C (*n=4*)	15°C (*n=2*)	22°C (*n=4*)	
C16:0	8.5±0.1	8.8±0.1	9.4±0.1	8.8	a ab c b
C16:1w9	5.7±0.2	5.4±0.1	6.5±0.1	6.0±0.1	ab a c b
C17:0	1.0±0.5	0.7	1.0±0.4	0.7	
C17:1w9	1.9	2.2±0.1	1.7	1.9	b c a b
C18:0	5.5	5.8±0.2	5.4±0.1	6.0±0.1	ab bc a c
C18:1w9	24.4±1.0	23.9±0.2	22.4±0.7	24.2±0.5	b ab a ab
C18:2w6	0.4±0.1	2.1	0.4	0.4	a b a a
C18:3w6	0	0	0	0	
C18:3w3	0	0	0	0	
C20:1w9	0.8	0.7	0.8	0.6	c b c a
C20:2w6	1.9±0.1	1.9	1.6±0.1	1.6	b b a a
C20:4w6	4.4±0.1	3.9±0.2	4.0±0.1	4.3	b a a ab
C20:5w3	28.5±0.5	28.8±0.4	30.7±0.6	28.7±0.1	a a b a
C22:5w3	0	0	0	0	
C22:6w3	15.7±0.4	14.5±0.4	15.0±0.3	15.6±0.1	b a ab b
Saturated	15.4±0.5	15.7±0.3	16.3±0.6	16.0±0.3	
Monounsat.	32.8±0.8	32.2	31.4±0.6	32.7±0.9	
Polyunsat.	51.1±1.3	51.5±0.3	51.8±1.2	51.0±0.6	
Ratio S/U	0.18±0.01	0.19	0.20±0.01	0.19	
C 16	14.2±0.1	14.2±0.1	15.9	14.8±0.2	a a c b
C 17	2.9±0.5	2.9±0.1	2.7±0.5	2.6	
C 18	30.3±0.8	31.8±0.4	28.2±0.7	30.6±0.5	b b a b
C 20	35.7±0.7	35.5±0.3	37.1±0.8	35.2±0.1	ab ab b a
C 22	15.9±0.4	14.6±0.6	15.2±0.3	16.0±0.5	ab a ab b
w3	44.5±1.0	43.5	46.0±0.9	44.8±0.4	ab a b ab
w6	6.7±0.3	8.0±0.3	5.9±0.3	6.2±0.2	b c a ab

membranes of 15°C, 8°C and 5°C-acclimated crabs. No differences were found between the fatty acid composition of TPL fractions from *Carcinus maenas* acclimated to different temperatures.

A comparison between the fatty acid composition of the TPL (Tables 4.4-4.7) in either species and the average fatty acid composition of the fish used as food (Table 4.1) revealed a few major differences. The levels of 16:0, 20:0, 20:1 and 22:6 were lower and the levels of 17:1, 18:0, 18:1, 20:4 and 20:5 were higher in crab membranes than in fish extract. It was assumed that the two weeks of starvation prior to membrane preparation for lipid analysis would reduce the influence of variations in the lipid composition of diet and the amount of food ingested.

4.3.2.2. Phosphatidylcholine (PC)

Tables 4.8-4.13 show the summary of the fatty acid analysis on the PC fraction from plasma membranes of *Cancer pagurus* and *Carcinus maenas* acclimated in winter (Tables 4.8 and 4.9), spring (Tables 4.10 and 4.11) and autumn (Tables 4.12 and 4.13).

In the winter experiment, the 15°C-acclimated crabs of both species had higher levels of C18:0 and lower levels of C16:1 in the PC fraction of their plasma membranes, compared to 5°C-acclimated crabs (Tables 4.8 and 4.9). These differences, combined with other smaller and not always significant variations, summed up as significantly higher levels of saturated fatty acids and lower levels of polyunsaturated fatty acids in the membranes of 15°C-acclimated crabs, and higher S/U ratios. The levels of monounsaturated fatty acids were reduced by warm-acclimation only in *Cancer pagurus* (Table 4.8).

In the spring experiment the 15°C and 22°C-acclimated crabs of both species had higher levels of C18:0 than the 5°C and 8°C-acclimated ones (Tables 4.10 and 4.11). The levels of C20:5 were lower only in the 15°C-acclimated crabs compared with the 5°C and 8°C-acclimated ones (both species). The 22°C-acclimated crabs had similar levels of C20:5 with the cold-acclimated crabs. In *Cancer pagurus*, the levels

Table 4.8. Fatty acid composition (relative percentages, means±S.D.) of phosphatidylcholine (**PC**) separated from lipid extracts of muscle plasma membranes of *Cancer pagurus* acclimated in **winter**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order **a<b**

FATTY ACID	ACCLIMATION TEMP.		STAT. ANALYSIS
	5°C (n=4)	15°C (n=3)	
C16:0	15.4±1.3	17.4±0.3	
C16:1w9	9.9±0.6	6.8±0.1	b a
C17:0	1.2±0.1	1.6±0.3	a b
C17:1w9	2.5±0.2	3.0±0.3	a b
C18:0	1.7±0.3	4.0±0.2	a b
C18:1w9	23.9±1.6	21.9±0.1	
C18:2w6	2.8±0.3	2.4±0.3	
C18:3w6	0.3±0.1	0.6±0.2	
C18:3w3	0.6±0.2	0.2±0.3	
C20:1w9	1.2±0.2	1.6±0.2	a b
C20:2w6	0.9±0.1	1.1±0.2	
C20:4w6	3.9±0.1	2.9±0.2	b a
C20:5w3	23.8±1.0	19.5±0.4	b a
C22:5w3	0.1	0.1±0.1	
C22:6w3	7.2±1.6	7.5±0.4	
Saturated	20.6±0.6	29.7±1.5	a b
Monounsat.	37.7±1.9	34.3±0.4	b a
Polyunsat.	40.7±2.5	35.4±1.6	b a
Ratio S/U	0.26±0.01	0.43±0.03	a b
C 16	25.3±1.8	24.2±0.4	
C 17	3.7±0.4	4.6±0.5	a b
C 18	29.4±2.0	29.1±0.2	
C 20	30.9±1.0	25.6±0.8	b a
C 22	7.9±1.8	8.4±0.4	
w3	31.9±2.6	27.3±1.2	b a
w6	8.8±0.3	7.8±0.6	b a

Table 4.9. Fatty acid composition (relative percentages, means±S.D.) of phosphatidylcholine (**PC**) separated from lipid extracts of muscle plasma membranes of *Carcinus maenas* acclimated in **winter**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order **a<b**

FATTY ACID	ACCLIMATION TEMP.		STAT. ANALYSIS
	5°C (n=4)	15°C (n=4)	
C16:0	13.7±1.7	15.4±0.7	
C16:1w9	10.3±0.4	7.0±1.2	b a
C17:0	1.4±0.3	1.5±0.1	
C17:1w9	2.9±0.6	3.6±0.2	
C18:0	2.8±0.2	4.0±0.6	a b
C18:1w9	21.9±0.7	24.7±0.8	a b
C18:2w6	2.3±0.5	2.4±0.4	
C18:3w6	0.2±0.2	0.3	
C18:3w3	0.8±0.3	0.5±0.1	
C20:1w9	1.6±0.2	1.3±0.2	
C20:2w6	1.5±0.4	1.0±0.1	
C20:4w6	2.3±0.2	2.8±0.2	a b
C20:5w3	22.3±3.0	19.5±1.8	
C22:5w3	0.2±0.1	0.2±0.2	
C22:6w3	10.9±1.2	8.5±1.9	
Saturated	20.9±0.7	24.6±2.3	a b
Monounsat.	37.0±0.7	38.3±2.7	
Polyunsat.	41.0±1.3	36.7±4.4	
Ratio S/U	0.27±0.01	0.33±0.04	a b
C 16	24.0±1.3	22.4±1.9	
C 17	4.3±0.8	5.1±0.2	
C 18	28.0±0.3	31.9±0.7	a b
C 20	28.4±2.5	25.5±1.9	
C 22	11.5±1.1	9.7±2.9	
w3	34.2±2.0	29.0±4.0	
w6	6.8±0.6	7.6±0.8	

Table 4.10. Fatty acid composition (relative percentages, means±S.D.) of phosphatidylcholine (**PC**) separated from lipid extracts of muscle plasma membranes of *Cancer pagurus* acclimated in **spring**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order a≤ab≤b, b≤bc≤c

FATTY ACID	ACCLIMATION TEMPERATURE				STAT. ANALYSIS
	5°C (*n*=4)	8°C (*n*=4)	15°C (*n*=4)	22°C (*n*=6)	
C16:0	16.6±0.9	16.7±1.7	17.8±1.1	16.2±0.8	ab ab b a
C16:1w9	8.2±0.9	9.0±1.4	9.2±2.6	7.2±1.1	
C17:0	1.8±0.2	0.8±0.3	1.5±0.1	1.1±0.3	c a b a
C17:1w9	3.0±0.7	1.5±0.3	2.4±0.3	1.6±0.4	b a b a
C18:0	1.6±0.4	1.3±0.5	2.3±0.2	2.7±0.6	a a b b
C18:1w9	24.1±2.6	24.1±1.2	23.8±0.7	26.9±3.0	ab ab a b
C18:2w6	2.4±0.1	1.3±0.3	2.0±0.5	1.3±0.4	b a b a
C18:3w6	0.8±0.3	0.2±0.1	0.7±0.3	0.2±0.1	b a b a
C18:3w3	0	0.1±0.1	0.1±0.1	0	
C20:1w9	1.7±0.3	1.1±0.4	1.3±0.2	1.0±0.3	b a ab a
C20:2w6	0.8±0.2	0.5	0.8±0.4	1.4±0.3	a a a b
C20:4w6	3.2±0.2	2.9±0.1	3.1±0.2	3.2±0.7	
C20:5w3	23.4±2.1	26.3±0.8	19.9±1.3	23.1±1.5	b c a b
C22:5w3	1.1±1.3	0.2±0.1	0.1±0.1	0.3±0.3	b a a ab
C22:6w3	9.1±1.0	11.3±1.7	10.0±0.3	12.3±2.4	a ab ab b
Sat	21.2±1.5	19.8±1.8	23.6±0.3	20.6±0.7	a a b a
Monounsat.	37.1±1.2	35.8±2.9	37.0±3.4	36.6±2.9	
Polyunsat.	41.2±1.4	43.6±1.2	38.4±3.6	42.2±3.0	ab b a b
Ratio S/U	0.27±0.03	0.25±0.03	0.31±0.01	0.26±0.01	a a b a
C 16	24.8±1.7	25.7±1.0	27.1±1.8	23.4±1.3	ab bc c a
C 17	4.9±0.9	2.3±0.4	3.9±0.2	2.7±0.6	c a b a
C 18	28.8±1.8	27.0±1.1	28.9±0.7	30.7±3.1	ab a ab b
C 20	29.6±2.1	31.2±0.7	25.6±1.7	28.9±0.9	bc c a b
C 22	10.3±0.9	11.9±1.6	11.6±1.5	12.9±2.4	a ab ab b
w3	33.7±1.8	38.1±1.5	30.9±2.2	35.7±2.5	ab c a bc
w6	7.5±0.8	5.5±0.3	7.6±1.5	6.5±1.2	b a b ab

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Table 4.11. Fatty acid composition (relative percentages, means±S.D.) of phosphatidylcholine (**PC**) separated from lipid extracts of muscle plasma membranes of *Carcinus maenas* acclimated in **spring**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order **a≤ab≤b, b≤bc≤c**

FATTY ACID	ACCLIMATION TEMPERATURE				STAT. ANALYSIS
	5°C (*n*=4)	8°C (*n*=4)	15°C (*n*=4)	22°C (*n*=2)	
C16:0	16.2±0.6	17.4±0.8	18.0±1.6	15.4±0.7	a ab b a
C16:1w9	8.4±0.2	6.7±0.9	7.5±0.7	5.1±0.7	c b bc a
C17:0	1.6±0.1	0.9±0.2	1.8±0.1	0.7	b a c a
C17:1w9	3.3±0.1	1.3±0.3	3.5±0.3	1.1±0.2	b a b a
C18:0	2.2±0.2	1.8±0.3	3.1±0.3	3.4±0.3	b a c c
C18:1w9	24.3±1.6	24.7±1.0	26.0±2.1	29.7±3.9	a a ab b
C18:2w6	2.1±0.5	0.9±0.6	2.3±0.4	0	b a b a
C18:3w6	0.1±0.1	0.1±0.1	0	0	
C18:3w3	0.9±0.4	0.2±0.1	1.5±0.4	0.1	b a c a
C20:1w9	1.5±0.1	1.1±0.2	2.2±0.6	1.1±0.1	a a b a
C20:2w6	1.7±0.6	1.4±0.2	2.1±0.4	1.2±0.3	ab a b a
C20:4w6	3.5±0.6	3.0±0.6	3.1±0.3	3.7±0.6	
C20:5w3	22.4±0.5	26.5±1.8	19.1±3.7	25.2±4.1	ab c a bc
C22:5w3	0	0	0.1±0.2	0.1±0.1	
C22:6w3	8.7±0.9	12.2±2.2	8.2±1.7	12.4±0.6	a b a b
Saturated	22.0±0.7	20.7±1.0	24.3±2.1	19.8±0.8	a a b a
Monounsat.	37.8±1.7	33.8±1.7	39.3±3.2	37.0±4.9	ab a b ab
Polyunsat.	39.8±1.7	44.7±2.3	36.5±4.8	42.7±4.5	ab b a ab
Ratio S/U	0.28±0.01	0.26±0.02	0.32±0.04	0.25±0.01	a a b a
C 16	24.6±0.5	24.1±1.1	25.4±2.2	20.6±0.01	b b b a
C 17	4.9±0.1	2.2±0.4	5.3±0.4	1.8±0.2	b a b a
C 18	29.6±0.9	27.6±1.0	32.9±2.6	33.2±3.5	ac a bc c
C 20	29.5±2.1	32.0±2.1	27.2±3.3	31.3±4.9	ab b a ab
C 22	9.2±1.1	12.7±2.2	8.5±1.8	12.6±0.5	a b a b
w3	32.2±0.7	39.3±1.8	28.9±5.2	37.9±3.7	ab c a bc
w6	7.5±1.6	5.4±0.9	7.5±0.9	4.9±0.9	b a b a

Table 4.12. Fatty acid composition (relative percentages, means±S.D.) of phosphatidylcholine (**PC**) separated from lipid extracts of muscle plasma membranes of *Cancer pagurus* acclimated in **autumn**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order a≤ab≤b, b≤bc≤c

FATTY ACID	ACCLIMATION TEMPERATURE				STAT. ANALYSIS
	5°C (*n*=2)	8°C (*n*=4)	15°C (*n*=2)	22°C (*n*=4)	
C16:0	14.4±0.3	15.7±1.0	13.1±0.1	13.9±0.3	ab b a a
C16:1w9	7.7±0.2	8.1±1.0	7.8±0.1	6.7±0.8	
C17:0	1.4±0.2	0.7±0.4	0.8	0.7±0.1	
C17:1w9	1.3±0.2	1.0±0.2	1.4	0.9±0.3	
C18:0	0.8	1.2±0.2	2.7±0.8	1.8±0.2	a a b ab
C18:1w9	20.4±0.4	24.0±0.7	21.6±0.7	28.5±1.1	a b a c
C18:2w6	3.6±0.8	2.4±0.5	2.4±0.1	1.7±1.3	
C18:3w6	0.1	0	0.1±0.1	0	
C18:3w3	0	0	0	0	
C20:1w9	0.8±0.1	0.8±0.1	1.5±0.3	0.8	a a b a
C20:2w6	0.9±0.1	1.0±0.1	1.1±0.3	2.0±0.4	a a a b
C20:4w6	4.0±0.6	3.0±0.3	3.9±0.7	4.4±0.3	ab a ab b
C20:5w3	24.4±1.4	24.8±1.1	24.8±0.1	25.1±1.2	
C22:5w3	0.1±0.1	0.5±0.1	0.6±0.3	0.1	a b b a
C22:6w3	16.4±0.4	13.8±1.6	16.5±0.2	12.4±1.4	b ab b a
Saturated	17.5±0.1	18.6±1.6	17.4±0.7	17.0±0.7	
Monounsat.	30.2±0.4	33.8±1.7	32.3±0.5	36.9±1.6	a b ab c
Polyunsat.	51.3±0.3	46.6±0.5	50.3±1.2	45.9±2.2	b a b a
Ratio S/U	0.22	0.23±0.02	0.21±0.01	0.21±0.01	
C 16	22.0±0.7	23.8±0.4	20.9±0.2	20.6±1.0	a b a a
C 17	2.7±0.6	1.8±0.6	2.2	1.5±0.4	b ab ab a
C 18	24.9±1.7	27.6±1.0	26.8±2.1	32.0±0.2	a b ab c
C 20	30.7±2.8	29.9±1.7	31.4±1.9	32.3±1.2	
C 22	17.9±1.2	15.1±1.8	18.1±0.1	12.7±1.4	bc ab c a
w3	41.4±1.1	39.9±0.5	42.9±0.2	37.8±0.7	c b c a
w6	9.9±0.8	6.7±0.9	7.4±1.4	8.1±1.7	b a ab ab

Table 4.13. Fatty acid composition (relative percentages, means±S.D.) of phosphatidylcholine (**PC**) separated from lipid extracts of muscle plasma membranes of *Carcinus maenas* acclimated in **autumn**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order **a≤ab≤b**

FATTY ACID	ACCLIMATION TEMPERATURE				STAT. ANALYSIS
	5°C (*n*=2)	8°C (*n*=4)	15°C (*n*=2)	22°C (*n*=4)	
C16:0	11.4±0.1	14.5±0.8	14.2	13.6±0.3	a b b b
C16:1w9	6.4±0.3	7.6±0.2	7.7±0.5	7.8±1.4	
C17:0	1.2±0.2	0.2±0.1	1.2±0.4	0.6±0.4	b a b ab
C17:1w9	1.4±0.1	2.2±0.2	1.9±0.1	1.6±0.3	a b ab a
C18:0	1.1±0.1	2.0±0.2	2.2	2.3±0.3	a b b b
C18:1w9	26.0±0.3	26.3±1.1	27.6±0.2	29.8±1.6	a a ab b
C18:2w6	0.3±0.1	2.1±0.7	1.0±0.1	0.2±0.1	a b a a
C18:3w6	0	0	0	0	
C18:3w3	0	0	0	0	
C20:1w9	0.6±0.1	0.7±0.1	0.7±0.3	0.5±0.1	
C20:2w6	1.0±0.2	1.3±0.2	1.1±0.2	1.4±0.1	
C20:4w6	2.6±0.1	2.9±0.2	2.6	3.1±0.3	a ab a b
C20:5w3	28.0±0.6	25.1±0.9	26.2±0.3	25.1±1.1	b a ab a
C22:5w3	0.5±0.2	0.1±0.1	0.5	0.1±0.1	b a b a
C22:6w3	17.3±0.7	12.8±0.7	11.5±1.4	13.0±1.6	b a a a
Saturated	14.7±0.1	17.6±1.0	18.4±0.8	17.1±0.4	a b b b
Monounsat.	34.4±0.4	36.8±1.2	37.9±0.8	39.7±3.0	a ab ab b
Polyunsat.	50.1±0.5	44.7±0.7	42.9±1.8	43.1±2.5	b a a a
Ratio S/U	0.17	0.22±0.02	0.23±0.01	0.21±0.01	a b b b
C 16	17.9±0.5	22.1±1.0	21.9±0.8	21.3±1.1	a b b b
C 17	2.6	2.4±0.1	3.1±0.5	2.3±0.4	ab a b a
C 18	27.5±0.7	30.5±1.5	30.8±0.3	32.3±1.8	a b ab b
C 20	32.5±0.9	30.2±1.2	30.9±0.8	30.2±1.2	
C 22	18.0±1.4	13.2±0.9	12.0±1.9	13.2±1.5	b a a a
w3	46.0±0.5	38.4±0.8	38.3±1.5	38.3±2.3	b a a a
w6	4.1	6.3±0.8	4.7±0.3	4.7±0.3	a b a a

of C22:6 increased with acclimation temperature. The same trend was observed in *Carcinus maenas* PC, with the exception of the 8°C-acclimated group. In both species, the 15°C-acclimated crabs had higher S/U ratios, resulted from higher levels of saturated and lower levels of polyunsaturated fatty acids. Rather surprising, the S/U ratio of 22°C-acclimated crabs was similar to those of cold-acclimated crabs.

The autumn experiment revealed little differences between the *Cancer pagurus* acclimation groups and a significant difference only between the 5°C-acclimated *Carcinus maenas* and the other three acclimation groups. In more detail, in *Cancer pagurus* PC differences were observed at C18:0 level, which increased with acclimation temperature, at C22:6 level, which was lower in 22°C acclimated crabs, and at total monounsaturated fatty acid level, which was higher in the 22°C-acclimated crabs. No difference in S/U ratios was observed. In *Carcinus maenas* the 5°C-acclimated crabs had lower levels of C16:0 and C18:0, and higher levels of C20:5 and C22:6, which summed up as lower levels of saturated and higher levels of polyunsaturated fatty acids and lower S/U ratios than the other acclimation groups.

4.3.2.3. Phosphatidylethanolamine (PE)

Tables 4.14 and 4.15 show the summary of the fatty acid analysis on the PE fraction from plasma membranes of *Cancer pagurus* and *Carcinus maenas* acclimated in winter. Tables 4.16 and 4.17 show the corresponding results for the spring experiment and Tables 4.18 and 4.19 for the autumn experiment.

In the winter experiment the 15°C-acclimated crabs, of both species, had increased levels of C16:0 and C17:0, and decreased levels of C18:1 and C20:5 compared to the 5°C-acclimated crabs (Tables 4.14 and 4.15). *Cancer pagurus* had also higher levels of C18:0 and lower levels of C20:4 in PE from warm-acclimated crabs. The total saturated fatty acids were significantly higher and the polyunsaturated fatty acids significantly lower in 15°C-acclimated *Cancer pagurus* and *Carcinus maenas* compared to the 5°C-acclimated crabs, leading to significantly higher PE S/U

Table 4.14. Fatty acid composition (relative percentages, means±S.D.) of phosphatidylethanolamine (**PE**) separated from lipid extracts of muscle plasma membranes of *Cancer pagurus* acclimated in **winter**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order **a<b**

| FATTY ACID	ACCLIMATION TEMP.		STAT. ANALYSIS
	5°C (*n*=4)	15°C (*n*=4)	
C16:0	8.2±1.0	14.8±0.7	a b
C16:1w9	5.0±0.6	5.6±1.3	
C17:0	1.4±0.2	2.6±0.5	a b
C17:1w9	3.3±0.4	4.6±1.8	
C18:0	7.1±0.5	12.3±1.8	a b
C18:1w9	22.2±1.4	17.5±0.8	b a
C18:2w6	2.1±0.2	2.4±0.3	
C18:3w6	0.6±0.1	0.9±0.5	
C18:3w3	0.5±0.1	0.3±0.3	
C20:1w9	1.7±0.1	2.0±0.5	
C20:2w6	0.9±0.1	1.1±0.2	
C20:4w6	5.2±0.5	3.1±0.6	b a
C20:5w3	28.9±2.0	16.7±2.8	b a
C22:5w3	0.3±0.1	0.1±0.1	
C22:6w3	11.5±1.7	9.8±2.2	
Saturated	18.0±1.4	33.5±1.9	a b
Monounsat.	32.3±1.9	30.5±3.9	
Polyunsat.	51.2±2.6	35.9±5.2	b a
Ratio S/U	0.22±0.02	0.51±0.04	a b
C 16	13.2±1.6	20.4±1.5	a b
C 17	4.8±0.5	7.3±2.3	
C 18	32.6±2.1	33.4±2.5	
C 20	37.5±2.7	23.4±3.3	b a
C 22	12.6±1.6	11.1±2.2	
w3	41.2±2.2	27.0±5.3	b a
w6	10.0±0.9	8.8±0.3	b a

Table 4.15. Fatty acid composition (relative percentages, means±S.D.) of phosphatidylethanolamine (**PE**) separated from lipid extracts of muscle plasma membranes of *Carcinus maenas* acclimated in **winter**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order **a<b**

FATTY ACID	ACCLIMATION TEMP.		STAT. ANALYSIS
	5°C (*n*=4)	15°C (*n*=4)	
C16:0	6.1±0.2	10.4±0.2	a b
C16:1w9	5.1±0.2	5.7±0.2	a b
C17:0	1.2±0.1	2.6±0.3	a b
C17:1w9	2.3±0.2	4.8±0.3	a b
C18:0	7.6±0.5	10.8±0.3	a b
C18:1w9	18.9±0.4	15.4±1.0	b a
C18:2w6	1.8±0.1	2.6±0.2	a b
C18:3w6	0.4±0.1	0.5±0.2	
C18:3w3	0.4±0.1	0.6±0.1	a b
C20:1w9	1.6±0.2	1.6±0.1	
C20:2w6	0.6±0.1	0.9±0.1	a b
C20:4w6	3.0±0.1	3.5±0.4	
C20:5w3	33.8±1.4	23.0±0.2	b a
C22:5w3	0.3±0.1	0.4±0.3	
C22:6w3	15.3±0.7	13.2±0.4	b a
Saturated	15.4±0.7	24.8±0.4	a b
Monounsat.	27.8±0.6	28.5±1.1	
Polyunsat.	56.0±1.6	46.2±1.0	b a
Ratio S/U	0.18±0.01	0.33±0.01	a b
C 16	11.1±0.2	16.1±0.2	a b
C 17	3.5±0.3	7.4±0.1	a b
C 18	29.1±0.7	29.8±0.9	
C 20	39.5±1.0	29.8±0.5	b a
C 22	15.7±0.8	14.7±0.5	
w3	49.8±1.9	37.6±0.7	b a
w6	6.3±0.3	8.6±0.4	a b

Table 4.16. Fatty acid composition (relative percentages, means±S.D.) of phosphatidylethanolamine (**PE**) separated from lipid extracts of muscle plasma membranes of *Cancer pagurus* acclimated in **spring**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order a≤ab≤b, b≤bc≤c

FATTY ACID	ACCLIMATION TEMPERATURE				STAT. ANALYSIS
	5°C (*n=4*)	8°C (*n=3*)	15°C (*n=4*)	22°C (*n=6*)	
C16:0	9.6±0.5	8.0±0.7	10.2±0.4	8.9±0.8	bc a c ab
C16:1w9	4.4±0.6	4.4±1.0	5.4±1.0	3.4±0.5	ab ab b a
C17:0	1.5±0.6	1.1±0.1	2.2±0.1	1.1±0.2	b a c a
C17:1w9	3.7±0.2	1.4±0.1	4.2±0.3	1.8±0.3	b a c a
C18:0	6.9±1.0	9.1±2.1	10.0±0.7	9.0±1.3	a ab b ab
C18:1w9	25.7±4.5	23.6±2.2	21.4±1.4	21.4±2.0	b ab a a
C18:2w6	1.7±0.6	0.4±0.4	2.0±0.8	0.6±0.4	b a b a
C18:3w6	0.6±0.3	0.4±0.1	1.3±0.8	0.3±018	a a b a
C18:3w3	0	0	0	0	
C20:1w9	2.3±0.6	1.7±0.2	2.7±1.2	1.5±0.2	ab a b a
C20:2w6	1.6±1.5	0.3±0.1	1.9±1.1	0.8±0.1	ab a b ab
C20:4w6	3.3±0.2	3.4±0.1	4.7±0.5	4.1±0.8	a a b ab
C20:5w3	22.2±0.1	27.8±0.4	20.2±2.6	28.8±1.1	a b a b
C22:5w3	0.3±0.3	1.2±0.4	1.2±0.5	0	a b b a
C22:6w3	13.2±1.2	14.1±0.7	10.5±0.4	17.1±3.0	ab b a c
Saturated	18.8±0.1	18.2±2.0	23.1±1.8	19.1±0.8	a a b a
Monounsat.	38.0±0.8	31.2±2.5	33.7±1.0	28.1±2.1	c b b a
Polyunsat.	43.2±0.7	49.8±0.9	42.7±2.3	52.0±3.2	a b a b
Ratio S/U	0.23	0.23±0.03	0.30±0.03	0.24±0.02	a a b a
C 16	14.0±1.1	12.4±1.1	15.6±1.0	12.3±1.2	ab a b a
C 17	5.2±0.4	2.5±0.2	6.4±0.4	2.8±0.4	b a c a
C 18	35.0±6.4	33.6±1.8	34.6±2.9	31.3±2.5	
C 20	29.6±2.1	33.4±0.5	30.2±1.8	35.3±1.0	a b a c
C 22	14.1±0.7	17.3±1.1	12.4±1.3	17.4±3.1	ab bc a c
w3	35.8±0.9	45.1±1.2	31.9±2.3	46.1±2.8	b c a c
w6	7.8±0.8	4.6±0.4	10.9±1.9	5.9±0.8	b a c a

Table 4.17. Fatty acid composition (relative percentages, means±S.D.) of phosphatidylethanolamine (**PE**) separated from lipid extracts of muscle plasma membranes of *Carcinus maenas* acclimated in **spring**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order a≤ab≤b, b≤bc≤c

FATTY ACID	ACCLIMATION TEMPERATURE				STAT. ANALYSIS
	5°C (*n*=4)	8°C (*n*=4)	15°C (*n*=4)	22°C (*n*=1)	
C16:0	7.5±0.8	6.9±0.7	12.4±2.2	7.0	a a b -
C16:1w9	4.8±0.8	3.7±0.2	5.9±0.3	3.5	b a c -
C17:0	1.5±0.6	0.8	2.6±0.2	0.7	b a c -
C17:1w9	3.2±0.8	1.2±0.2	5.4±0.3	1.0	b a c -
C18:0	5.9±1.4	6.2±0.9	7.8±0.5	6.7	a ab b -
C18:1w9	21.9±1.9	23.9±1.0	20.1±0.7	16.1	bc c b -
C18:2w6	2.7±1.0	1.0±0.8	3.4±0.6	0.2	b a b -
C18:3w6	0.3±0.4	0.2±0.2	0	0	
C18:3w3	1.4±1.4	0.2±0.1	2.3±0.7	0.3	ab a b -
C20:1w9	2.9±1.4	1.6±0.3	3.2±0.7	1.4	ab a b -
C20:2w6	2.0±0.8	0.9±0.1	2.1±0.6	0.7	b a b -
C20:4w6	5.2±1.1	3.7±0.6	4.0±0.3	4.3	b a ab -
C20:5w3	26.2±1.2	30.7±1.5	18.1±2.8	36.9	b c a -
C22:5w3	0.1±0.1	0.1±0.1	0	0.237	ab b a -
C22:6w3	12.5±1.1	17.5±1.7	10.1±2.2	14.3	a b a -
Saturated	16.3±1.9	13.9±1.3	24.7±3.7	14.4	a a b -
Monounsat.	32.8±0.9	30.4±0.9	34.6±0.9	22.0	b a c -
Polyunsat.	50.9±2.4	54.4±2.8	40.6±4.1	56.8	b b a -
Ratio S/U	0.20±0.03	0.16±0.02	0.33±0.07	0.18	a a b -
C 16	10.3±1.5	10.6±0.7	18.3±2.5	10.5	a a b -
C 17	4.7±1.4	1.9±0.3	8.0±0.5	1.8	b a c -
C 18	32.2±0.9	31.0±1.9	33.6±1.2	23.2	bc b c -
C 20	37.0±2.9	36.9±1.9	28.4±1.9	43.2	b b a -
C 22	12.9±1.3	17.7±2.0	10.4±2.5	14.5	a b a -
w3	40.3±1.3	48.5±1.9	30.6±4.6	51.7	b c a -
w6	10.6±2.7	5.3±0.5	10.0±0.9	5.1	b a b -

Table 4.18. Fatty acid composition (relative percentages, means±S.D.) of phosphatidylethanolamine (**PE**) separated from lipid extracts of muscle plasma membranes of *Cancer pagurus* acclimated in **autumn**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order a≤ab≤b, b≤bc≤c

FATTY ACID	ACCLIMATION TEMPERATURE				STAT. ANALYSIS
	5°C (*n*=2)	8°C (*n*=4)	15°C (*n*=2)	22°C (*n*=4)	
C16:0	6.2±0.1	8.1±1.0	7.3±0.4	6.7±0.7	a b ab ab
C16:1w9	4.4±0.1	4.3±0.9	3.8±0.1	3.0±0.3	ab b ab a
C17:0	0.9	1.6±0.3	0.9±0.2	0.8±0.2	a b a a
C17:1w9	1.4±0.2	1.2±0.2	1.3	1.3±0.5	
C18:0	7.1±0.9	8.6±1.3	7.4±0.6	6.1±0.5	ab b ab a
C18:1w9	22.2±0.3	22.5±1.2	22.60±0.5	24.2±1.0	
C18:2w6	1.2±0.9	0.9±0.7	1.1±0.2	0.2	
C18:3w6	0	0.2±0.1	0.1±0.1	0	
C18:3w3	0	0	0	0	
C20:1w9	1.7	1.8±0.1	1.2±0.1	1.2±0.4	ab b a a
C20:2w6	0.9	1.1±0.2	0.6±0.1	1.5±0.4	ab bc a c
C20:4w6	4.5±0.1	4.2±0.5	3.7	5.6±0.7	ab a a b
C20:5w3	30.5±1.8	28.3±2.0	31.0±0.6	26.0±2.0	b ab b a
C22:5w3	0.3	0.8±0.4	0.8±0.1	0	ab b b a
C22:6w3	18.1±0.3	14.2±1.0	17.1±0.2	22.9±1.4	b a b c
Saturated	14.3±1.3	19.6±0.7	15.9±0.3	13.7±1.3	ab c b a
Monounsat	29.7±0.9	29.8±1.7	28.9±0.7	29.7±0.3	
Polyunsat	55.9±2.2	50.4±1.9	55.2±1.1	56.3±1.6	b a b b
Ratio S/U	0.17±0.02	0.25±0.01	0.19	0.16±0.02	ab c b a
C 16	10.6±0.1	12.4±2.1	11.1±0.8	9.7±1.0	ab b ab a
C 17	2.3±0.3	2.8±0.5	2.3±0.2	2.2±0.7	
C 18	30.6±3.1	32.2±2.5	31.3	30.4±0.8	
C 20	37.7±2.8	35.4±2.7	36.5±0.8	34.4±2.3	
C 22	18.7±0.7	15.7±1.4	18.7±0.4	23.0±1.4	b a b c
w3	49.1±3.0	44.0±1.1	49.7±1.3	49.0±1.0	b a b b
w6	6.9±0.9	6.4±1.3	5.5±0.3	7.3±1.0	

Table 4.19. Fatty acid composition (relative percentages, means±S.D.) of phosphatidylethanolamine (**PE**) separated from lipid extracts of muscle plasma membranes of *Carcinus maenas* acclimated in **autumn**: significantly different groups (p≤0.05) are marked with different letters; the group means increase in the order a≤ab≤b, b≤bc≤c

FATTY ACID	ACCLIMATION TEMPERATURE				STAT. ANALYSIS
	5°C (*n*=2)	8°C (*n*=4)	15°C (*n*=2)	22°C (*n*=4)	
C16:0	4.5	4.8±0.3	5.3±0.3	6.9±0.8	a a a b
C16:1w9	4.0	3.1±0.2	3.3±0.1	4.5±0.6	bc a ab c
C17:0	0.9±0.1	0.5	0.4	1.0±0.5	ab a ab b
C17:1w9	1.5±0.1	2.5±0.3	1.6±0.3	2.0±0.7	
C18:0	5.7±0.1	6.8±0.8	5.5	5.9±1.1	
C18:1w9	20.2±0.1	20.8±1.2	21.1±0.5	19.2±1.6	
C18:2w6	0.3±0.1	1.9±0.6	0.5±0.1	0.3±0.2	a b a a
C18:3w6	0	0	0	0	
C18:3w3	0	0.1	0	0.1±0.1	
C20:1w9	1.3	1.1±0.2	1.3±0.2	0.8±0.1	b ab b a
C20:2w6	1.0	0.9±0.1	0.7±0.1	0.8±0.1	b ab a ab
C20:4w6	4.7	3.8±0.3	3.7	3.8±0.2	b a a a
C20:5w3	34.6±0.1	34.3±1.5	34.0±0.5	33.1±1.0	
C22:5w3	0.5±0.3	0	0.1	0	b a a a
C22:6w3	20.4	18.4±0.3	22.1±0.6	21.3±1.4	b a b b
Saturated	11.2±0.6	12.2±0.7	11.5±0.6	13.9±1.9	a ab ab b
Monounsat.	27.0±0.2	27.5±0.9	27.3±1.0	26.5±0.9	
Polyunsat.	61.8±0.4	59.7±1.0	61.2±0.4	59.5±1.0	b a ab a
Ratio S/U	0.13±0.01	0.14±0.01	0.13±0.01	0.16±0.03	a ab ab b
C 16	8.4	7.9±0.4	8.6±0.4	11.4±0.5	a a a b
C 17	2.4±0.1	3.0±0.3	2.1±0.4	3.0±1.0	
C 18	26.2±0.2	29.5±1.2	27.2±0.5	25.5±1.0	a b a a
C 20	41.6±0.1	40.1±1.6	39.8±0.4	38.6±1.2	b ab ab a
C 22	21.2±0.6	18.7±0.2	22.2±1.0	21.4±1.5	b a b b
w3	55.8±0.5	53.1±1.8	56.3±0.3	54.6±0.7	b a b ab
w6	6.0±0.1	6.6±0.9	4.9±0.1	4.9±0.3	ab b a a

ratios in the warm-acclimated crabs. The monounsaturated fatty acids decreased in *Cancer pagurus* and increased in *Carcinus maenas* with warm-acclimation.

In the spring experiment the 15°C-acclimated crabs of both species had significantly higher S/U ratios than the cold-acclimated crabs and the 22°C-acclimated ones. The difference was due to higher levels of saturated fatty acids and lower levels of polyunsaturates. In *Cancer pagurus* PE, the total monounsaturates decreased with acclimation temperature. The variations in individual fatty acids with acclimation temperature did not follow a consistent pattern in either species (Tables 4.16 and 4.17). In *Cancer pagurus* C18:1 decreased with acclimation temperature and C22:6 was higher in 22°C-acclimated crabs. In *Carcinus maenas* there was also a decrease in C18:1 with acclimation temperature.

In autumn, in *Cancer pagurus* PE there were a few significant differences between the acclimation groups: C20:5 decreased with the acclimation temperature, and C22:6 was higher in 22°C-acclimated crabs (Table 4.18). The highest S/U ratio was found in the 8°C-acclimated crabs, due to higher levels of saturated fatty acids and lower levels of polyunsaturated fatty acids than in the other three acclimation groups, between which there were no significant differences. In *Carcinus maenas* C16:0 increased and C20:4 decreased with acclimation temperature, leading to slightly higher levels of saturated fatty acids and a higher S/U ratio in the PE from membranes of 22°C-acclimated crabs (Table 4.19)

4.3.2.4. ω3 and ω6 Fatty Acids

In winter, the main decrease in polyunsaturated fatty acids with acclimation temperature was at ω3 fatty acids level. The extent of the difference between acclimation groups was greater in PE than in PC (Tables 4.8, 4.9, 4.14 and 4.15). The ω6 fatty acids decreased slightly with acclimation temperature in *Cancer pagurus* and increased in *Carcinus maenas*. Also *Carcinus maenas* PE had more ω3 and less ω6 fatty acids than *Cancer pagurus* PE.

In spring in both species the 8°C and 22°C-acclimated crabs had higher levels of ω3 and lower levels of ω6 fatty acids than the 5°C and 15°C-acclimated groups, in PC and PE Tables 4.10, 4.11, 4.16 and 4.17). No consistent patterns of variation with acclimation temperature could be found in the autumn experiment (Tables 4.12, 4.13, 4.18 and 4.19).

4.3.2.5. Comparison Between the PC and PE Class

Figures 4.5 and 4.6 show a comparison of the S/U ratios of PC and PE fractions from *Cancer pagurus* and *Carcinus maenas*, respectively, and of their variation with acclimation temperature.

The S/U ratio of PC was higher than that of PE, in most cases where there was a significant difference between the two phospholipid classes. Only in 15°C-acclimated *Cancer pagurus*, in the winter experiment (Figure 4.5.c) the S/U ratio of PE was significantly greater than that of PC. The difference between PC and PE was more often significant, and greater, in *Carcinus maenas* (Figure 4.6) than in *Cancer pagurus* (Figure 4.5).

The variations in the S/U ratio with acclimation temperature, where any, were usually greater for the PE class than for the PC class. Examples illustrating this statement are the difference found in the winter experiment between the 5°C- and the 15°C-acclimated groups, in both species (Figures 4.5.c. and 4.6.c), and the difference found in the spring experiment between the 15°C-acclimated group and the other three acclimation groups, in *Carcinus maenas* (Figure 4.6.a).

The relative percentages of each major category of fatty acids (saturated, monounsaturated and polyunsaturated) in PC and PE were also plotted against acclimation temperature (Figures 4.7-4.12). In both species, the PC class had higher levels of monounsaturated fatty acids and lower levels of polyunsaturated fatty acids than PE. The differences were usually greater in *Carcinus maenas* (Figures 4.10-4.12) than in *Cancer pagurus* (Figures 4.7-4.9). The levels of saturated fatty acids in PC

Figure 4.5. The effect of acclimation temperature on the saturated/unsaturated (S/U, means±S.D.) fatty acid ratio in **PC** and **PE** separated from lipid extracts of muscle plasma membranes of *Cancer pagurus* acclimated in (a) **spring**, (b) **autumn** and (c) **winter**
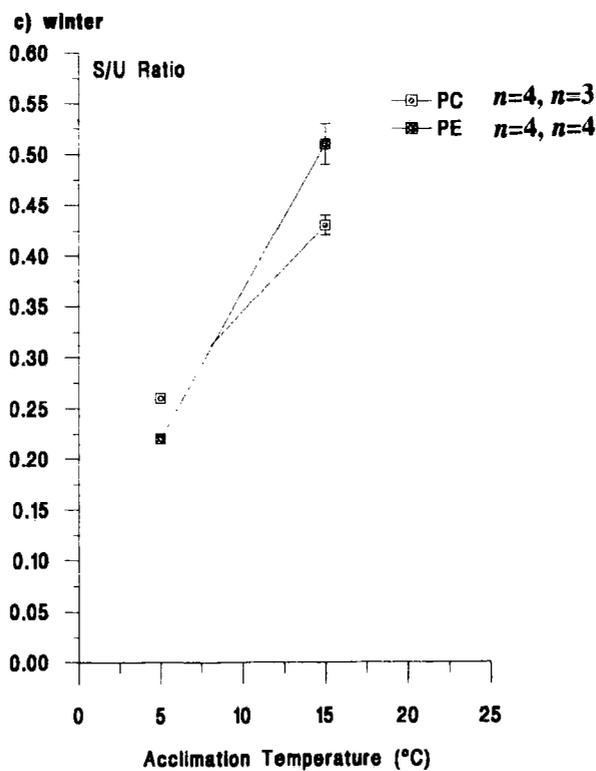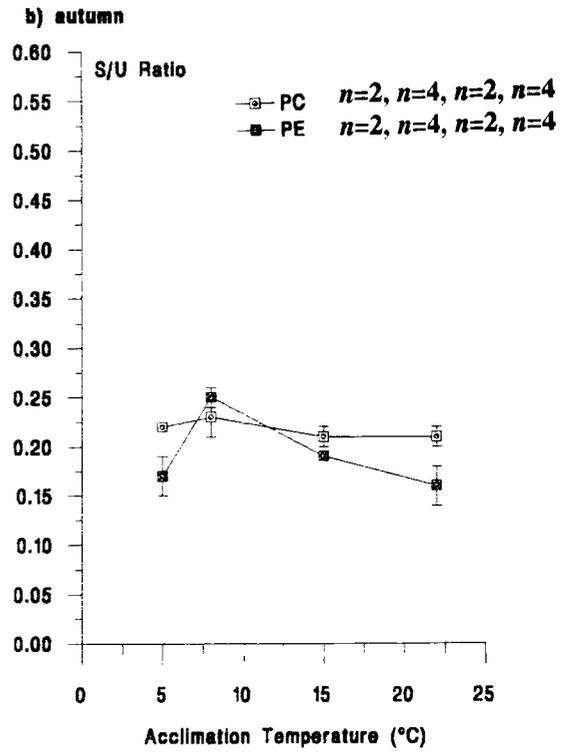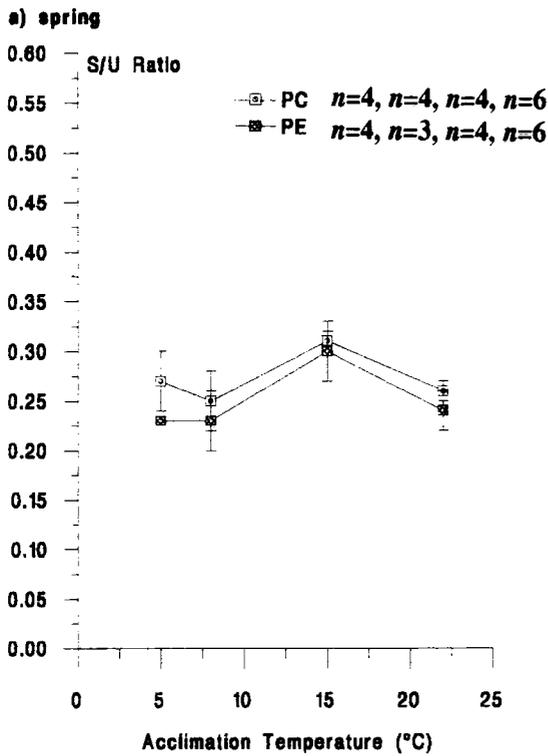
Figure 4.6. The effect of acclimation temperature on the saturated/unsaturated (S/U, means±S.D.) fatty acid ratio in **PC** and **PE** separated from lipid extracts of muscle plasma membranes of *Carcinus maenas* acclimated in **(a)** spring, **(b)** autumn and **(c)** winter

a) spring

b) autumn

c) winter
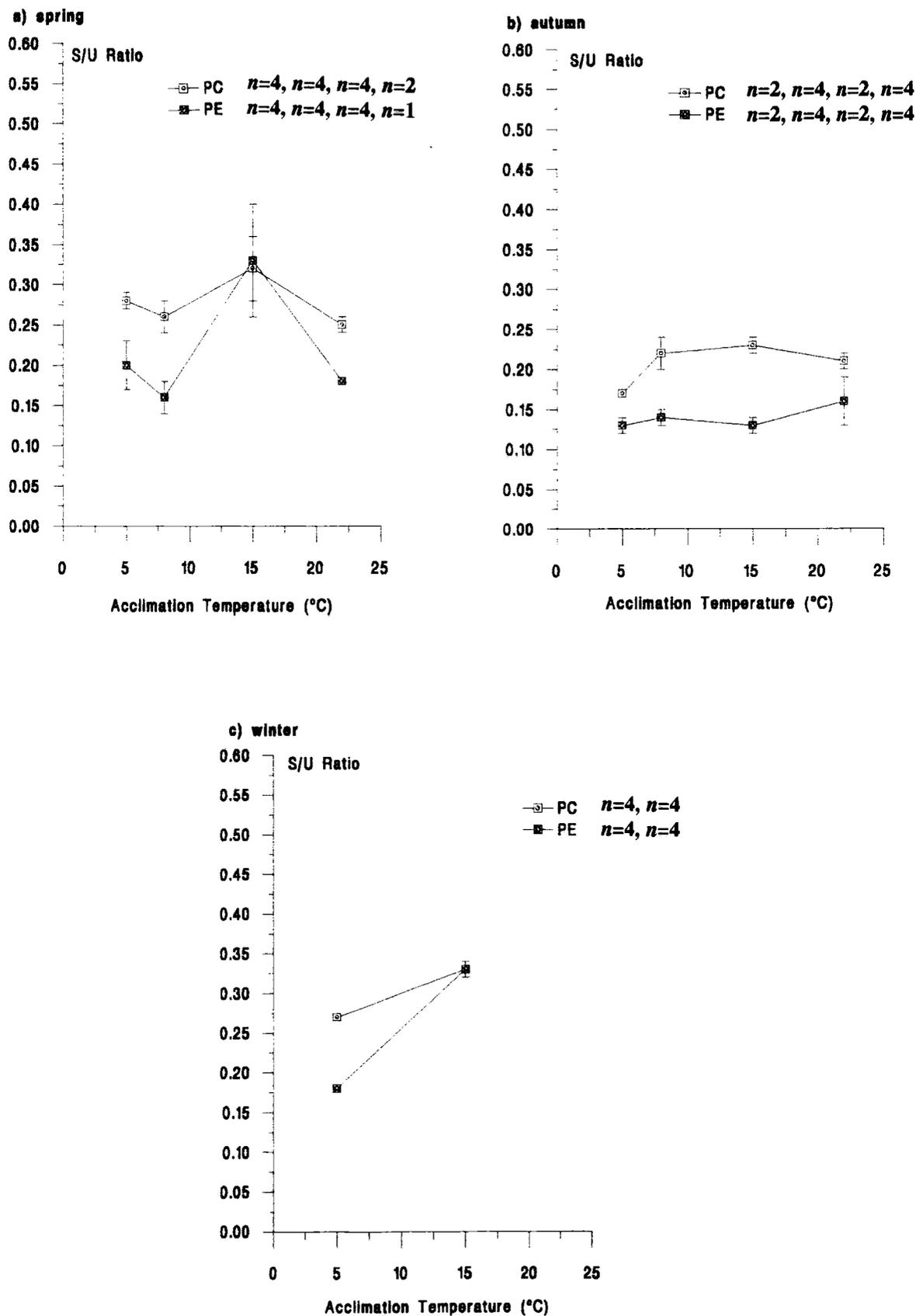
Figure 4.7. The effect of acclimation temperature on the relative percentage of total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in PC (*n*=4, *n*=3) and PE (*n*=4, *n*=4) separated from lipid extracts of muscle plasma membranes of *Cancer pagurus* acclimated in **winter** (means±S.D.)
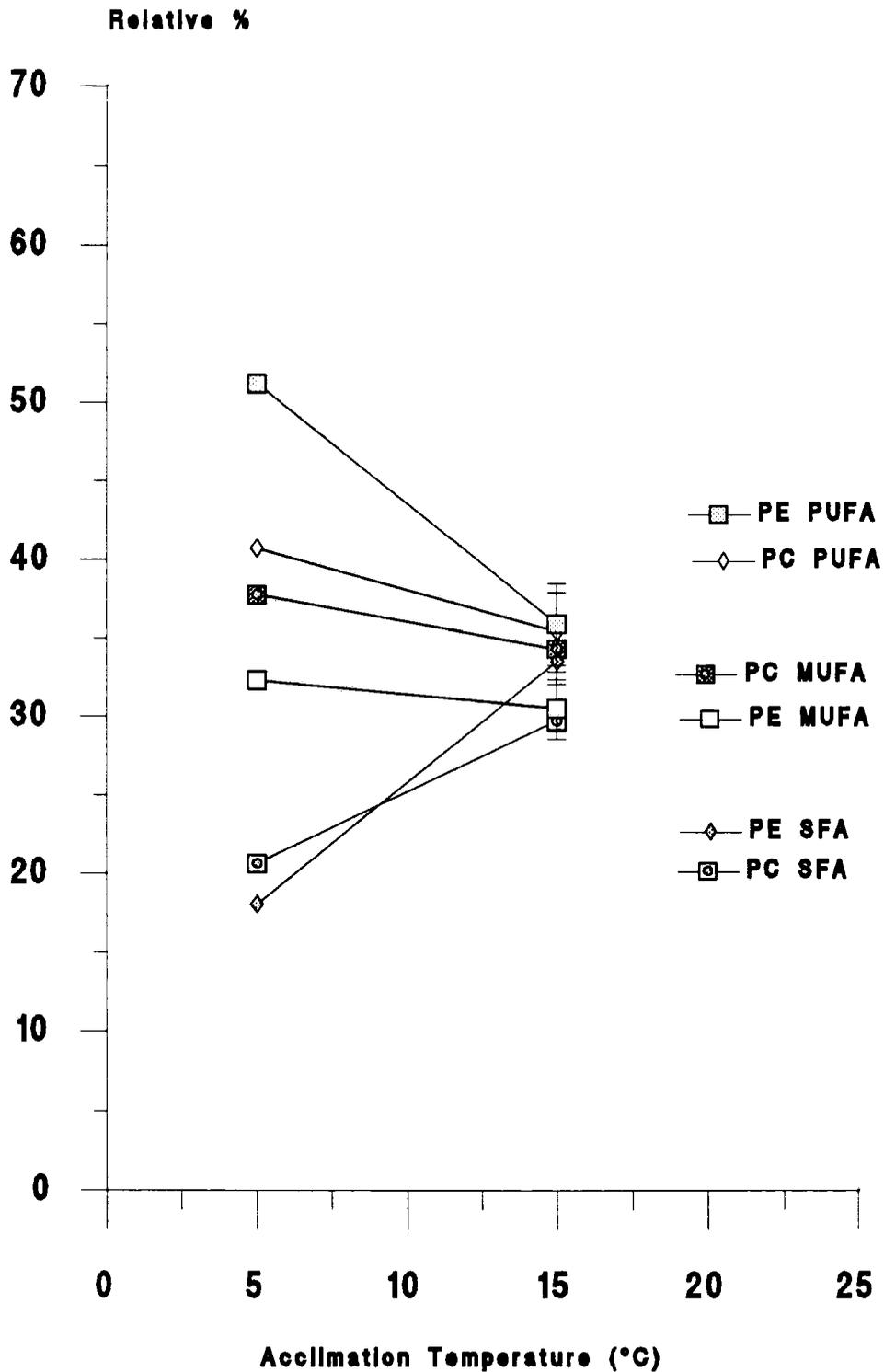
Figure 4.8. The effect of acclimation temperature on the relative percentage of total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in PC ($n=4$, $n=4$, $n=4$, $n=6$) and PE ($n=4$, $n=3$, $n=4$, $n=6$) separated from lipid extracts of muscle plasma membranes of *Cancer pagurus* acclimated in **spring** (means±S.D.)
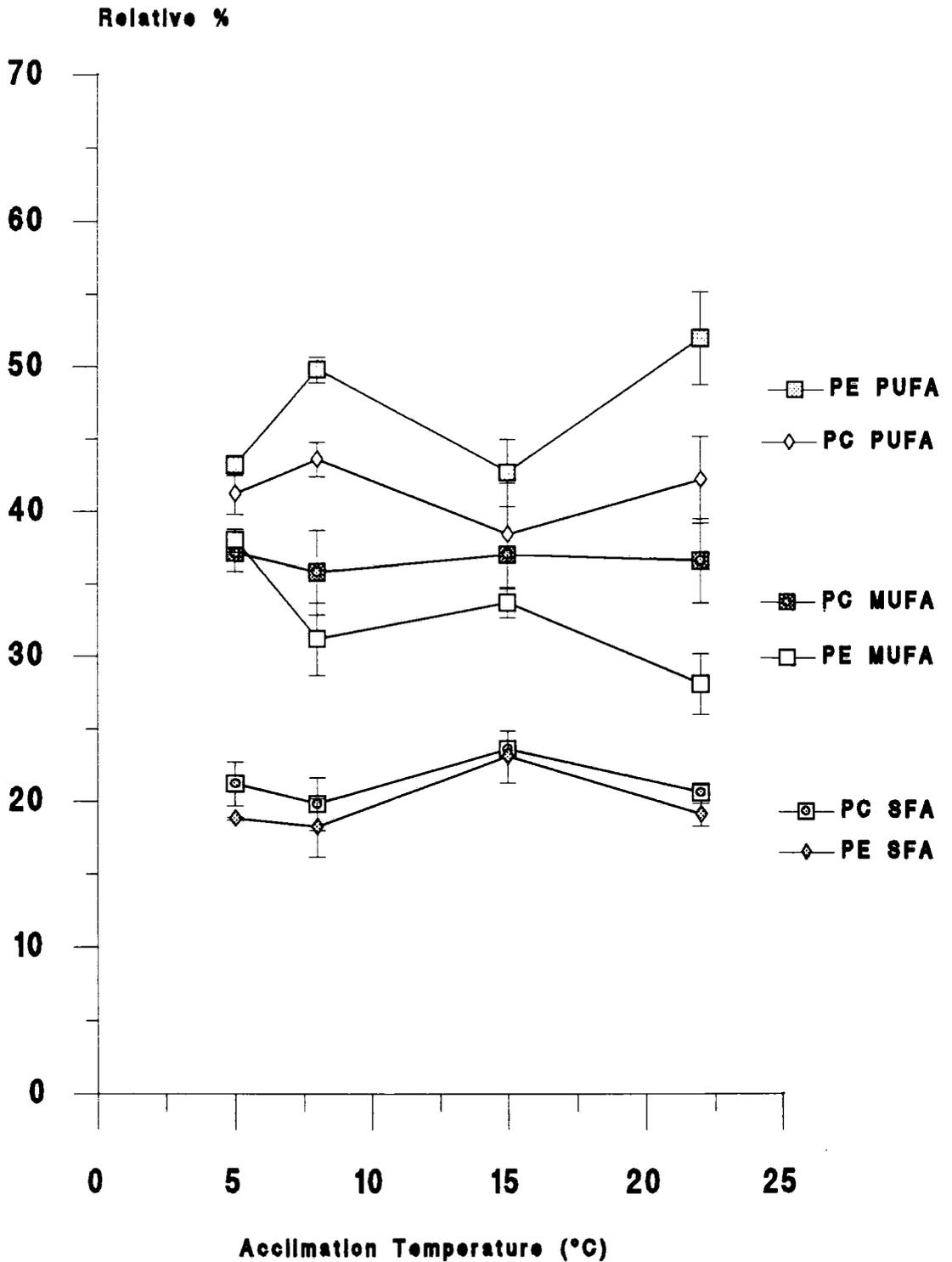
Figure 4.9. The effect of acclimation temperature on the relative percentage of total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in PC (*n*=2, *n*=4, *n*=2, *n*=4) and PE (*n*=2, *n*=4, *n*=2, *n*=4) separated from lipid extracts of muscle plasma membranes of *Cancer pagurus* acclimated in **autumn** (means±S.D.)
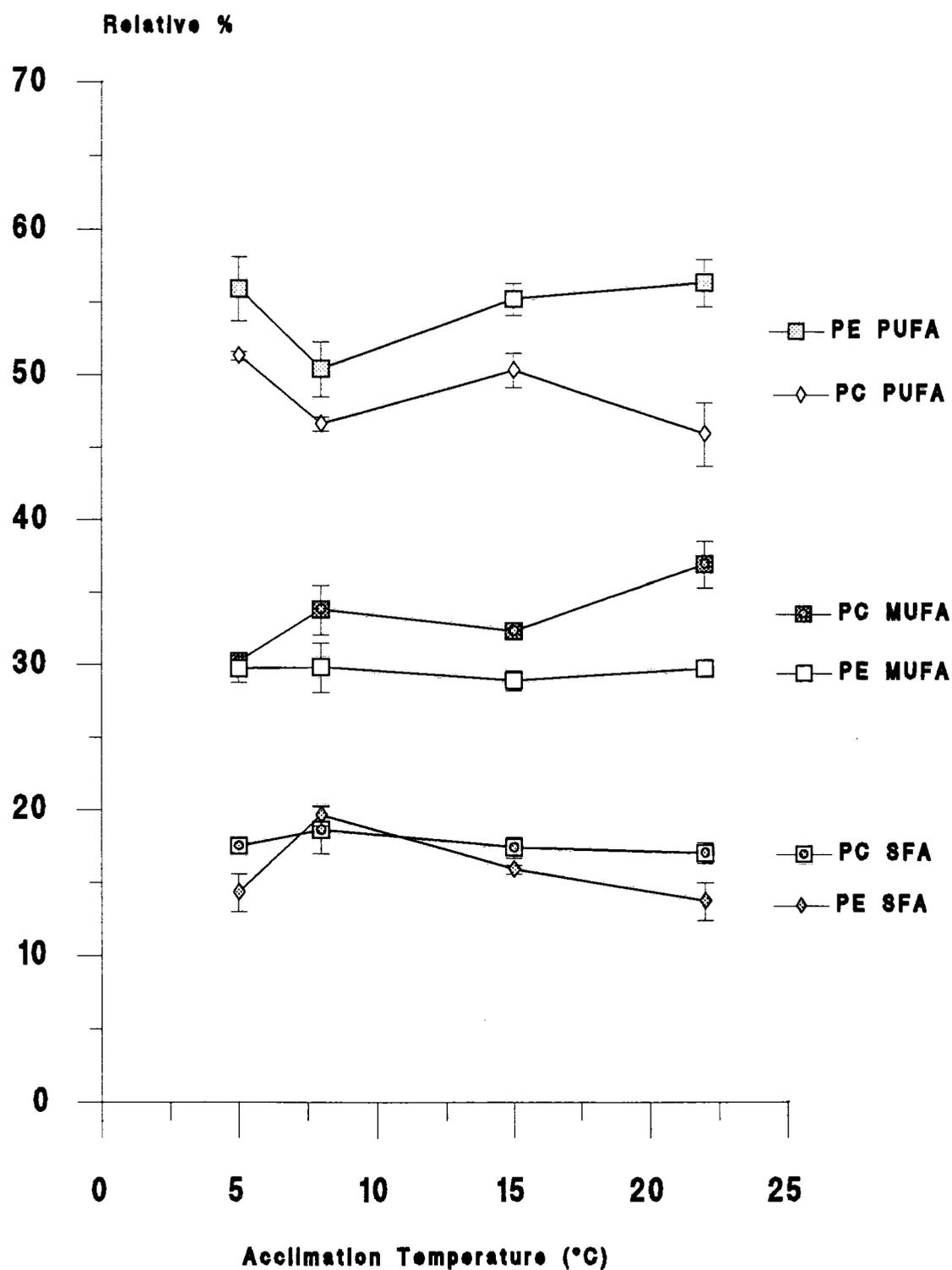
Figure 4.10. The effect of acclimation temperature on the relative percentage of total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in PC ($n=4$, $n=4$) and PE ($n=4$, $n=4$) separated from lipid extracts of muscle plasma membranes of *Carcinus maenas* acclimated in **winter** (means±S.D.)
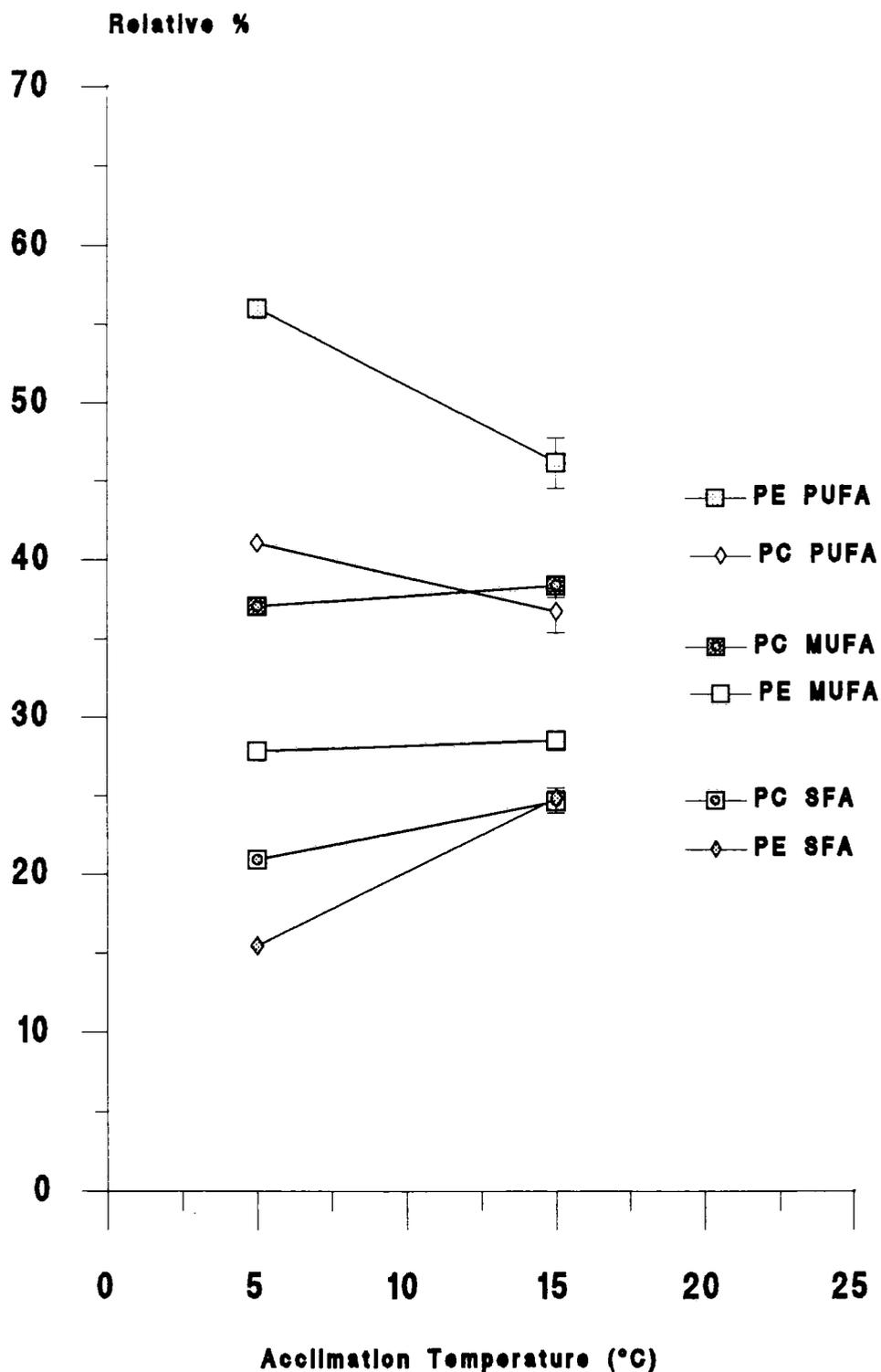
Figure 4.11. The effect of acclimation temperature on the relative percentage of total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in PC (*n*=4, *n*=4, *n*=4, *n*=2) and PE (*n*=4, *n*=4, *n*=4, *n*=1) separated from lipid extracts of muscle plasma membranes of *Carcinus maenas* acclimated in **spring** (means±S.D.)
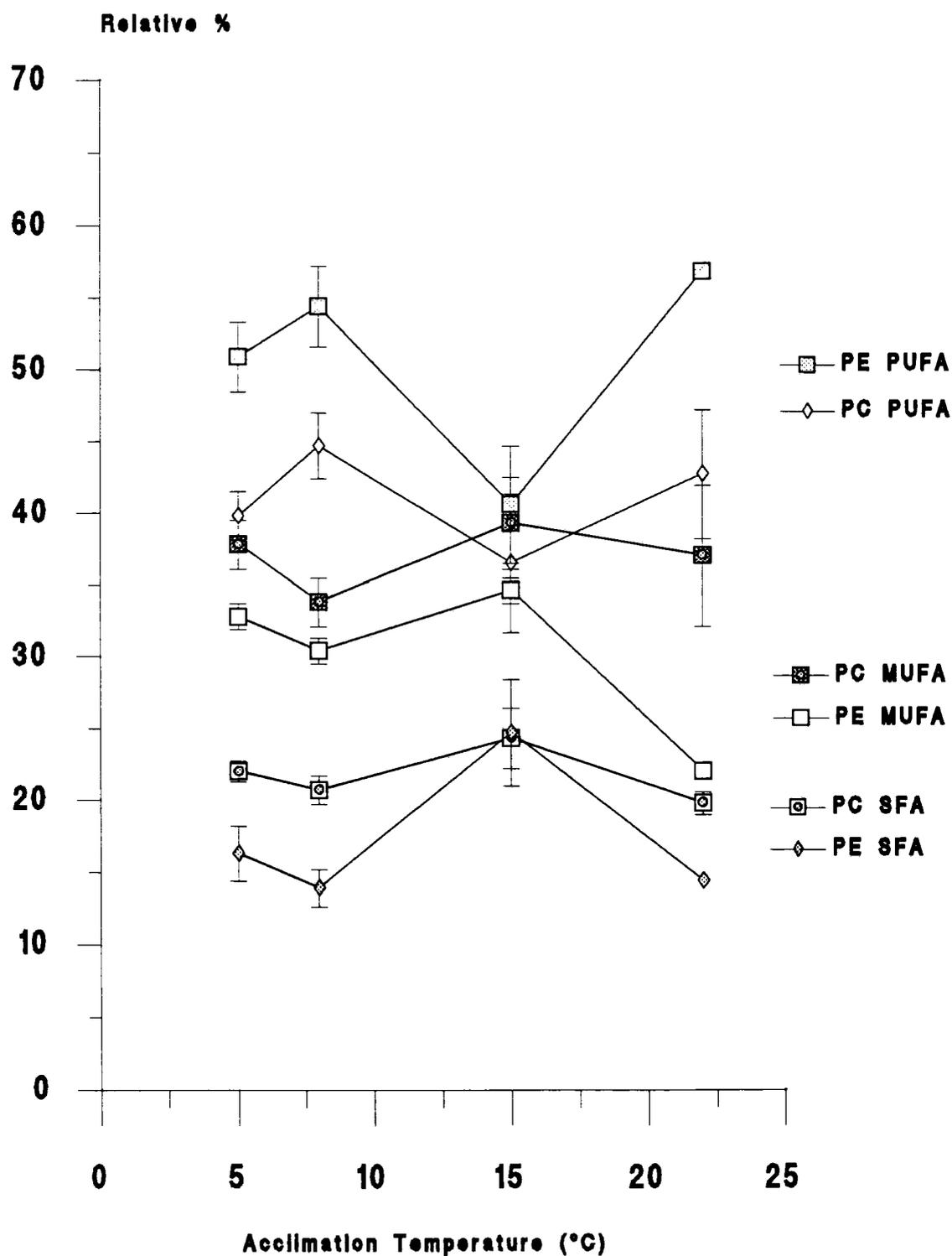
Figure 4.12. The effect of acclimation temperature on the relative percentage of total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in PC (*n*=2, *n*=4, *n*=2, *n*=4) and PE (*n*=2, *n*=4, *n*=2, *n*=4) separated from lipid extracts of muscle plasma membranes of *Carcinus maenas* acclimated in **autumn** (means±S.D.)
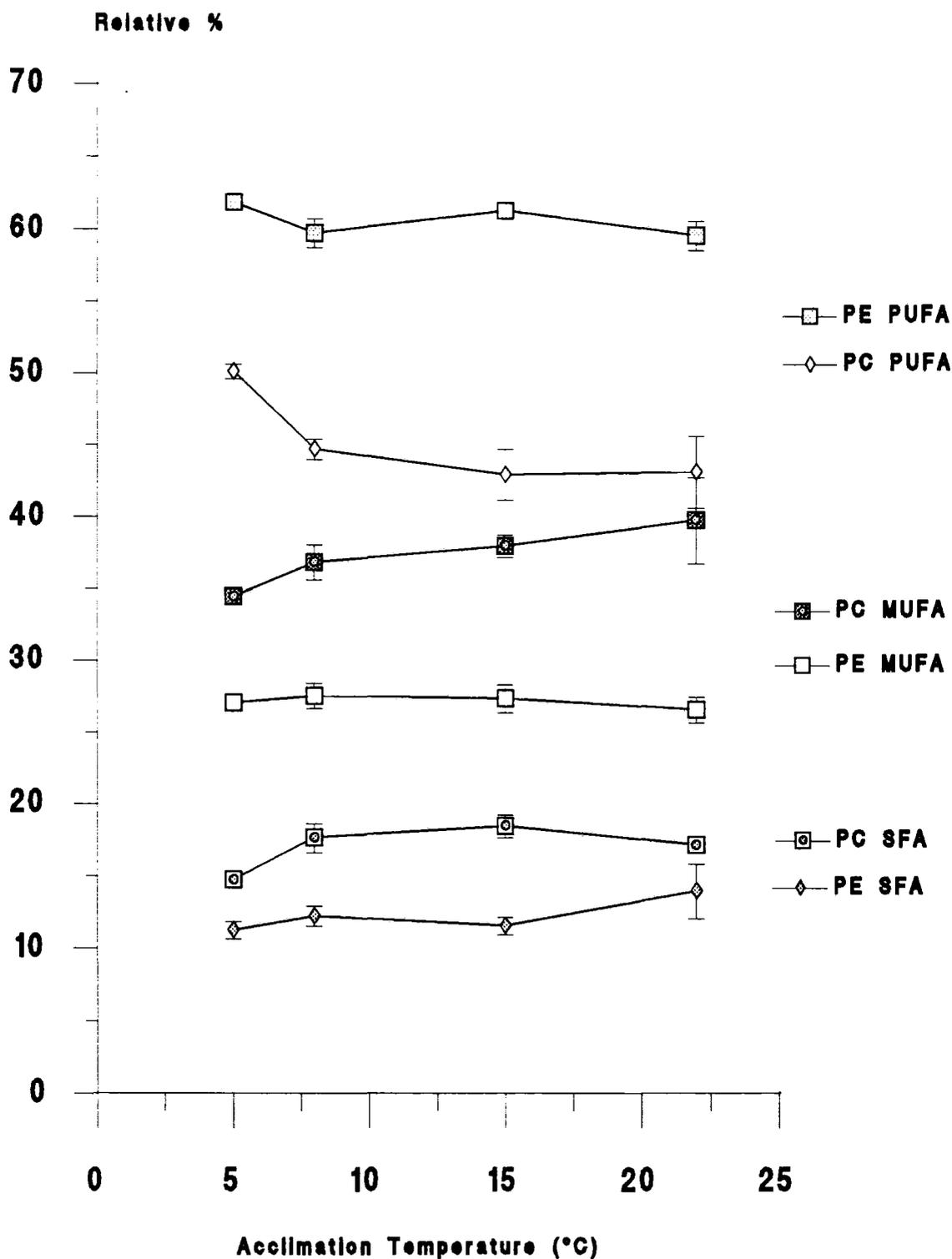
and PE were similar in *Cancer pagurus* and slightly higher in the PC class in *Carcinus maenas*.

The variations in the relative percentages of polyunsaturated and saturated fatty acids with acclimation temperature followed similar patterns in PC and PE, but with a greater extent of changes in PE. A few differences were observed between the variations of the relative percentages of monounsaturated fatty acids with acclimation temperature in spring and autumn. In spring, the PC monounsaturates did not significantly vary between acclimation groups, while the PE monounsaturates decreased with acclimation temperature, in both species (Figures 4.8 and 4.11). In autumn, the PC monounsaturates increased with acclimation temperature, while the PE monounsaturates did not vary (Figures 4.9 and 4.12).

4.3.2.6. Seasonal Comparison

The statistical analysis of data from the spring and autumn experiments, showed that both species had higher levels of saturated fatty acids and lower levels of polyunsaturated fatty acids in spring than in autumn, relatively independent of the temperature at which they were acclimated. The monounsaturated fatty acids were higher in spring in some cases.

The data from the winter experiment, although incomplete, were in most cases similar to those of the spring experiment, with the exception of the monounsaturated and polyunsaturated fatty acids levels in PE of 5°C-acclimated crabs, which were similar to those in the autumn experiment. The S/U ratios of PC and PE from *Cancer pagurus* acclimated to 15°C in winter were higher than those found in spring.

The average values for individual fatty acids and for categories of fatty acids are already shown in Tables 4.10-4.13 and 4.16-4.19, and the graphs in Figures 4.7-4.12 were printed on the same scale to allow an estimation of the extent of the above mentioned differences. Figures 4.13 and 4.14 illustrate a direct seasonal comparison of the variations in the S/U ratio with acclimation temperature in *Cancer pagurus* and *Carcinus maenas*, respectively, which showed that in *Cancer pagurus* the increase in

Figure 4.13. Seasonal comparison between the variation of the saturated to unsaturated (S/U) fatty acid ratio of **(a) PC** and **(b) PE** from plasma membranes of *Cancer pagurus* with acclimation temperature (means±S.D.)

a) PC

b) PE
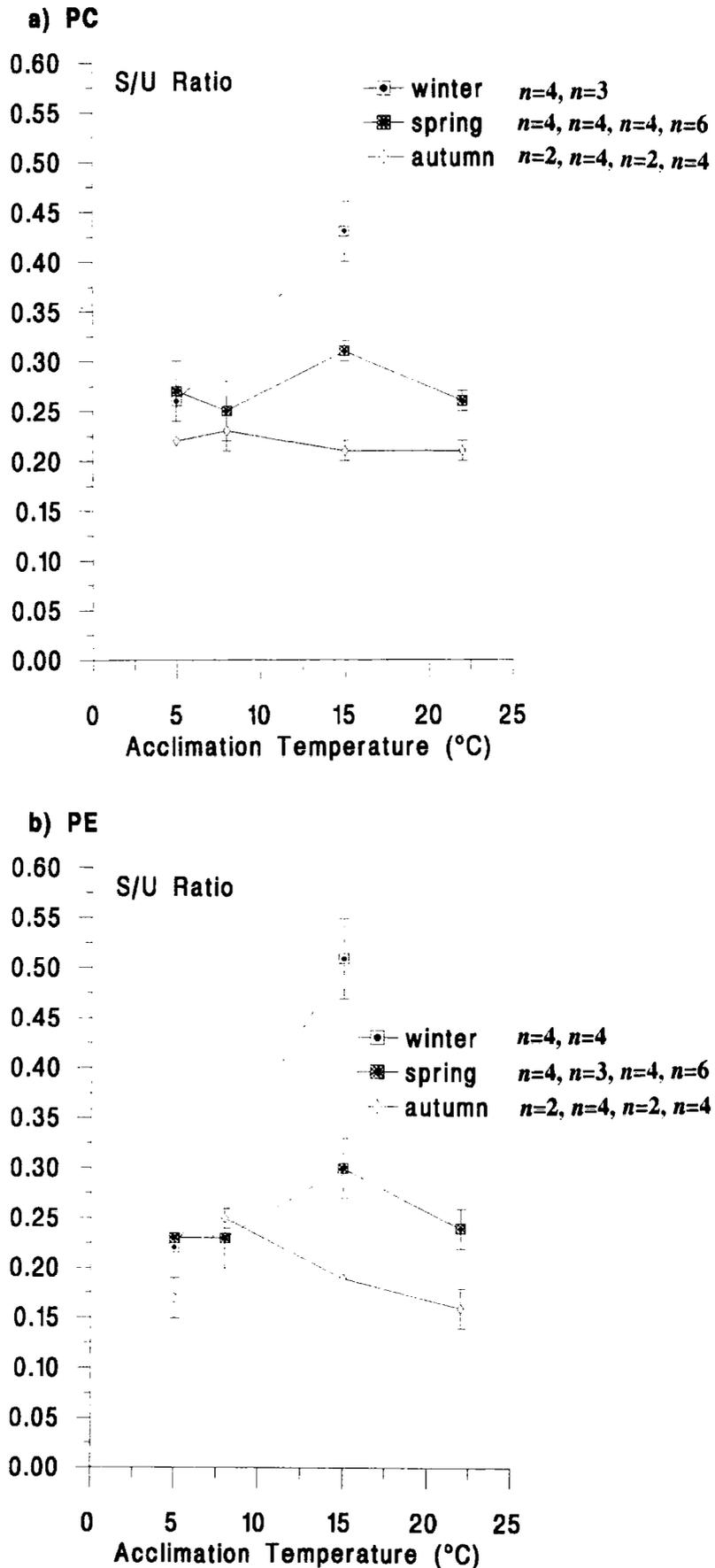
Figure 4.14. Seasonal comparison between the variation of the saturated to unsaturated (S/U) fatty acid ratio of **(a)** PC and **(b)** PE from plasma membranes of *Carcinus maenas* with acclimation temperature (means±S.D.)

a) PC

b) PE
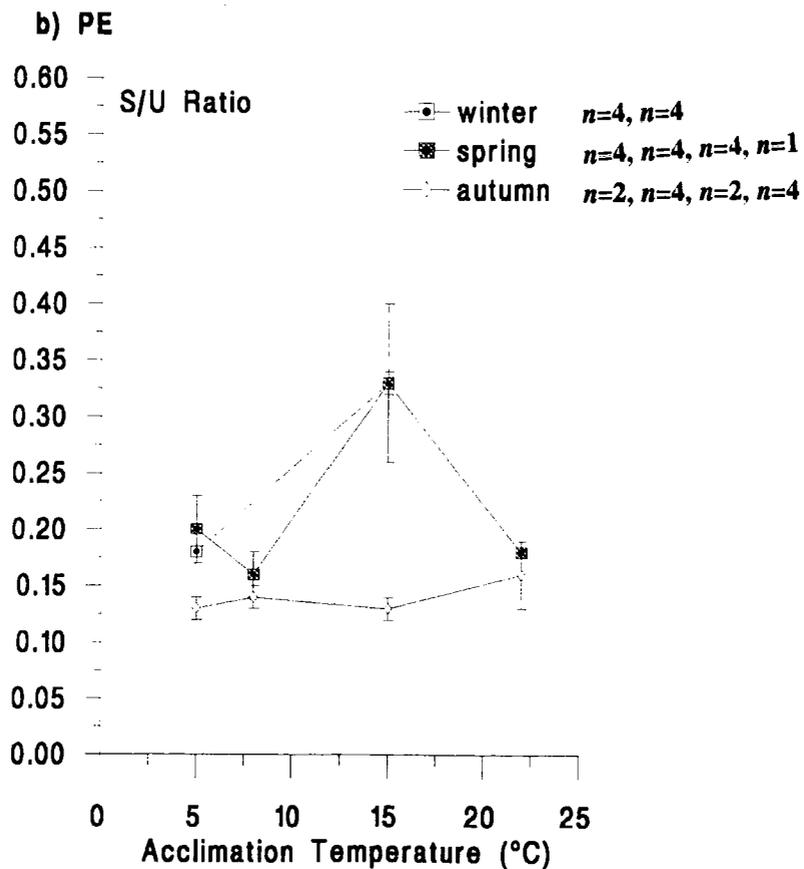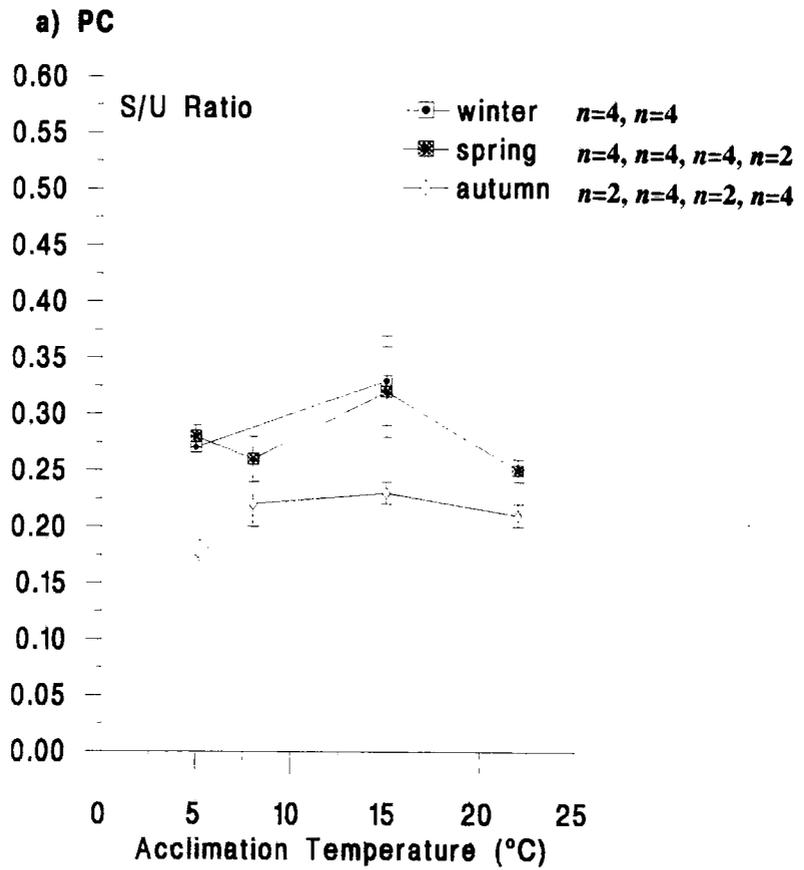
the S/U ratio following warm-acclimation, or 'scope for acclimation', was much greater in winter than in spring, while in *Carcinus maenas* the response was similar in both winter and spring. In both species in autumn there was very little variation in the S/U ratio with the acclimation temperature and the autumn S/U ratios were lower than in spring and winter, at nearly all acclimation temperatures (not in *Cancer pagurus* PE, at 8°C AT).

The levels of ω3 and ω6 varied very little from winter to spring. More seasonal differences were observed between the autumn crabs and the winter and spring ones. In 5°C and 15°C-acclimated crabs the ω3 fatty acids were higher in autumn, compared to winter and spring, in both PC and PE. The ω6 fatty acids were lower in autumn than in winter and spring in most cases. In the 8°C-acclimated *Cancer pagurus* the ω6 were higher in autumn. No seasonal variations were observed in the 22°C-acclimated *Cancer pagurus*. In 8°C and 22°C-acclimated *Carcinus maenas* no seasonal difference was observed at PC level, but the PE ω3 fatty acids were higher in autumn than in spring and winter.

4.4. Discussion

The results of this study on the influence of acclimation temperature on the lipid composition of plasma membranes isolated from crab leg muscle were not in good agreement with previous publications showing that in aquatic ectotherms cold-acclimation is associated with a graded increase in the unsaturation of the fatty acyl chains of membrane phospholipids and warm-acclimation with an increase in the levels of saturated fatty acids (Hazel, 1989, Chapelle, 1978) and that cholesterol levels are little influenced by temperature acclimation (Pruitt, 1990).

The basic experiment, which involved acclimating crabs at 'cold' and 'warm' temperatures, then isolating plasma membranes from the leg muscle and comparing their lipid compositions and fluidities, was repeated in different seasons for reasons other than looking for a seasonal influence on the acclimatory response. However,

when all data were put together, the main observation was that there was a significant difference between the responses observed in each season (winter, spring and autumn) and for this reason the results description was done separately for each season.

The cholesterol/phospholipid molar ratios were little influenced by acclimation temperature in the winter and spring experiments, but the levels of both total phospholipids and cholesterol appeared to be higher in cold-acclimated crabs. In the autumn experiment, the cholesterol/phospholipid ratio was found to be slightly higher in the 22°C-acclimated crabs. In *Carcinus maenas* this was due to a slight increase in cholesterol levels in warm-acclimated crabs. The total phospholipid levels varied in an inconsistent manner in both species. Chapelle *et al.* (1977) have noted slightly higher levels of total phospholipids in muscle and gills isolated from cold-acclimated *Carcinus maenas* which could result from changes in the total amount of particular phospholipid classes. Studies on fish (Hazel, 1989), crayfish (Pruitt, 1988) and shore-crab (Chapelle *et al.*, 1977) have shown that cold-acclimation was associated with increased percentages (with the total phospholipid as 100%) of PE, at the expense of PC. Due to low amounts of material, the assay of individual phospholipid classes was not possible, so the observed changes in the concentration of total phospholipids could not be related to changes in a particular phospholipid class.

The higher levels of cholesterol found in membranes of cold-acclimated crabs in the winter and spring experiments (greater difference in the spring experiment) can not be easily explained. According to previous studies, cholesterol does not appear to play an important role in the regulation of bulk membrane fluidity in crustaceans in response to changes in the environmental temperature and the data available from literature are not consistent (Pruitt, 1990). Cholesterol is the predominant sterol in the cellular membranes of crustaceans and can account for between 5 and 12% of the total membrane lipid (Krzynowek, Wiggon and Donahue, 1982). Gastaud (1977) found that sterol concentrations differed by less than 1% in the lipids of planktonic crustaceans from Arctic and temperate seas and Cossins (1976) found no significant difference in the phospholipid to cholesterol ratio of crayfish acclimated to 4°C and

25°C. The cholesterol content of membranes from digestive gland cells of scallops was higher in a Mediterranean Sea than in an Antarctic species (Viarengo *et al.*, 1994). Significant decreases in the levels of cholesterol after cold-acclimation were found in membranes of carp liver mitochondria (Wodtke, 1978) and flounder erythrocyte plasma membranes (Grove Sørensen, 1993), but not in carp red muscle mitochondria (Wodtke, 1981). Crockett and Hazel (1995) showed that cold acclimation did not change the cholesterol content of basolateral membranes, but increased it (expressed relative to protein, or to total polar lipid) in brush border membranes from the intestinal epithelia of rainbow trout.

Our results suggest that the modulation of the concentration of total cholesterol and phospholipids in the plasma membranes of the two species of marine crab studied plays a role in the thermal adaptation of these plasma membranes, at least when the crabs are subjected to laboratory acclimation for a longer period at constant temperatures. The concentrations of cholesterol and total phospholipid were calculated per gram of fresh weight of muscle tissue from which the plasma membranes were prepared. Because all the tissue collected from one crab had to be used for plasma membrane preparation and then lipid extraction, it was not possible to determine the dry weight of the muscle, or of the plasma membrane preparation.

The fatty acid analysis was carried out on the total phospholipid and, of the individual phospholipid classes, only on PC and PE. Chapelle (1977) showed that PC represented approximately 60% and PE approximately 20% of the total phospholipid in a total lipid extract of *Carcinus maenas* muscle.

The fatty acid composition of plasma membranes of both *Cancer pagurus* and *Carcinus maenas* was in agreement with the general pattern described by Chapelle (1986) for Crustacean species: the major saturated acid was 16:0, the major monounsaturated acid 18:1, the major polyunsaturated acid 20:5; there was a high degree of unsaturation and a predominance of long chain length fatty acids; the contents of C16 and C18 polyunsaturated acids were low and 20:5 was present in greater amounts than 22:6, relation which appears to be characteristic of the larger

decapods (Chapelle, 1986). Other characteristics with which our results were in agreement were that the phospholipids of Crustaceans generally contain low ω6 but high ω3 levels of polyunsaturated fatty acids, and that PE is more unsaturated than PC (Chapelle, 1986). Similar compositional patterns have been reported recently for the prawn *Penaeus japonicus* by Muriana, Ruiz-Gutierrez and Bolufer (1993) and for species of shrimps (Farkas *et al.*, 1994). Muriana, Ruiz-Gutierrez and Bolufer (1993) showed that the main lipids in muscle tissue were phospholipids and free cholesterol and that each phospholipid class investigated (PC, PE, SM and PI) had a characteristic fatty acid composition.

In preliminary measurements of the fatty acid composition of individual phospholipid classes we have also analysed other phospholipid classes than PC and PE, namely LPC, SM, PS+PI, and PA (data not shown) and observed large and consistent differences in the fatty acid profiles between these classes. However, these other phospholipids were present in much lower amounts than PC and PE and the ranges of variation in the relative proportion of individual fatty acids between preparations were even larger than for PC and PE and their analysis was not pursued.

The variations in the fatty acid composition of the plasma membrane phospholipids with acclimation temperature were dependent on the season in which the experiment was carried out and on the range of acclimation temperatures used, and consequently were not easy to interpret.

The winter experiment (when we used only 5°C and 15°C as the cold and warm acclimation temperatures) revealed a typical homeoviscous acclimation response: significant differences were found between the fatty acid composition of PC and PE from cold- and warm-acclimated crabs, summed up as an overall reduction of the saturated/unsaturated fatty acid ratio with cold acclimation, greater in PE than in PC. The lower value of the S/U ratio in membranes of cold-acclimated crabs was due to significantly lower levels of saturated and higher levels of polyunsaturated fatty acids. The difference in the levels of monounsaturated fatty acids was less marked and suggested a possible difference between the two species investigated: in *Cancer*

pagurus monoenes tended to increase at low acclimation temperatures, while in *Carcinus maenas* the opposite trend was observed.

Because no fluidity measurements were carried out on membranes from the winter experiment (the membrane preparations were used entirely for lipid extraction) the experiment was repeated in spring. Fluidity measurements on membranes prepared from crabs acclimated in the summer of the previous year had not revealed any difference between 5°C and 15°C-acclimated crabs, so a higher 'warm'-acclimation temperature was introduced (22°C) and a slightly higher 'cold'-acclimation temperature (8°C), because crabs seemed to become noticeably less active after 3 weeks at 5°C (casual observations).

The spring experiment revealed much smaller differences between acclimation groups. These were not consistent at individual fatty acids level, but summed up as the 5°C, 8°C and 22°C-acclimated groups having similar S/U ratios, and lower than those of the 15°C-acclimated crabs. This difference resulted from higher levels of saturated fatty acids and lower levels of polyunsaturated fatty acids in the PC and PE of 15°C-acclimated crabs. The extent of the difference was larger in PE than in PC for both species, and larger in *Carcinus maenas* PE than in *Cancer pagurus* PE. The only graded variation with acclimation temperature was observed in the level of monounsaturated fatty acids in *Cancer pagurus* PE, which decreased with acclimation temperature.

The autumn experiment did not reveal any consistent changes in the S/U ratio of *Cancer pagurus* membranes. The levels of PC monoenes increased with acclimation temperature in both species. In addition, in *Carcinus maenas* PC and PE the levels of saturated fatty acids increased slightly with acclimation temperature and the 5°C-acclimated crabs had lower S/U ratios than all the other groups.

The results of the winter experiment and, to some extent, those of the spring experiment agreed with those reported by other authors. It has been shown that acclimation to low temperatures increased the degree of membrane unsaturation in crayfish species (Cossins, 1976, Farkas and Nevenzel, 1981, Pruitt, 1988), the shore

crab *Carcinus maenas* (Chapelle, 1978), barnacles (Cook and Gabbott, 1972) and amphipods (Dawson, Morris and Lockwood, 1984). In fact, none of these studies has been carried out on isolated membranes, of a defined type and level of purification, therefore direct comparison was difficult.

The results reported in the paper of Chapelle (1978) on *Carcinus maenas* acclimated to 7°C, 14°C and 27°C were considered in more detail. The S/U ratio of total phospholipid from 7°C-acclimated crabs was lower (0.23) than those of the 14°C and 27°C-acclimated crabs (0.27 and 0.26, respectively). The S/U ratio of PC increased with acclimation temperature (0.33 in 7°C-acclimated crabs and 0.43 and 0.41 in 14°C and 27°C-acclimated crabs, respectively), but the S/U ratio of PE, although lower than that of PC, did not vary with acclimation temperature (0.20-0.21 at all three acclimation temperatures). The greatest changes with acclimation temperature occurred in the levels of polyunsaturated fatty acids (decreased) and of monounsaturated fatty acids (increased). There was little difference between the S/U ratios of 14°C and 27°C-acclimated crabs, despite a difference of 13°C in acclimation temperature, compared to the difference between the S/U ratios of 7°C and 14°C-acclimated crabs, suggesting that other mechanisms than the overall S/U ratio were involved in the acclimation to 27°C. This could be correlated with our findings about the fatty acid composition of membranes of 22°C-acclimated crabs, which was not different from that of the 8°C-acclimated crabs, but in some cases (spring experiment) the cholesterol/phospholipid molar ratio of the 22°C-acclimated membranes was higher than at lower acclimation temperatures.

The analysis of the changes in the chain length distribution did not reveal the expected consistent patterns, that the cold-acclimated groups had higher concentrations of long chain, polyunsaturated fatty acids and the warm-acclimated groups had higher concentrations of short chain saturated fatty acids (Thompson, 1983).

When fatty acids were grouped by the series to which they belong, ω3 or ω6, it appeared that in the winter experiment the large reduction in the levels of

polyunsaturated fatty acids observed in the 15°C-acclimated crabs was due mainly to a decrease in the level of ω3 fatty acids, while in the spring experiment the differences in the levels of ω3 and ω6 fatty acids due to acclimation temperature itself were low compared to the differences between the groups acclimated to 5°C and 15°C and those acclimated to 8°C and 22°C. Of the two major ω3 acids, C20:5 and C22:6, the C20:5 varied more with acclimation temperature. In a few cases, the C22:6 was higher in the 22°C-acclimated crabs (*Cancer pagurus* PC and PE, spring, and only PE, autumn).

In his review on aspects of phospholipid metabolism in crustaceans in relation to changes in environmental temperatures and salinities, Chapelle (1986) suggested that, although there was little information on the origin and the role of ω3 acids in marine crustaceans, the ω3 fatty acid requirements might represent one of the adaptation mechanisms in marine animals subjected to environmental fluctuations. The structure of the ω3 fatty acids allows a greater degree of unsaturation than the ω6 or ω9 and this may contribute to a greater plasticity of membrane structure.

The findings of the spring experiment were difficult to explain without a study on the temperature dependence of the activities and specificities of the enzymes involved in the regulation of the membrane phospholipid fatty acid composition and saturation. A study on the effects of temperature acclimation on the composition of the fatty acyl - Coenzyme A pool in liver of rainbow trout (Hazel and Livermore, 1990) suggested that the temperature dependence of substrate specificities for the enzymes of phospholipid synthesis and turnover plays a primary role in determining the acyl chain composition of membrane lipid following a shift in temperature. Tocher and Sargent (1990) found that acclimation temperature influenced the extent of incorporation of ω3 and ω6 polyunsaturated fatty acids in fish cells in culture, but did not affect differently the two series. Their conclusion was that the direct kinetic effect of temperature on the activity of the enzymes involved in the regulation of the fatty acid composition of membrane phospholipids is an important factor and suggested

that this effect is dependent on the level of stress a certain acclimation temperature represents.

Chapelle (1986) reviewed information showing that environmental temperature strongly influences various aspects of phospholipid metabolism in marine crustaceans, such as the distribution of PC between hemolymph and tissues in *Carcinus maenas* (Chapelle, Brichon and Zwingelstein, 1982), the velocity of total phosphatide renewal in *Carcinus maenas* tissues, which was higher at warmer temperatures (Chapelle *et al.*, 1977) and also the velocity of synthesis of individual phospholipid classes, each having a different temperature dependence (Chapelle *et al.*, 1977). The biosynthesis of PC increased more with temperature than that of PE (Chapelle *et al.*, 1977) and it was proposed (Chapelle, 1986) that the different temperature activations of the biosynthesis of PC and PE can represent an important supplementary process of adaptation to environmental temperature, together with the N-methylation pathway which would convert PE into PC.

It can be speculated that at different acclimation temperatures, different mechanisms of regulation of lipid composition and membrane fluidity are prevalent, for example a regulation via changes in the unsaturation of fatty acids at 8°C and 15°C, and a regulation via modulation of the PC/PE ratio and the cholesterol/phospholipid ratio at 22°C.

A few reports on differences in lipid composition between species of invertebrates evolutionarily adapted to different temperatures are available. Farkas *et al.* (1994) compared the lipid composition of two species of shrimp living at different latitudes: *Pandulus borealis* (Norway, 2°C) and *Parapandulus sp.* (Egypt, 27-30°C) and showed that the major dominating fatty acids were C16:0, C18:0, C18:1, C20:4, C20:5 and C22:6 in both species, independent of their geographic location and inhabiting temperature. Moreover, the S/U ratio of total phospholipid, PC and PE did not show any notable difference between species. The cold-adapted species had increased levels of C18:1 and C20:5, whereas the warm-adapted species had increased levels of C18:0 and C20:4, particularly in PE. They suggested that the increased level

of C20:5 in cold-adapted species might have dietary origin, because in cold water bodies the diatomaceae rich in this fatty acid predominate, while C18:1 might be the result of its preferential formation. Docosahexaenoic acid (C22:6) levels were similar in cold- and warm-adapted species.

Viarengo *et al.* (1994) reported that cold- (Antarctic) and warm- (Mediterranean Sea) adapted species of scallops differed in their ratios of short/long and straight/branched fatty acid chains and also in the cholesterol content of the membranes of digestive gland cells.

A few differences were observed in the acclimatory response of *Cancer pagurus* and *Carcinus maenas*. The usually larger extents of variations with acclimation temperature in the phospholipid or cholesterol contents and in the fatty acid composition of membrane phospholipids suggested that *Carcinus maenas* has a somehow greater adaptive capacity than *Cancer pagurus*. This agreed with the theory that animals that live in a relatively constant environment have less capacity for acclimation than animals that live in environments with seasonal cycles (Prosser and Heath, 1991). *Cancer pagurus* is known as a stenothermal species, that prefers habitats with a relatively constant temperature, as opposed to the eurythermal *Carcinus maenas* which is exposed to large variations in temperature with the tidal cycle and from one season to another. However, the results of our experiments showed that both species were able to adapt to all the four temperatures investigated, although *Cancer pagurus* living in the North Sea around the shores of Great Britain is not very likely to experience a temperature as high as 22°C in natural conditions. There was no major difference in the adaptive strategies of the two species, apart from different directions of variation in the monounsaturated fatty acids in some cases.

A consistent difference was that the cholesterol/ phospholipid molar ratios were lower in *Cancer pagurus* than in *Carcinus maenas*, in all seasons, in all acclimation groups. The differences in the cholesterol to phospholipid ratios were due

mainly to higher concentrations of phospholipids in the *Cancer pagurus* plasma membranes.

To the best of our knowledge there are no other studies comparing the lipid composition of a stenothermal and a eurythermal species of marine decapod crustaceans.

Crustacean species with different over-wintering strategies have been compared. Pruitt (1988) studied a winter-active (*Cambarus bartoni*) and a winter-dormant (*Orconectes propinquus*) species of crayfish and showed that both species were able to change the overall degree of saturation of membrane lipids in response to temperature, but the overall adaptation to temperature in membrane composition was related to their over-wintering strategy. The level of saturates was significantly reduced at low temperatures in the winter-dormant but not in the winter active species. In PE, the proportion of monoenes increased with cold acclimation only in the winter-active species (Pruitt, 1988). Cold acclimation increased the ω6 in PE in both species, and also the ω3 in the total phospholipids in both species (Pruitt, 1988). According to other studies, the increase in ω3 was characteristic only for winter-active species. Farkas (1979) and Farkas, Nemecz and Csengeri (1984) observed such an increase with cold acclimation only in winter-active species of planktonic crustaceans. In a winter-dormant planktonic crustacean, *Daphnia magna*, cold temperature had little or no effect on the degree of total phospholipid or phosphatide specific unsaturation (Farkas, Nemecz and Csengeri, 1984). The most striking difference between the winter-active and the winter-dormant species of crayfish was that the winter dormant species did not alter the relative proportions of its phospholipid classes, while in the winter active species cold-acclimation induced a significant increase in the relative proportion of PE, SM and PI and a decrease in PC (Pruitt, 1988).

Apart from the seasonal differences in the overall response to acclimation temperature discussed above, a major seasonal difference between the fatty acid

composition of the plasma membrane phospholipids was observed in both species. The plasma membranes from crabs caught in spring contained significantly higher concentrations of saturated and short chain monounsaturated fatty acids than those from crabs caught in autumn, while in autumn the concentrations of long chain polyunsaturated fatty acids were significantly higher than in spring. The saturated to unsaturated fatty acid ratios were significantly higher in spring than in autumn, in most acclimation groups. The fatty acid composition of membranes from crabs caught in winter were closer to those of crabs caught in spring, suggesting that the accumulation of polyunsaturated fatty acids in autumn reported also for other species (Farkas and Herodek, 1964) does not last throughout the winter. The most marked seasonal differences in individual fatty acids were at the C22:6 (ω3) level, which was significantly higher in autumn in both species. The winter levels of C22:6 were intermediary between the autumn and spring ones in a few cases, and similar to spring levels in other cases. In both *Cancer pagurus* and *Carcinus maenas* the levels of ω3 fatty acids were lower in spring in the 5°C and 15°C-acclimated groups. At cold acclimation temperatures, the ω6 fatty acids were higher in autumn, and at warm acclimation temperatures, the ω6 group was higher in spring.

Seasonal differences in fatty acid composition of membrane phospholipids have been reported by other authors. Farkas and Herodek (1964) found that freshwater planktonic crustaceans accumulated long unsaturated fatty acids during the decrease in temperature in autumn, which disappeared in spring. Cook and Gabbott (1972) reported that barnacles were most cold-tolerant in winter and least cold-tolerant in summer and these differences were correlated with seasonal changes in the degree of unsaturation of the body lipids.

It was interesting that in our experiments the seasonal differences were not altered by the three weeks of laboratory acclimation. Layne, Claussen and Manis (1987) also found that in crayfish heat resistances were typically highest at certain points during the summer or fall, and cold resistances were highest in winter, despite laboratory acclimation to constant temperature. The same was observed in other

aquatic vertebrates, even when the laboratory acclimation periods were of up to several weeks (Layne, Claussen and Manis, 1987). Significant increases in the levels of docosahexaenoic acid (C22:6) in autumn or winter, or with-cold acclimation have been reported by Farkas, Storebakken and Bhosle (1988), Farkas (1979) and Chapelle (1978) in crustaceans and by several authors in fish (Farkas and Csengeri, 1976, Cossins, 1977, Cossins and Prosser, 1978, Grove Sørensen, 1990). Farkas (1979) proposed a generalization that 'fish and crustaceans alike, manipulate their level of docosahexaenoic acid to maintain membrane integrity and functions in the cold, and their ability to accumulate this fatty acid influences seasonal occurrence and perhaps geographical distribution of these animals'. A recent study of Buda *et al.* (1994) concluded that the gross amount of C22:6 did not play a major role in membrane adaptation to temperature. It was proposed that certain molecular species of PE (Buda *et al.*, 1994) or PC (Hazel *et al.*, 1991) containing C22:6 may play a more important role in adjusting the membrane physical properties to temperature, rather than the total amount of C22:6.

The seasonal comparison also showed that for *Cancer pagurus*, the cholesterol/ phospholipid molar ratio was much higher in autumn in the 22°C-acclimated group, due to higher amounts of cholesterol. The plasma membranes of warm-acclimated *Carcinus maenas* had higher amounts of both cholesterol and phospholipids in autumn and a lower molar ratio. In cold-acclimated groups, the concentrations of cholesterol and phospholipids were higher in spring, in both species, but the molar ratios were not significantly different between seasons. Tsai, Chen and Tsai (1984) reported that in the blue crab *Callinectes sapidus* the cholesterol content of muscle (total tissue) was low compared to hepatopancreas and gonads, and there was an obvious trend for reduction of both cholesterol and total lipid concentrations from August to September (in mature males).

The long chain polyunsaturated fatty acids accumulated before winter could make the crabs more able to tolerate sudden decreases in temperature, and the saturated fatty acids accumulated before summer more able to tolerate rapid increases

in temperature. One would expect such a seasonal adaptation to be greater in a eurythermal species, but our results do not demonstrate a major difference between the seasonal variations in *Cancer pagurus* and *Carcinus maenas*.

The fact that in *Cancer pagurus* and *Carcinus maenas* plasma membranes the fatty acid composition of phospholipids appeared to be influenced more by complex seasonal adaptations than by laboratory acclimation to constant temperature suggested that in these species the fatty acids may play a more important role in the rapid adjustments of membrane fluidity to variations in environmental temperature, by processes of rearrangement between phospholipid classes or in different molecular species of the same class, without new synthetic requirements. Hazel and Landrey (1988b) suggested that modulation of phospholipid molecular species composition may provide a mechanism for effecting rapid homeoviscous adjustments in membrane architecture, while the changes in the proportions of species containing long chain polyunsaturated fatty acids are considerably slower. Farkas *et al.* (1994) also argued against the role of saturated/unsaturated fatty acid ratio in membrane lipid adaptation and proposed that the reshuffling of fatty acyl chains between phospholipid molecules, to produce specific molecular species has a more important role.

Chapter 5. Temperature Acclimation of the Neuromuscular Function

5.1. Introduction

Adaptation of the nervous system to temperature is a major component of the thermal adaptation of animals (Prosser and Nelson, 1981) because vital integrative processes, such as coordinated motor activity, sensory input and learning depend on the well-functioning of the entire nervous system. Each component function of the nervous system, such as synaptic transmission and axonal conduction, is influenced by changes in temperature, therefore ectotherms had to develop mechanisms for balancing the temperature effects on the processes involved when nervous system functions were challenged by fluctuations in environmental temperature (Macdonald, 1994). The cold and heat tolerance of an organism, and ultimately its ability to survive in conditions of altered temperature, have been suggested to depend on the cold and heat tolerance of the 'weakest link', which may be a particular cellular structure and/or function (Lagerspetz, 1987).

The interneuronal and neuromuscular synapses have long been among the presumed 'weakest-links', whose perturbation would cause a general loss of nervous integration and control of both autonomic and neuromuscular activity (Cossins and Bowler, 1987). Behavioural disturbances, starting with hyperexcitability and followed by uncoordinated swimming, loss of equilibrium and ultimately coma and respiratory failure, were observed in goldfish during cooling or heating of the entire fish or only of the cerebellum to extreme temperatures (Friedlander, Kotchabhakdi and Prosser, 1976, Cossins, Friedlander and Prosser, 1977). These effects, presumed to be related to synaptic block, occurred in the sequence given above and their critical temperatures were adaptively modified by thermal acclimation. Block of synaptic transmission is significantly more sensitive to heating and cooling than is cessation of axon conduction (Lagerspetz, 1974). Kivivuori (1980) found that in the crayfish *Astacus*

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astacus the isolated nerve cord was still showing neuronal activity at 36°C, while simple reflexes like those maintaining the respiratory movements of scaphognathites stopped at 32°C, walking at 30°C, and the more complex righting reflex at 26°C. Studies on the neuromuscular function in frog (Grainger, 1973, Jensen, 1972) and crayfish (White, 1983) showed that the transmission between the motor nerve cells and the muscle cells, at synapse level, was more sensitive to heat and cold than the functions of either nerve or muscle cells and that inhibitory synapses were more sensitive than excitatory ones (Macdonald, 1990). The experiments on goldfish also suggested that the inhibitory synapses were more sensitive than the excitatory ones in some parts of the central nervous system (Friedlander, Kotchabhakdi and Prosser, 1976). These observations seemed to indicate that the more synapses there are in a control pathway, the less temperature resistant is the function (Prosser and Nelson, 1981, Lagerspetz, 1987).

Several examples have demonstrated that excitable membranes can adapt or acclimate their function (Macdonald, 1988 and 1990) and biophysical properties (Cossins, 1994) to extreme thermal conditions. Cossins, Friedlander and Prosser (1977) showed that the changes in behavioural resistance to temperature observed in goldfish during thermal acclimation were correlated in direction and overall time course with changes in the fluidity of brain synaptosomes, which in turn appeared to be determined by changes in the saturation of membrane phospholipids. However, little evidence supports the idea of a direct causal relationship between the changes observed in the lipid bilayer composition or physical state and the functional adaptation of excitable membranes (Macdonald, 1990).

The effects of temperature acclimation on the neuromuscular function and on related processes have been extensively studied in crustaceans. Examples of well-studied species are the Pacific shore-crab *Pachygrapsus crassipes* (Stephens, 1990, review) and various crayfish species, such as *Austropotamobius pallipes* (Gladwell, Bowler and Duncan, 1975), *Astacus leptodactylus* (Harri and Florey, 1977, 1979), *Procambarus clarkii* (White, 1983) and *Astacus astacus* (Kivivuori, Lehti and

Lagerspetz, 1990, Kivivuori, Laine and Lagerspetz, 1984, Kivivuori and Lagerspetz, 1982). These are all eurythermal species that remain active over wide ranges of temperatures and their acclimation to different temperatures caused compensatory shifts in the thermal resistance and temperature dependence of neuromuscular function, with optimal activities usually occurring around the acclimation temperature (Stephens, 1990). Relatively few stenothermal species have been studied. Blundon (1989) compared the response to cold-acclimation in a warm-water stenotherm, the stone crab *Menippe mercenaria*, with the eurythermal blue crab, *Callinectes sapidus* and also in northern and southern populations of the same species. The hypotheses to test were that, within the same species, the neuromuscular response to temperature changes depends on the range of temperature experienced by each population and that, between species, animals that frequently experience large temperature fluctuations are more able to cope with such changes than animals which live in a relatively constant thermal environment. Testing the second hypothesis is also one of the objectives of the work presented in this chapter and thesis. Blundon (1989) concluded that the main difference between the stenothermal and the eurythermal species studied was in the time course of the cold-acclimation process. The stenothermal species required a gradual shift in temperature, over a long period of time (6 months outdoors, following the seasonal decrease in temperature). The eurythermal species achieved similar compensation of the neuromuscular function after only 4 weeks of laboratory acclimation.

Possible mechanisms involved in the temperature acclimation of neuromuscular function, such as adaptive changes in the electrical properties and in the functional characteristics of the pre-synaptic or post-synaptic membranes, and their relation to alterations in the chemical composition or biophysical state of these membranes, have also been studied (Gladwell, Bowler and Duncan, 1975, Cossins, Friedlander and Prosser, 1977, White, 1983, Macdonald, 1988, Macdonald, 1990).

Decapod crustaceans are particularly suitable for studying the temperature acclimation of neuromuscular function because of the particularities of their neuromuscular system, compared to that of vertebrates.

The most characteristic difference between the neuromuscular system of decapod crustacea (also arthropods, in general) and that of vertebrates is that in vertebrates each muscle is innervated by a number of motor nerve fibres, all with similar properties, whereas in Crustacea only a very few efferent axons, each with a well-defined effect, form the supply for the muscles (Wiersma, 1961). Many decapod crustacean muscles are innervated by three axons, the slow and fast motor axons and an inhibitory axon. These terms do not refer to their conduction speeds, but to the rapidity of the contractions resulting from their stimulation. Excitation of the inhibitory neurone suppresses contractions caused by the firing of the excitatory motor neurones. An individual muscle fibre may be innervated by two or more axons (polyneuronal innervation) and all are multiterminally innervated (Aidley, 1989).

The innervation scheme for the distal muscles in legs of Brachyura Decapods, group which includes *Cancer pagurus* and *Carcinus maenas*, was established by Wiersma and Ripley (1952) (Figure 5.1.a).

The small number of motor axons in Crustacea demands a control mechanism for muscular contraction differing from that of successive recruitment of motor units in the higher vertebrates. In many arthropod muscles the cell membranes are electrically excitable, but the postsynaptic electrical responses are not of an 'all-or-nothing' type, as in vertebrate muscle fibres.

The vertebrate neuromuscular junction is specialised for 'one-for-one' transmission with a high safety factor, where the release of neurotransmitter is much higher than that necessary for inducing the muscle contraction or for triggering the action potential in the next neuron (Cunningham and Hyde, 1995) and is little affected by temperature acclimation. Tiiska and Lagerspetz (1994) showed that in the peripheral nerve terminals of the frog *Rana temporaria* the rate of exocytosis of synaptic vesicles and the rate constant of conformational changes of receptor-channel

Figure 5.1. a) The innervation scheme for distal muscles in legs of Brachyura Decapods (from Wiersma and Ripley, 1952); **A** = adductor, **B** = bender, **C** = closer, **E** = extensor, **F** = flexor, **O** = opener, **S** = stretcher; _____ (full lines) = excitatory axon, ------- (dotted lines) = inhibitory axon
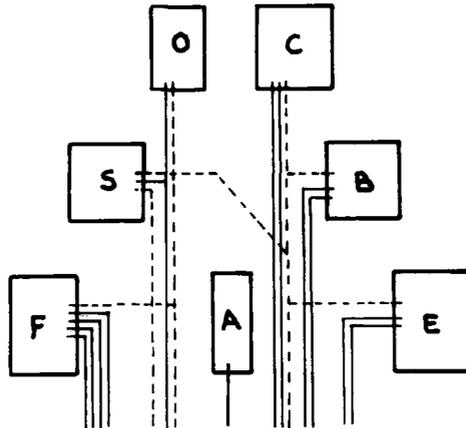
b) The segments of a decapod crustacean leg
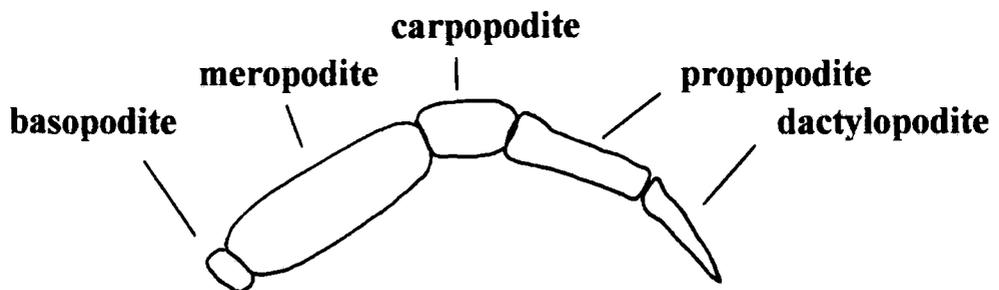
molecules were not affected by temperature acclimation. Harper, Shelton and Watt (1989) did not find any difference in the channel closing rate constants in extraocular muscle from cold and warm-acclimated carp, either, but they found a slight acclimation effect on the duration of the growth phase of the miniature end plate currents recorded at low temperatures, probably due to differences in the presynaptic neurotransmitter release.

In contrast, in crustaceans the postsynaptic responses are graded and the size of the postsynaptic potential is determined by the amount of neurotransmitter released. The exocytosis of neurotransmitter is considered to be the main temperature-sensitive part of synaptic transmission (Macdonald, 1988) and it is also dependent on which axon, fast or slow, is active, on the frequency of the excitatory nerve impulses, which affects the degree of membrane depolarization by summation and facilitation of the junction potentials, on the activity of the inhibitory axon and on the neuromodulator axon, where this exists (Aidley, 1989).

The purpose of the work presented in this chapter was to investigate the influence of temperature acclimation on the temperature dependence of some parameters of neuromuscular function in walking legs of *Cancer pagurus* and *Carcinus maenas*.

The parameters measured were the conduction velocity in axons of leg nerve, using an extracellular recording technique, and the closer muscle resting membrane potential and the amplitudes, facilitation and latency of the excitatory junction potentials evoked in the closer muscle fibres by electrical stimulation of the excitatory axon, using microelectrode techniques.

The axonal conduction is a phenomenon of intermediate complexity and accessible for study in a wide variety of animals, with relatively simple experimental set-ups. The conduction velocity is influenced by the capacitance and resistance of the membrane, and by the internal resistance of the cell, which in turn depend on axon diameter and myelination. The formula describing the inter-relation between these

factors in determining the conduction velocity was originally deduced by Huxley (1959) and subsequently refined by Easton and Swenberg (1975). This formula (Macdonald, 1994) includes also the temperature coefficients of these parameters and also, and most important for the temperature-dependence studies, the temperature coefficients of the changes that occur in the ionic permeability and conductance of the axonal membrane. Harper et al. (1990) demonstrated a great extent of thermal acclimation of the axonal conduction velocity in carp nerve, in both myelinated and unmyelinated axons. The temperature dependences of the conduction velocity displayed discontinuity points, which were shifted by temperature acclimation in a compensatory manner (Harper et al., 1990).

The resting membrane potential reflects the conductance state of the membrane through which Na^+, K^+ and other ions passively diffuse, and the activity and coupling of the (Na^+,K^+)-ATPase pump (Macdonald, 1990). Cossins, Bowler and Prosser (1981) showed that thermal adaptation of goldfish involved changes in the thermal stability of the synaptic (Na^+,K^+)-ATPase, which were correlated to changes in the fluidity of the synaptic membranes. Temperature acclimation of the activity of other ATPases may be involved in the modulation of the electrical properties of excitable membranes following temperature acclimation. Lagerspetz and Skyttä (1979) found a perfect temperature compensation of the Na^+ transport across frog skin, measured as short-circuit current, when they compared frogs acclimated to 6°C, 12°C and 23°C, which appeared to be due to an inverse temperature compensation in the activity of a Mg^{2+}-ATPase, while the activity of the (Na^+,K^+)-ATPase from the same membranes showed little temperature compensation.

The amplitude of the excitatory junction potentials is determined by the amounts of neurotransmitter released at the neuromuscular synapse and by membrane cable properties, such as capacitance and resistance (Blundon, 1989).

The effect of cold- and warm-acclimation on the temperature dependence of these membrane-related parameters and the time-course of the acclimation-induced changes were sought. Compensatory changes in the dependence of temperature of

these parameters following temperature acclimation, in order to maintain an efficient neuromuscular function over a wider range around the acclimation temperature, have been reported for many crustacean species (Stephens, 1990, Macdonald, 1990), as detailed above, but the time course of these changes has not been studied. The compensation in the myofibrillar ATPase activity of fish skeletal muscle has been used by other authors as a criterion for the completion of the temperature acclimation process in fish species (Harper, Shelton and Watt, 1989, Harper et al., 1990, Heap, Watt and Goldspink, 1985, Johnston, Davison and Goldspink, 1975).

The microelectrode technique, used here for measuring the resting membrane potential and the closer muscle response to stimulation of the innervating axons, was developed in the late 1940s by Ling and Gerard (1949), and further refined and used for a variety of studies of the ionic basis of resting and action potentials and of synaptic transmission (Hall, 1992). The fine tip (approx. 0.5 µm diameter) of the micropipette electrode does not irreversibly damage the cell membrane and although the technique has certain drawbacks of electrical and technical order, most of these can be overcome and do not alter the versatility of the technique, which has evolved from measuring various parameters of single cells, to the patch-clamp technique which allows measurement of single ionic channels (Sakmann and Neher, 1983) and to ion-selective intracellular electrodes for measuring intracellular ionic concentrations (Standen, Gray and Whitaker, 1987).

As shown above in Figure 5.1.a, the closer muscle in Brachyura crabs is innervated by two motor axons, one fast and one slow, and an inhibitory axon (Wiersma and Ripley, 1952). The fibre structure of the closer muscle has been described in detail for various species of crustaceans, including crayfish (Günzel, Galler and Rathmayer, 1993), lobster (Govind, Budd and Atwood, 1981) and crab (Atwood, 1963, Maier, Pette and Rathmayer, 1986, Tse, Govind and Atwood, 1983). The best studied are the fibres of the closer muscle of the crab *Eriphia spinifrons* (Rathmayer and Maier, 1987, Read and Govind, 1993). These were classified in four groups, I to IV, according to their specific biochemical and histochemical properties

and combinations of several parameters such as innervation pattern, facilitation properties of the excitatory junction potentials and existence and extent of pre- and post-synaptic inhibition (Maier, Pette and Rathmayer, 1986). When the fine structure of these fibre types was investigated (Read and Govind, 1993) it appeared that all four types had a fine structure characteristic of crustacean slow muscle. In crayfish closer muscle only three major groups of fibres were distinguished, which differed in histochemistry, electrophysiology and morphology (Günzel, Galler and Rathmayer, 1993).

5.2. Materials and Methods

5.2.1. Animals

Crabs (adult, intermoult) caught in April 1994 from the North Sea, near Hartlepool, were maintained in tanks with aerated filtered sea water in thermostated rooms (12 hrs light-12 hrs dark). Cold-acclimated crabs were kept for 3 weeks at 8°C. Warm-acclimated crabs were kept a day or two at 8°C, to settle in laboratory conditions, then moved for a week at 15°C, then directly to 22°C for 3 weeks. The crabs were fed weekly on frozen fish (commercial source) and the water changed the day after feeding.

5.2.2. Conduction Velocity Measurements

Measurements were carried out on leg nerve isolated from the meropodite (Figure 5.1.b) of walking legs from cold- and warm-acclimated crabs. During the dissection, the excised limbs were bathed in freshly prepared crab saline: 470 mM NaCl, 8 mM KCl, 20 mM $CaCl_2$, 10 mM $MgCl_2$ and 5 mM HEPES, pH 7.2 at 20°C (Stephens and Atwood, 1982). This buffer has a low temperature coefficient of -0.015 pH units per °C (Dawson et al., 1986), therefore the pH was allowed to vary freely with temperature.

The dissection was carried out under a binocular dissecting microscope. The leg was fixed to a piece of wood, with cyanoacrylate glue, with the carpopodite (Figure 5.1.b) pointing upwards. A large window was cut in the exoskeleton of the meropodite using a drill, the underlining cuticle and muscle were removed, then the nerve was freed of connections with the remaining muscle by running the tip of a fire polished glass electrode under it. The loose (proximal) end of the nerve and the end near the joint with the carpopodite (distal) were tied with surgical thread, then the nerve was cut at the distal end and transferred to a small volume of crab saline and maintained at a temperature equal to the acclimation temperature of the crab from which the leg had been excised.

The experimental set-up used for the extracellular recordings is schematically represented in Figure 5.2.a. The length of the nerve (up to 2.5 cm) was arranged between the stimulating electrodes and the recording electrodes, with a grounded monopolar electrode placed close to the stimulating electrodes to minimise stimulation spread (Harper et al., 1990). The silver wire electrodes were glued to a thin glass microscope slide (approximately 2 mm distance inter-paired electrodes), to maintain a fixed distance between the stimulating and the recording electrodes (1.7 cm). The glass slide was placed on a metallic plate, the temperature of which was controlled by internal circulation of water pumped from a water-bath fitted with a temperature control unit Tempunit TU-16D (Techne), linked to a temperature programmer unit TP-16 (Techne). The heat transfer between the glass slide and the metallic plate was facilitated by a thin layer of thermoconductive paste.

The nerve was kept moist by covering it with a thin strip of filter paper which had one end immersed in a small glass container with crab saline that was maintained at the same temperature with the nerve during the experiments (on the same controlled-temperature plate). The stimulating electrodes were connected to a battery-operated stimulus isolation unit (Digitimer Ltd., model DS 2A), triggered by a signal from a CFP Stimulator 8127, which produced square-wave, constant-voltage pulses of 50 µs width, with a 1 ms delay, at a frequency of 10 Hz. The amplitude of

Figure 5.2. a) Schematic representation of the experimental set-up used for the axonal conduction velocity measurements;
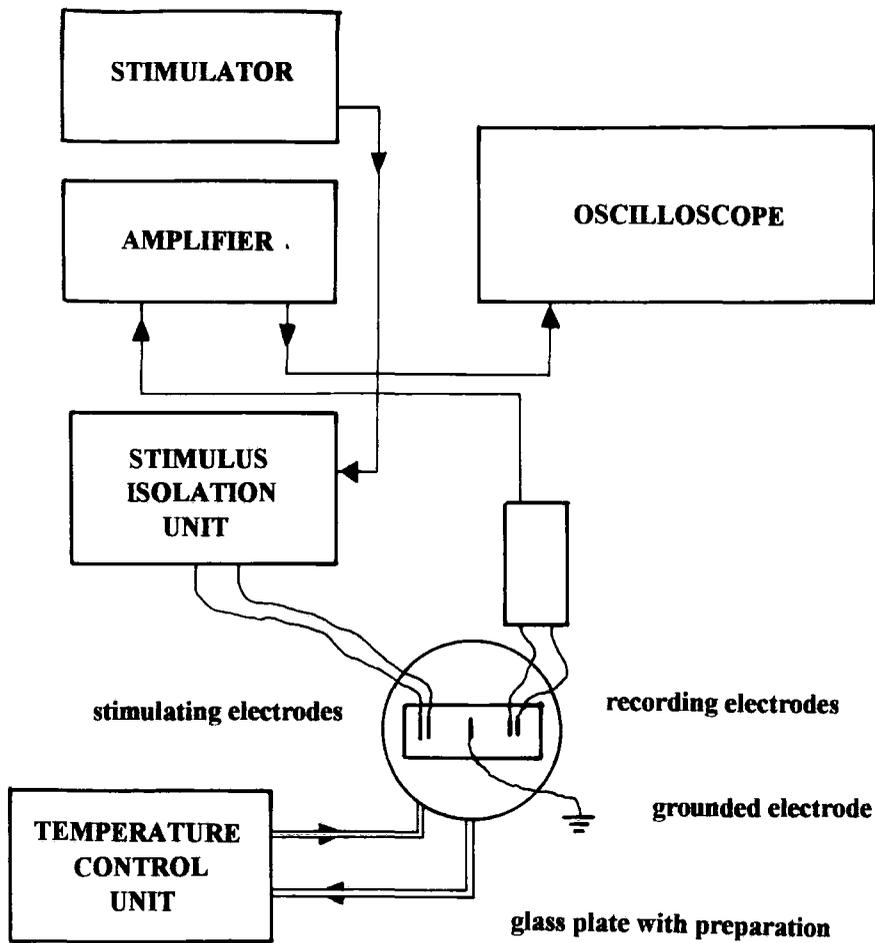
b) Trace of an action potential on the oscilloscope screen in an extracellular recording
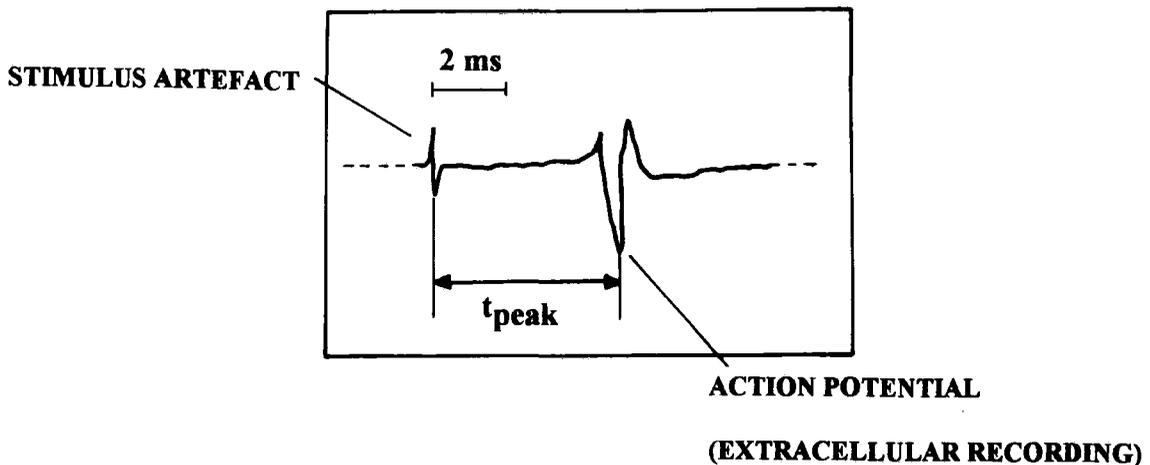
the pulses was gradually increased (range 0-7 volts) until stable action potentials were observed on the oscilloscope screen (the electrical activity caused by the evoked action potentials was transmitted via the recording electrodes to an amplifier, then visualised on the oscilloscope screen).

The axon measured was not precisely identified: the fibre, or fibres that were the first to produce a steady train of action potentials during the slow increase of the stimulation pulse, at low temperature, were measured over the whole range of temperature.

The time between the stimulus artefact and the peak of the action potential (t_{peak}, of the order of ms) was read from the oscilloscope screen (Figure 5.2.b) and used to calculate the conduction velocity (CV) with the following formula:

$CV = d / t_{peak}$, in m/s, where d was the distance between the stimulating and the recording electrodes (0.017 m) and t_{peak} was expressed in seconds.

The conduction velocity in nerves isolated from cold- and warm-acclimated crabs was measured over a range of temperatures from 5 to 35°C (±0.1°C), at a rate of temperature increase of 0.5°C/minute. Axons from warm-acclimated crabs were slowly cooled and all measurements were taken during warming. The temperature of the nerve was monitored with a thermocouple fixed with BlueTac to the glass slide as close as possible to the nerve.

5.2.3. Resting Membrane Potential and Excitatory Junction Potential Measurements

Measurements of the resting membrane potential of the dactylopodite closer muscle fibre and recordings of the excitatory junction potentials evoked in muscle fibres by stimulation of the excitatory axon were carried out on neuromuscular preparations from excised walking legs of cold- or warm-acclimated crabs, over a range of temperature from 5°C to 25°C (±0.1°C).

The dissection was performed under a binocular dissection microscope. The excised leg was glued on a side onto a wood block and a large window was cut in the

exoskeleton of the meropodite using a drill. The top muscle was carefully removed to expose the nerve. The middle part of the nerve was freed from the connections with the underlying muscle using the tip of a fire polished glass electrode and then the nerve was lifted on a thin glass rod and the bundle was split longitudinally in two thinner bundles with the same glass electrode. During the dissection, crab saline was frequently pipetted on the exposed nerve and muscle. A small window (approximately 2x2 mm) was cut in the propopodite, on the lateral side, just below the joint with the dactylopodite, where the muscle fibres are not attached to the exoskeleton (Cunningham, personal communication), in order to expose a small area of the closer muscle. A fine-tip drill was used for cutting this window and the procedure had to be executed with great care, to produce minimum of damage to the underlying muscle fibres, on which the recordings were to be made. The dactylopodite was held in closed position during the drilling, with the muscle in flexed state, not reaching the level of the drilling site.

The leg was pinned in a small plastic bath, with the bottom covered in a 0.5 cm thick layer of Sylgard (polymer resin, Dow Corning), filled with crab saline (see 5.2.2. for composition and pH). The dactylopodite was pinned down firmly, in stretched position (Figure 5.3.a) to prevent movement during nerve stimulation.

The microelectrode technique was used for the measurement of the resting membrane potentials and the recording of excitatory junction potentials. Glass microelectrodes with a resistance of 20-50 MΩ were pulled in standardized conditions, using an electrode puller (Harvard), and filled with a solution of 3M K(CH$_3$COO)$^-$ and 100 mM KCl (Standen, Gray and Whittaker, 1987). The potassium acetate was used instead of the potassium chloride because in case of leakage into the cell, the acetate ion could be metabolized by the cell, and it would not alter the membrane potential, as the chloride ion would (Coombs, Eccles and Fatt, 1955). The electric contact between the solution in the electrode and the recording apparatus was ensured by a silver-silver chloride wire. The microelectrode was held in a

Figure 5.3. a) The positioning of the neuromuscular preparation used for measuring the resting membrane potential of the closer muscle and the parameters of the excitatory junction potentials evoked in the closer muscle by electrical stimulation of the excitatory axon (microelectrode technique);

MICROELECTRODE

STIMULATING
ELECTRODES

REFERENCE ELECTRODE

BATH WITH CRAB SALINE SOLUTION

b) Schematic representation of the experimental set-up used for measuring the resting membrane potential of the closer muscle and the parameters of the excitatory junction potentials evoked in the closer muscle by electrical stimulation of the excitatory axon (microelectrode technique)

STIMULATOR

AMPLIFIER

OSCILLOSCOPE

INTERFACE

COMPUTER

STIMULUS
ISOLATION UNIT

microelectrode

stimulating electrodes

TEMPERATURE
CONTROL UNIT

reference electrode

PREPARATION

140
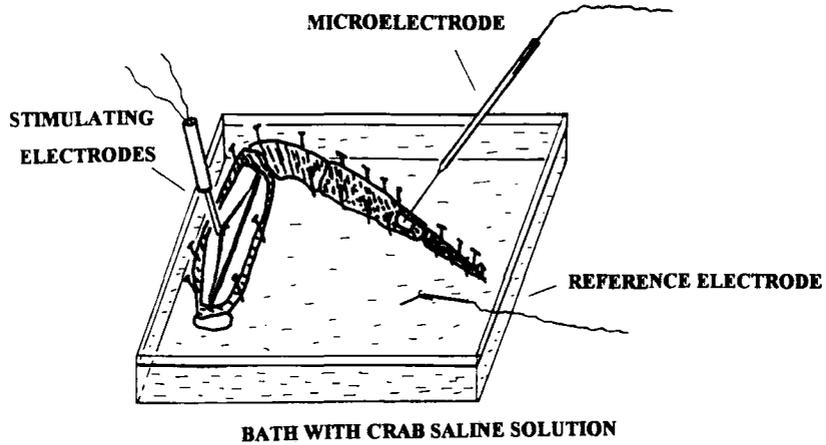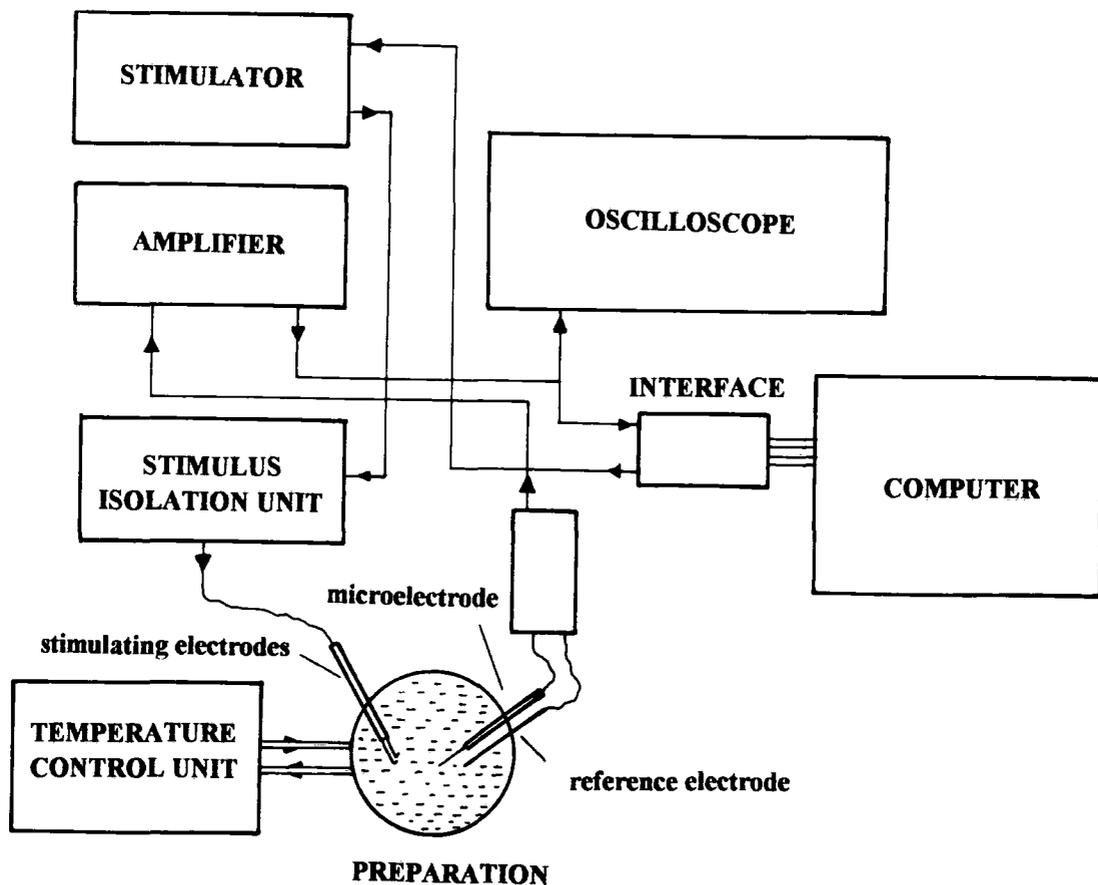
micromanipulator and its tip was carefully introduced in a muscle fibre. A reference electrode was inserted in the solution in the bath with the neuromuscular preparation (Figure 5.3.a).

One of the two half-bundles of the leg nerve was hooked on the stimulating electrodes and a few stimulating pulses were sent through to check that at least one of the excitatory axons was present in the half-bundle chosen. The stimulating electrodes consisted of two platinum wires (0.3 mm diameter, Fisons) connected to a stimulator, triggered by the computer (PC compatible, MJN Technical services) connected to the experimental set-up (Figure 5.3.b). This produced either single pulses or paired pulses of the same amplitude (the lowest that produced a response, range 0-7 V, pulse width 0.2 ms), at an interval of 40 ms. The EJP evoked in the muscle fibre was transmitted through an amplifier to an oscilloscope, then to the computer. The whole experiment, apart from the temperature of the solution in the bath with the neuromuscular preparation, was controlled and recorded using the SCAN software (J. Dempster, Strathclyde Electrophysiological Software).

The temperature of the bath with the neuromuscular preparation was controlled by a Peltier block, and monitored with a battery operated thermocouple. Temperature was slowly (approx. 0.5°C/min) increased from 5°C to 25°C for preparations from cold-acclimated crabs, or slowly decreased from 25°C to 5°C for preparations from warm-acclimated crabs and sets of 16 records, which were averaged by the computer, were taken every 1-2°C in the single pulse stimulation mode first, then in the double pulse stimulation mode.

The muscle fibre resting membrane potential was read at each temperature, before triggering the EJP recording. Although reports of hysteresis in membrane potential exist in literature (Kivivuori, Lehti and Lagerspetz, 1990) preparations from warm-acclimated crabs had to be measured in this way because often initial cooling of the preparation produced cold-induced contractions of the leg which broke the electrode and damaged the muscle fibres. The temperature threshold of the cold-induced contractions was difficult to predict (usually around 8-10°C).

Records were stored on floppy disks and later reloaded for reading the amplitudes and the latencies of the EJPs. The typical shape of the EJPs recorded in double pulse stimulation mode is shown in Figure 5.4.

Facilitation was calculated as $F = (A_2-A_1)/A_1$ where A_1 is the amplitude of the EJP evoked by the first pulse and A_2 is the amplitude of the EJP evoked by the second pulse (Figure 5.4). The latency was measured as the period of time elapsed between the stimulation of the nerve and the start of the EJP in the muscle fibre (Figure 5.4).

5.2.4 Time-Course of Changes in Neuromuscular Function During Temperature Acclimation

Crabs from both species were initially acclimated at either 8°C or 22°C for three weeks, as described in Section 5.2.1. The 8°C-acclimated crabs were transferred for a week to 15°C, then to 22°C and walking legs were sampled before the transfer (day 0), after 4 and 7 days at 15°C, and then after 7, 14 and 21 days at the final acclimation temperature (22°C). Both species tolerated well the transfer to warmer temperatures, subsequent to cold-acclimation. The 22°C-acclimated crabs were transferred to lower temperatures and the same sampling timetable was followed, after 4 and 7 days at 15°C, then after 7, 14 and 21 days at the final acclimation temperature (8°C). Some of the *Carcinus maenas* survived this down-shift in acclimation temperature and a few measurements were recorded, but the 22°C-acclimated *Cancer pagurus* did not cope well with the transfer to colder temperatures and all of them died during the first or second week.

The legs sampled at the above mentioned time-intervals from crabs in course of acclimation to cold or warm temperatures, were used for measurements of resting membrane potential and recordings of excitatory junction potentials as described in 5.2.3.

Figure 5.4. Trace of a typical excitatory junction potential (**EJP**) recording (average of 16 individual records) in the double-pulse stimulation mode: the parameters measured on the records were the amplitude of the first pulse A_1 (non-facilitated), the amplitude of the second pulse A_2 (facilitated) and the **latency** (time between the stimulus and the beginning of the rise of the first EJP); the amount of facilitation (Fac) was calculated as Fac $= (A_2 - A_1)/A_1$
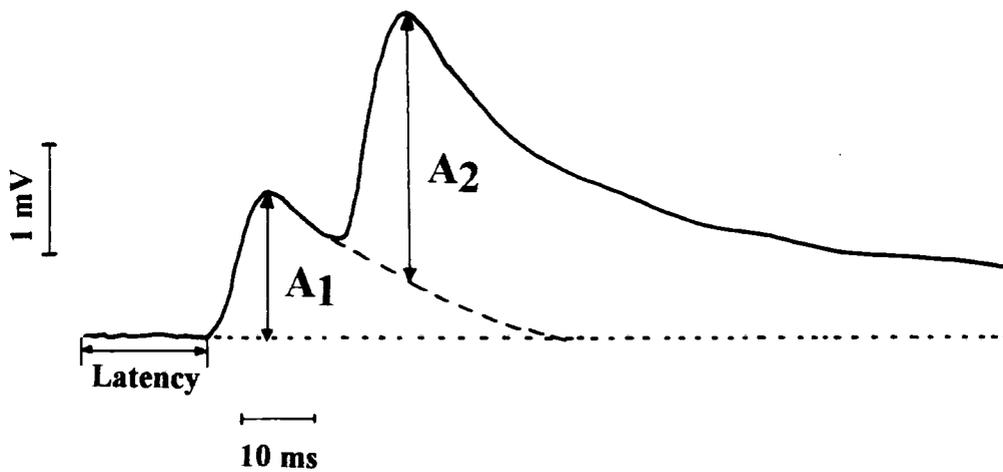
5.3. Results

5.3.1. Axonal Conduction Velocity

Figures 5.5 and 5.6 show the plots of axonal conduction velocity (in m/s) against temperature (in °C) for all the nerve preparations from legs of *Cancer pagurus* and *Carcinus maenas*, respectively. The width of the range of variation of the conduction velocities measured at a given experimental temperature was between 1 and 2 m/s. This could be due to the fact that the axon (or axons, where two fibres of the same nerve were measured) measured was not precisely identified, and also to individual variation in the diameter of the axons. There was little difference between the shape or slopes of curves for individual preparations of crabs from the same acclimation groups. Figure 5.5.a shows that in axons of 8°C-acclimated *Cancer pagurus* the conduction velocity increased continuously with temperature, up to a temperature at which the conduction was blocked. In only two of the six preparations measured the conduction velocity decreased slightly before the blockage temperature. The conduction velocity in axons of 22°C-acclimated *Cancer pagurus* (Figure 5.5.b) and 8°C- and 22°C-acclimated *Carcinus maenas* (Figure 5.6 a and b) had a discontinuous variation: it increased linearly with temperature up to a point around 17-22°C, above which it remained constant for a few degrees Celsius, then decreased just before the conduction blockage temperature.

Table 5.1 summarizes the temperature ranges over which maximum conduction velocity was measured, the temperatures of conduction blockage and the results of the comparison of the mean blockage temperatures between all the four groups (t-test). For both species, the average temperature of axonal conduction blockage was lower in the 22°C-acclimated crabs: 25.5°C in warm- compared to 29°C in cold-acclimated *Cancer pagurus* (not significant), and 28.5°C in warm- compared to 33°C in cold-acclimated *Carcinus maenas*, showing an inverse acclimation response. Also the maximum conduction velocities occurred over ranges of lower temperatures in warm-acclimated crabs: 18-25°C in warm-, compared to 26-31°C (the

Figure 5.5. The temperature dependence of axonal conduction velocity in individual preparations (isolated leg nerves) of *Cancer pagurus* **(a) 8°C-acclimated** (labelled as **Cp 8 1** to **Cp 8 6**) and **(b) 22°C-acclimated** (labelled as **Cp 22 1** to **Cp 22 5**, with **a** and **b** being two different axons in the same preparation)

a)
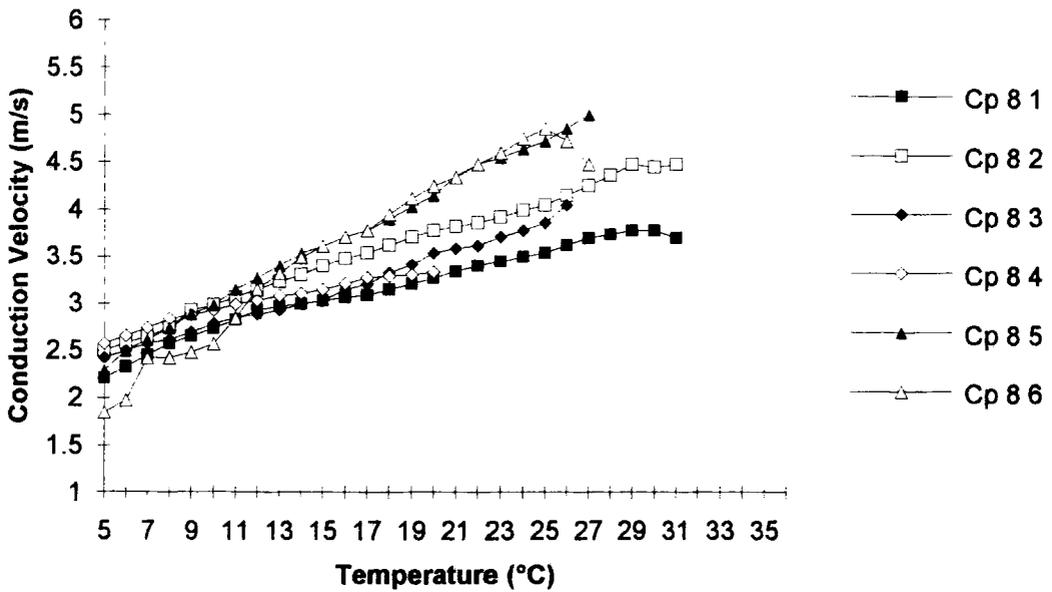
b)
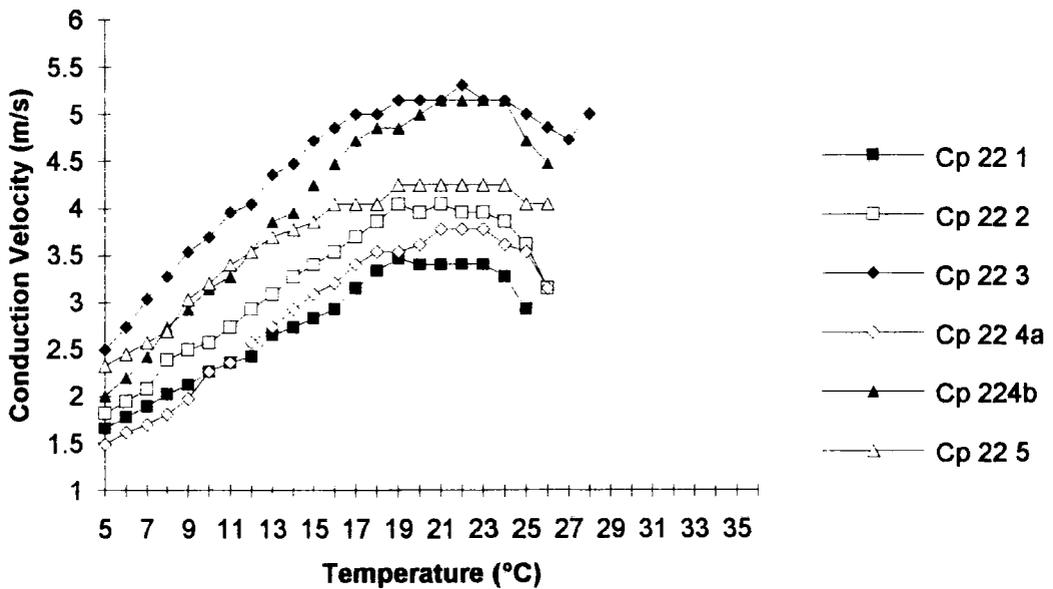
Figure 5.6. The temperature dependence of axonal conduction velocity in individual preparations (isolated leg nerves) of *Carcinus maenas* **(a) 8°C-acclimated** (labelled as **Cm 8 1** to **Cm 8 5**, with **a** and **b** being two different axons in the same preparation) and **(b) 22°C-acclimated** (labelled as **Cm 22 1** to **Cm 22 4**, with **a** and **b** being two different axons in the same preparation)

a)
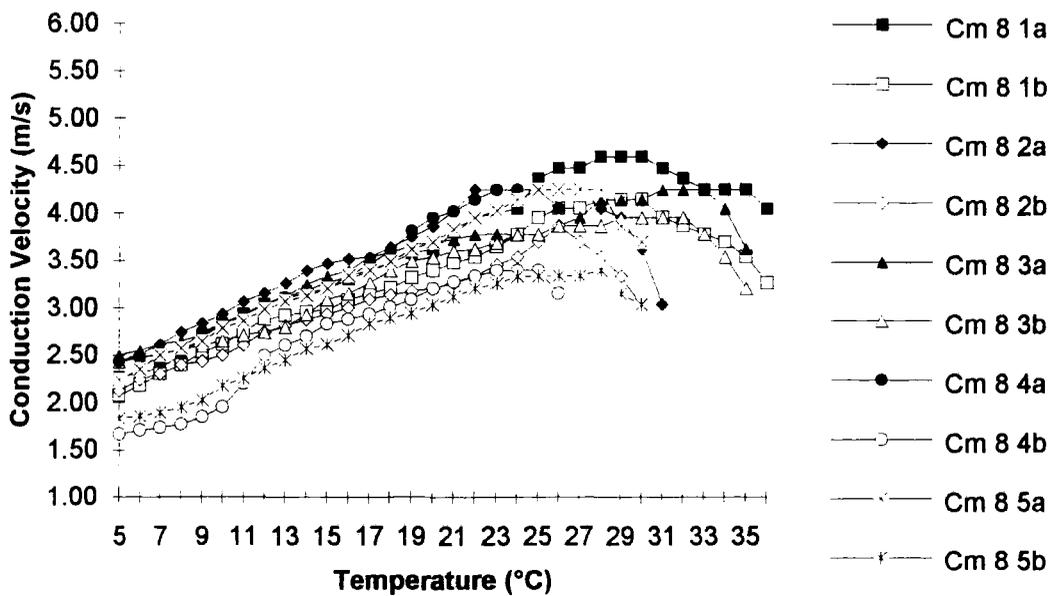
b)
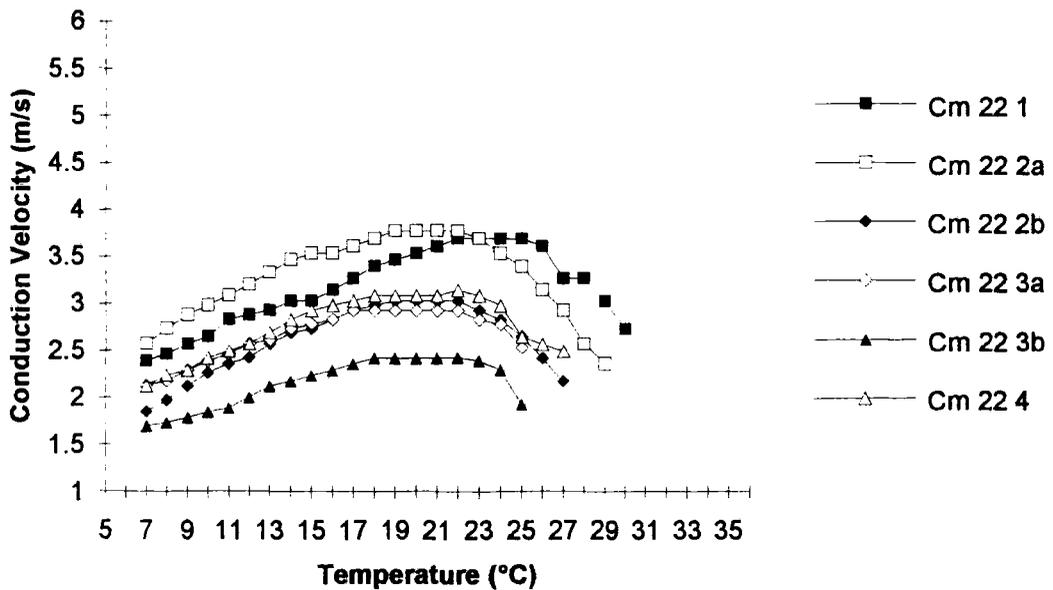
Table 5.1. Temperatures of **axonal conduction blockage** and temperature ranges of **maximum conduction velocity** in preparations of **8°C-acclimated** and **22°C-acclimated** *Cancer pagurus* and *Carcinus maenas* (means±S.D.; the one-way analysis of variance, combined with the least significant difference range test, compared the conduction blockage temperatures between all the four groups; significantly different groups are marked with different letters; the group means increase in the order **a** < **b**)

Species	Acclimation Temperature	Temperature of Conduction Blockage (°C)	n	t-test	Max Cond. Vel. Temperature Range
Cancer pagurus	8°C	29.0±2.8	6	a	-
	22°C	25.5±0.7	6	a	19 - 24°C
Carcinus maenas	8°C	33.0±4.2	10	b	24.5 - 30°C (±0.7) (±2.8)
	22°C	28.5±2.1	6	a	19.5 - 24.5°C (±2.1) (±2.1)

range of conduction blockage temperatures) in cold-acclimated *Cancer pagurus*, and 17-26°C in warm-, compared to 22-33°C in cold-acclimated *Carcinus maenas*.

A comparison between species showed that the axonal conduction in both cold- and warm-acclimated *Cancer pagurus* was more sensitive to warm temperatures than in cold-acclimated *Carcinus maenas*. The warm-acclimated *Carcinus* were also more thermally sensitive than the cold-acclimated *Carcinus maenas*, but no significant difference was found between them and the warm-acclimated *Cancer pagurus* (Table 5.1).

The plots of the average axonal conduction velocity against temperature are shown in Figure 5.7.a. These illustrate clearer the above mentioned differences in the shape of the temperature dependence curves between preparations from cold- and warm-acclimated crabs. Also, the averaged absolute values of conduction velocity were slightly higher in axons of *Cancer pagurus* than of *Carcinus maenas* at all temperatures above 8°C.

Figure 5.7.b shows only the average conduction velocities at 8°C and at 22°C for the cold and warm-acclimated crabs of the two species. For *Cancer pagurus* the axonal conduction velocity measured at 22°C was slightly higher in warm-acclimated crabs than in cold-acclimated crabs (not significant, $p = 0.2$), suggesting no compensation or an inverse acclimation, whereas in *Carcinus maenas* an acclimatory compensation was observed: the axonal conduction velocity measured at 22°C was significantly lower in warm-acclimated crabs than in cold-acclimated crabs ($p < 0.03$). By analogy with the homeoviscous responses calculated from the fluorescence polarization vs. temperature curves (Wodtke and Cossins, 1991, see also section 3.2.5), the efficacy of the acclimation response was calculated as

$$\%AR = [CV_{8AT}(22°C)-CV_{22AT}(22°C)]/[CV_{8AT}(22°C)-CV_{8AT}(8°C)] \times 100$$

where the low index shows the acclimation temperature (AT) and the value in °C given in parentheses shows the experimental temperature corresponding to the value

Figure 5.7. (a) The temperature dependence of the average (avg±S.D.) axonal conduction velocity in **8°C-acclimated** and **22°C-acclimated** *Cancer pagurus* (Cp) and *Carcinus maenas* (Cm)
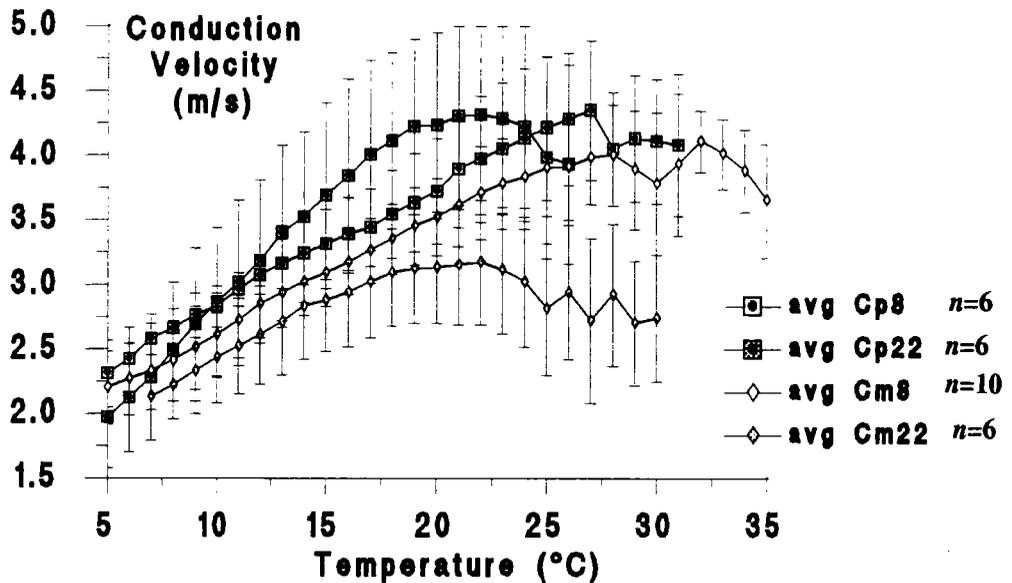
(b) The average conduction velocities (CV) at 8°C and at 22°C in 8°C- and 22°C-acclimated *Cancer pagurus* (Cp) and *Carcinus maenas* (Cm): the values used to calculate the efficacy of the acclimation response (%AR) as:

$$\%AR = [CV_{8AT}(22°C)-CV_{22AT}(22°C)]/[CV_{22AT}(22°C)-CV_{22AT}(8°C)] \times 100$$

(the low index shows the acclimation temperature (AT) and the value in °C given in parentheses shows the experimental temperature)
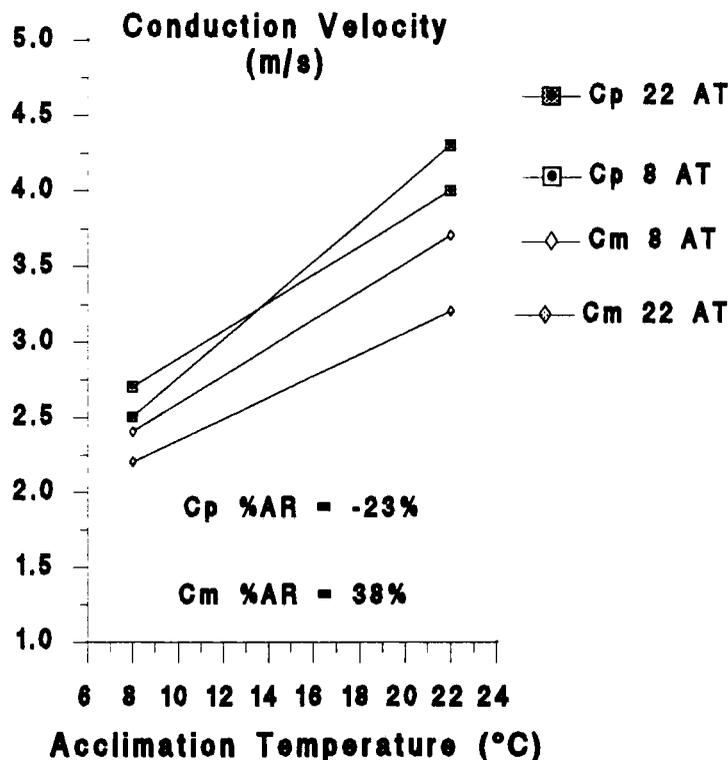
of the conduction velocity used in calculation. The values obtained were -23% for *Cancer pagurus* and 38% for *Carcinus maenas* (Figure 5.7.b).

The rates of increase in axonal conduction velocity with temperature were calculated for the linear portions of the average curves shown in Figure 5.7.a (Table 5.2) showing that there were little differences in the temperature dependence of conduction between acclimation groups and between the two species. The main difference was that in axons of warm-acclimated *Carcinus maenas* the rate of increase with temperature was slightly lower than in cold-acclimated *Carcinus maenas*, suggesting a certain degree of acclimation, while in axons of warm-acclimated *Cancer pagurus*, the rate of increase was faster than in cold-acclimated crabs, suggesting an inverse acclimation, results which parallel the calculated acclimation efficacies, %AR.

For the purpose of comparison with the variations in axonal conduction velocity with temperature and with acclimation temperature reported by other authors, the sets of averaged data used to plot the curves shown in Figure 5.7.a were normalized to the values at the acclimation temperature and plotted on the same graph with data from two other papers (Harper *et al.*, 1990 and Lagerspetz and Talo, 1967).

Figure 5.8 shows a comparison of our results, expressed as $1/t_{peak}$ and normalized, with the lines fitted by Harper *et al.* (1990) to their normalized data from measurements on non-myelinated vagus nerve fibres from cold (8°C) and warm (28°C) acclimated carp, *Cyprinus carpio*, in a semilog plot.

Figure 5.9. shows a comparison of our results, in m/s and normalized, with those of Lagerspetz and Talo (1967) on median and lateral giant fibres from cold (13°C) and warm (23°C) acclimated earthworm, *Lumbricus terrestris*.

Both figures (5.8 and 5.9) show that in cold acclimated preparations the rates of increase in conduction velocity with temperature are faster in carp and in earthworm axons than in crab axons, but in warm-acclimated preparations, at temperatures lower than the discontinuity points, there are little differences in the rates of increase in conduction velocity with temperature between crabs and carp or earthworm. Neither the carp nor the earthworm preparations displayed the decrease in

Table 5.2. Rates of increase in axonal conduction velocity with temperature in preparations of **8°C-acclimated** and **22°C-acclimated** *Cancer pagurus* and *Carcinus maenas*, calculated as the slope of the linear portions of the average conduction velocity vs. temperature curves (the average conduction velocity vs. temperature curves are shown in Figure 5.7a, the n values represent the number of preparations averaged before the regression analysis)

Species	Acclimation Temperature	n	Rate of Increase in Conduction Velocity
Cancer pagurus	8°C	6	0.10 m/s/°C
	22°C	6	0.13 m/s/°C
Carcinus maenas	8°C	10	0.09 m/s/°C
	22°C	6	0.08 m/s/°C

Figure 5.8. Comparison of our averaged axonal conduction velocity measurements on 8°C- and 22°C-acclimated *Cancer pagurus* (**Cp**), and *Carcinus maenas* (**Cm**) leg nerve (**Cp 8 N**, **Cp 22 N**, **Cm 8 N** and **Cm 22 N**) with the graphic estimation of the curves fitted to the measurements of **Harper** *et al.* (1990) on non-myelinated vagus nerve fibres from cold (**Vagus CA N**) and warm (**Vagus WA N**) acclimated carp, *Cyprinus carpio*; the data are expressed as **1/time-to peak** (ms^{-1}) and each curve has been **normalized** (**N**) against the value at the respective acclimation temperature, and plotted on semi-log scale (**CA** and **WA** = cold and warm acclimated, 8°C and 28°C, respectively, from Harper *et al.*, 1990)
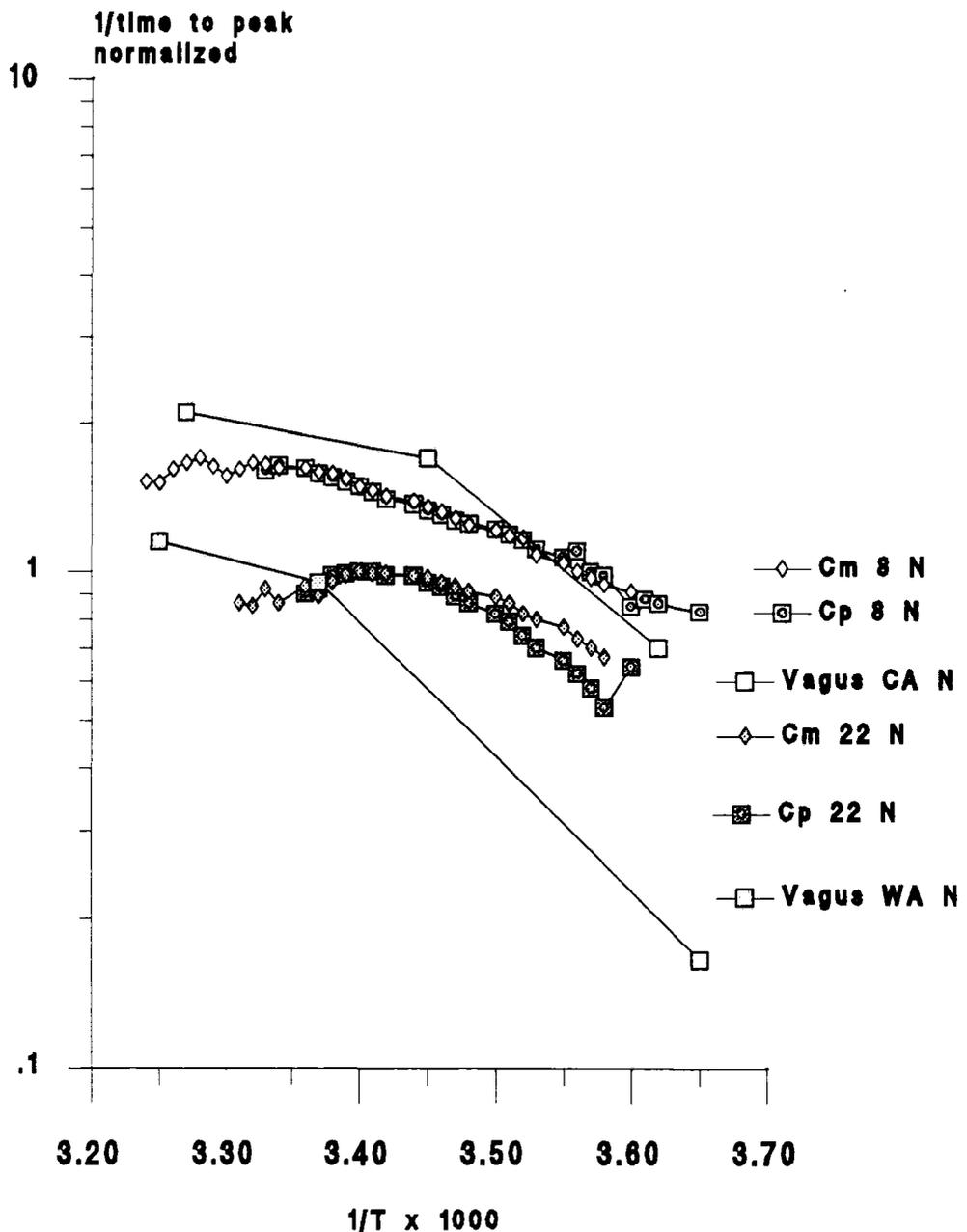
Figure 5.9. Comparison of our averaged axonal conduction velocity measurements on 8°C- and 22°C-acclimated *Cancer pagurus* (Cp), and *Carcinus maenas* (Cm) leg nerve (**Cp 8 N, Cp 22 N, Cm 8 N** and **Cm 22 N**) with the results of **Lagerspetz and Talo (1967)** on median and lateral giant fibres from cold (**CA Med N** and **CA Lat N**) and warm (**WA Med N** and **WA Lat N**) acclimated earthworm *Lumbricus terrestris* L.; the data are expressed in **m/s** and each curve has been **normalized** (**N**) against the value at the respective acclimation temperature (**CA** and **WA** = cold and warm acclimated, 13°C and 23°C, respectively, from Lagerspetz and Talo, 1967)
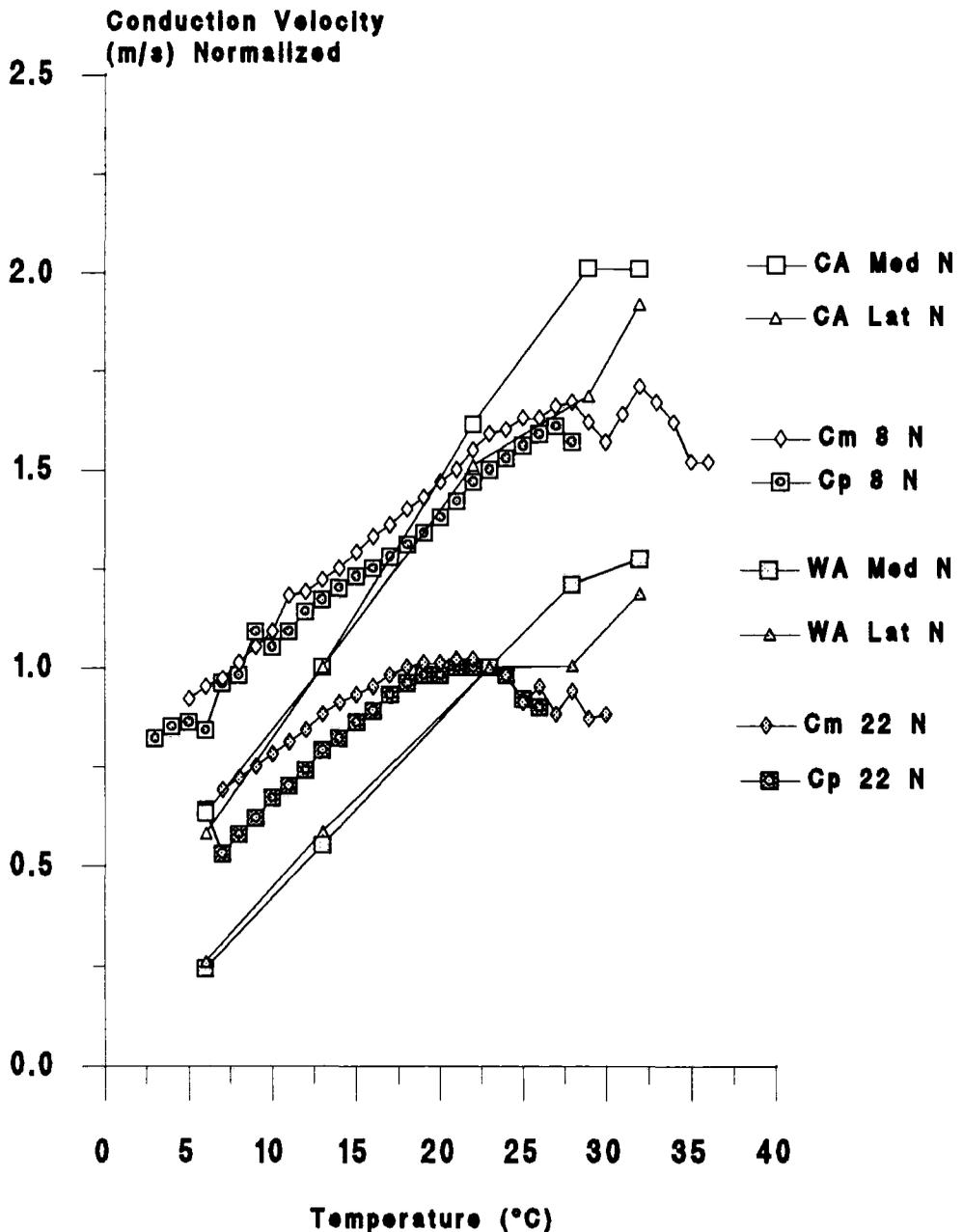
conduction velocity at warmer temperatures observed in crab preparations, although discontinuity points were observed in the temperature dependence of conduction velocity in carp nerve. These occurred at warmer temperatures in fibres from warm-acclimated carp and no inverse acclimation was observed.

5.3.2. Time-course of Temperature Acclimation-Induced Changes in Muscle Fibre Resting Membrane Potential

Warming the neuromuscular preparations from 4-6°C to 24-25°C hyperpolarized the muscle fibre membrane in all cases. Figures 5.10 and 5.11 show only the plots for the fully acclimated *Cancer pagurus* and *Carcinus maenas*, respectively. Figures 5.12-5.14 show the plots of all the measurements taken during the time-course experiment, for *Cancer pagurus* (Figure 5.12) and *Carcinus maenas* (Figures 5.13 and 5.14). Table 5.3 summarizes the values of the slopes of the linear regressions of membrane potential against temperature and the squared correlation coefficients for all the curves plotted in Figures 5.10-5.14.

Referring first to fully acclimated crabs, Figure 5.10 shows that in *Cancer pagurus* the muscle fibre membranes of 22°C-acclimated crabs were depolarized compared to those of 8°C-acclimated crabs and the membrane potential compensation was nearly perfect. The average values at the respective acclimation temperatures were -84±3.9 mV (n=3) in 8°C-acclimated *Cancer pagurus* and -81.3±1.9 mV (n=3) in 22°C-acclimated crabs, while the membrane potential measured at 22°C in preparations of cold-acclimated crabs was -91.8±3.4 mV. The membrane potential changed at a faster rate in preparations of warm-acclimated *Cancer pagurus*: -0.89 mV/°C, compared to -0.63 mV/°C in preparations of cold-acclimated *Cancer pagurus*.

In *Carcinus maenas* (Figure 5.11) the muscle fibre membranes of cold- and warm-acclimated crabs had similar resting potentials over the whole range of measurement temperatures: -75±6 mV (cold-acclimated, n=3) and -76±4.2 mV (warm-acclimated, n=6) at 8°C, and -88.3±1.8 mV (cold-acclimated) and -87±6.5 mV

Figure 5.10. The temperature dependence of the average **resting membrane potential (Mp)** of closer muscle fibres from **8°C-acclimated** (*n*=3) and **22°C-acclimated** (*n*=3) *Cancer pagurus* (the first number after Mp in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature; the standard deviations are not shown)

Figure 5.11. The temperature dependence of the average **resting membrane potential (Mp)** of closer muscle fibres from **8°C-acclimated** (*n*=3), **22°C-acclimated** (*n*=6) and **8°C-reacclimated** from 22°C *Carcinus maenas* (the first number after **Mp** in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature, d=down-shift in acclimation temperature; the standard deviations are not shown)
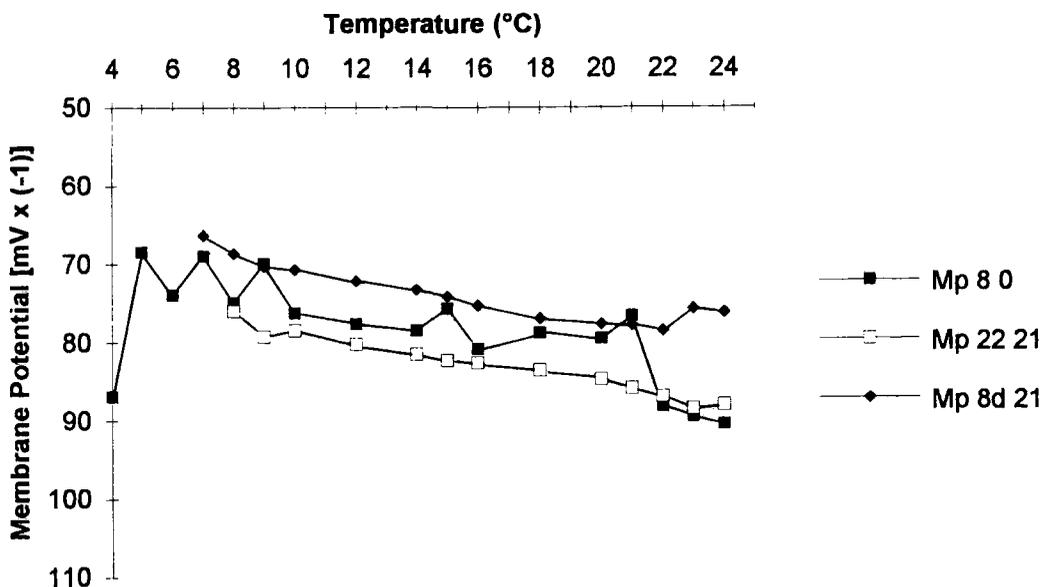
Figure 5.12. The temperature dependence of the average **resting membrane potential (Mp)** of closer muscle fibres from *Cancer pagurus* during acclimation from 8°C to 22°C (the first number after **Mp** in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature; Mp 8 0 *n*=3, Mp 15 7 *n*=4, Mp 22 7 *n*=2, Mp 22 14 *n*=1, Mp 22 21 *n*=3; the standard deviations are not shown)
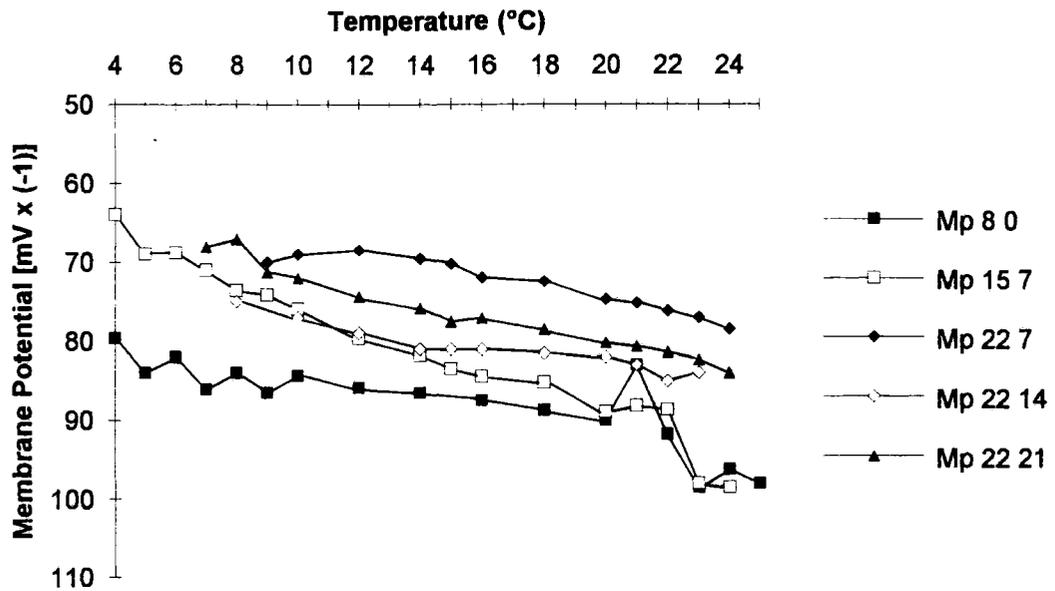
Figure 5.13. The temperature dependence of the average **resting membrane potential (Mp)** of closer muscle fibres from *Carcinus maenas* **during acclimation from 8°C to 22°C** (the first number after **Mp** in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature; Mp 8 0 *n*=3, Mp 15 4 *n*=2, Mp 15 7 *n*=2, Mp 22 7 *n*=3, Mp 22 14 *n*=3, Mp 22 21 *n*=6; the standard deviations are not shown)
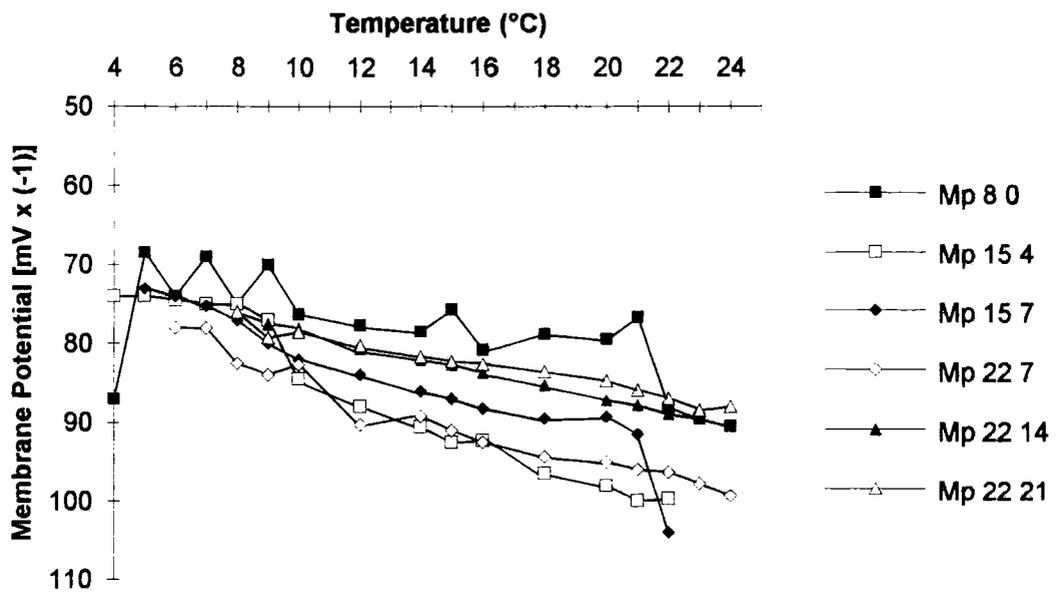
Figure 5.14. The temperature dependence of the average **resting membrane potential (Mp)** of closer muscle fibres from *Carcinus maenas* **during acclimation from 22°C to 8°C** (the first number after **Mp** in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature, d=down-shift in acclimation temperature; Mp 22 21 *n*=6, Mp 15d 4 *n*=2, Mp 15d 7 *n*=2, Mp 8d 7 *n*=1, Mp 8d 14 *n*=2, Mp 8d 21 *n*=3; the standard deviations are not shown)
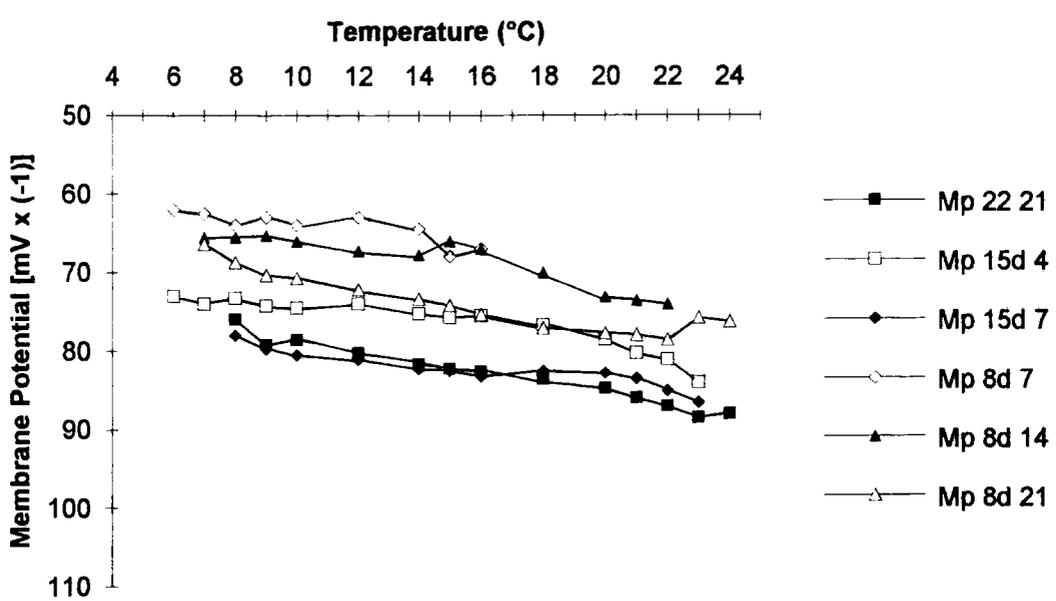
Table 5.3. Summary of the **linear regression analysis** of the average 'resting **membrane potential** vs. **temperature**' curves for acclimated and in-course of acclimation *Cancer pagurus* (*Cp*) and *Carcinus maenas* (*Cm*) (the *n* values represent the number of preparations averaged before the regression analysis; R^2 = squared correlation coefficient)

Time (weeks) & Acclim. Temperature	*Cp* *n*	*Cancer pagurus* slope (R^2)	*Cm* *n*	*Carcinus maenas* slope (R^2)
0 (8°C)	3	-0.63 mV/°C (0.68)	3	-0.66 mV/°C (0.42)
0.5 (15°C)			2	-1.67 mV/°C (0.96)
1 (15°C)	4	-1.46 mV/°C (0.96)	2	-1.37 mV/°C (0.89)
2 (22°C)	2	-0.64 mV/°C (0.90)	3	-1.12 mV/°C (0.95)
3 (22°C)	1	-0.56 mV/°C (0.92)	3	-0.88 mV/°C (0.99)
4 (22°C)	3	-0.89 mV/°C (0.96)	6	-0.69 mV/°C (0.97)
4.5 (15°C)			2	-0.52 mV/°C (0.84)
5 (15°C)			2	-0.41 mV/°C (0.87)
6 (8°C)			1	-0.47 mV/°C (0.71)
7 (8°C)			2	-0.58 mV/°C (0.83)
8 (8°C)			3	-0.59 mV/°C (0.85)

(warm-acclimated) at 22°C. The membranes of crabs re-acclimated to 8°C after warm-acclimation were slightly depolarized compared to those of warm-acclimated crabs: -68.7±6.1 mV at 8°C and -78.5±5.2 mV at 22°C (n=3). The rates of increase of membrane potential with temperature were similar in the muscle fibres of cold- and warm-acclimated *Carcinus maenas*, at -0.66 mV/°C and -0.69 mV/°C, respectively, and slightly lower in preparations of crabs re-acclimated to 8°C, at -0.59 mV/°C (Table 5.3.).

The results of the time-course experiment (Figures 5.12-5.14) showed that the temperature-dependence of the resting membrane potential was altered after transfer to a new temperature and it reached a new steady state after approximately two weeks at the new temperature.

In preparations of *Cancer pagurus* the transfer to warmer temperatures depolarized the muscle fibre membranes (Figure 5.12). After the first week at 15°C the membrane potential measured at 15°C (-83.5±2.5 mV) was close to the value measured at 8°C in the 8°-acclimated crabs (-84±3.9 mV), but the transfer to 22°C depolarized the membranes to much lower potentials (-76.3±4.8 mV at 22°C). After 2 weeks at 22°C the membranes were repolarized (-85 mV at 22°C) to values similar to those measured in cold-acclimated crabs at their acclimation temperature, and then slightly depolarized again after 3 weeks at 22°C (-81.3±1.9 mV), in crabs considered to be fully acclimated (shown also in Figure 5.10). Figure 5.15.a shows the values of the resting membrane potential measured at 8°C and at 22°C at different moments of time during the process of warm-acclimation in *Cancer pagurus*, as described above.

The transfer to warmer temperatures hyperpolarized the *Carcinus maenas* muscle membranes: -92.5±6.4 mV measured at 15°C, after 4 days at 15°C; -87±7.8 mV measured at 15°C, after a week at 15°C; -96.3±6.3 mV measured at 22°C after a week at 22°C, compared to -75±6 mV at 8°C in cold-acclimated crabs (Figure 5.13) and the rates of increase in membrane potential with temperature were faster than in preparations of cold-acclimated crabs (Table 5.3). The membranes appeared to be equilibrated at the warm acclimation temperature only after the second week at 22°C

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Figure 5.15. The **time-course** of the changes in **the resting membrane potential** (**Mp**) [only values at a cold (8°C) and at a warm (22°C) experimental temperature (**ET**) were plotted] of closer muscle fibre **(a) during** acclimation **from 8°C to 22°C** of *Cancer pagurus* and **(b) during** acclimation **from 8°C to 22°C and from 22°C to 8°C** of *Carcinus maenas* (the arrow marks the time of the down-shift in acclimation temperature)

(a) *Cancer pagurus*
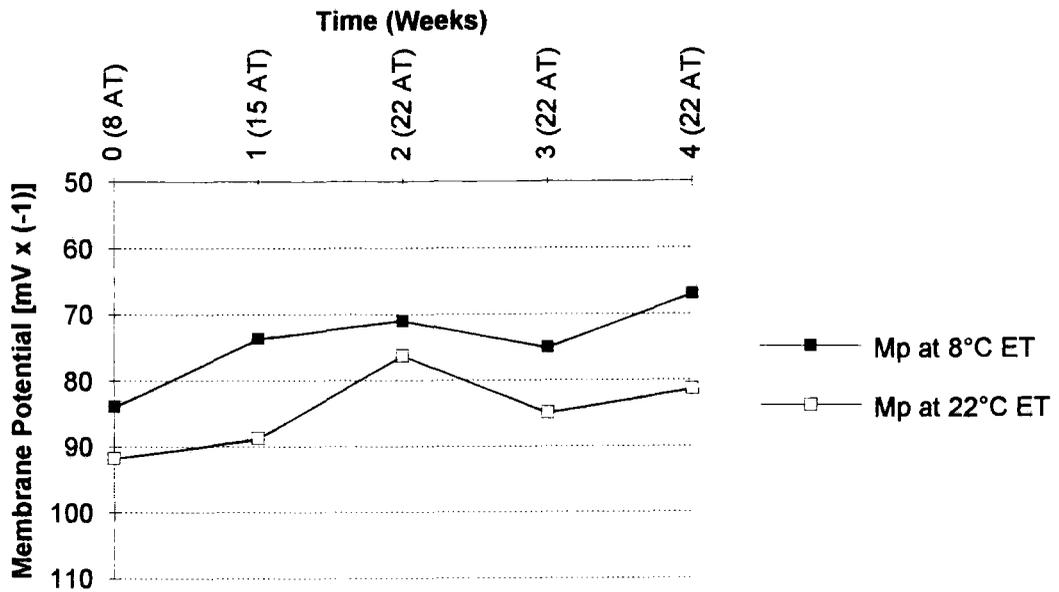
(b) *Carcinus maenas*
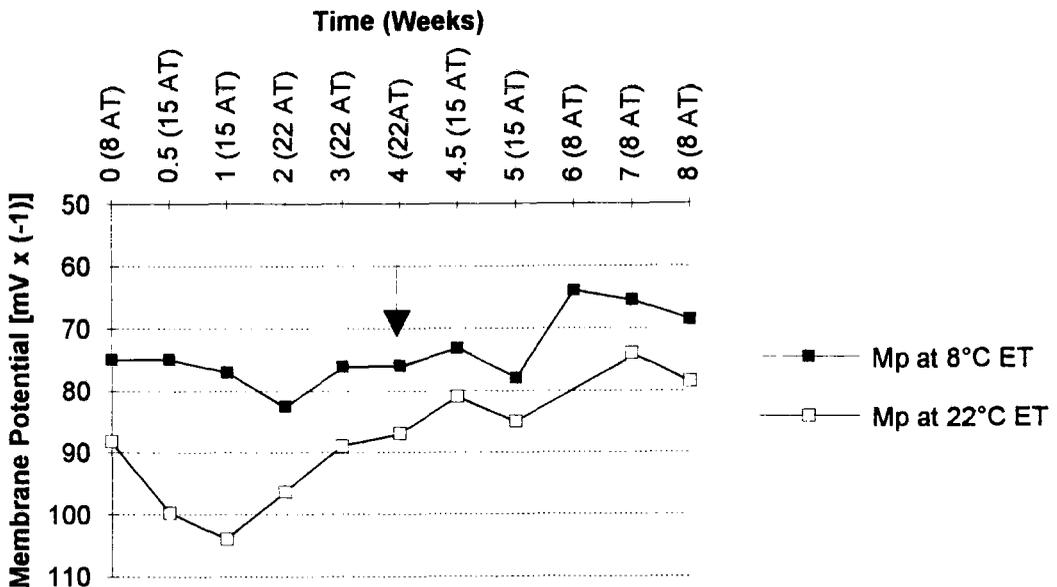
when the values measured at 22°C (-89±3.7 mV after 2 weeks and -87±6.5 mV after 3 weeks at 22°C) were close to those measured at 22°C in membranes of cold-acclimated crabs (-88.3±1.8 mV).

Cold-acclimation of 22°C-acclimated *Carcinus maenas* depolarized the muscle fibre membranes (Figure 5.14). Four days after transfer to 15°C the membranes were depolarized to -75.8±2.5 mV at 15°C and -81±1.4 mV at 22°C, compared to -82.3±5.8 at 15°C and 87±6.5 mV at 22°C in membranes of warm-acclimated crabs, but after 7 days at 15°C, the membrane potential was restored to values close to those measured in preparations of warm-acclimated crabs, i.e. -82.5±7.8 mV at 15°C and -85±7.1 mV at 22°C. Transfer to 8°C depolarized the membranes again (-64-65.5 mV at 8°C; -66-68 mV at 15°C; -74 mV at 22°C after 1 or 2 weeks at 8°C) with a slight recovery after 3 weeks at 8°C (-68.7±3.1 mV at 8°C; -74.2±3.8 mV at 15°C and -78.5±2.6 mV at 22°C). Figure 5.15.b shows the time-course of the changes in the resting membrane potential measured at 8°C and at 22°C during the processes of warm- and cold-acclimation in *Carcinus maenas*.

In all preparations the rate of increase in the resting membrane potential with temperature (Table 5.3.) was faster than that predicted by the Nernst equation (0.2 mV/°C).

5.3.3. Time-course of Temperature Acclimation of Excitatory Junction Potentials

The amplitudes of the excitatory junction potentials (EJP) evoked in the closer muscle fibres by the electrical stimulation of the excitatory axon in the meropodite were read from the individual records, at each experimental temperature. The values (in mV) were normalized as ratios to the value measured at 10°C, averaged and plotted against temperature.

The rather large variability between preparations and the small number of individual preparations measured at each acclimation temperature (and moment of time, for the following time-course description) did not allow a thorough statistical

analysis of the results and an accurate curve-fitting procedure. For the same reason, the standard deviation bars are not shown as these are rather large for some sets of data, up to 40% of the mean value, and they would obstruct the observation of the main trends in the temperature dependence of the parameters measured. The data are presented as point-to-point plots.

The notations used were:

- A_S for the amplitude of the single-pulse EJP;

- A_1 for the amplitude of the first EJP when a double-pulse stimulation was used; and

- A_2 for the amplitude of the second EJP (facilitated) in double-pulse stimulation, all measured as previously described.

Figures 5.16-5.18 show the temperature dependence of A_S, A_1 and A_2, respectively, in neuromuscular preparations of cold- and warm-acclimated *Cancer pagurus* (fully acclimated). Figure 5.19 shows the plots of facilitation vs temperature for cold- and warm-acclimated *Cancer pagurus*, to be followed at the same time with the variations in the amplitude of the facilitated EJP, A_2. Figures 5.20-5.23 show the same parameters for fully cold-, warm- and re-cold-acclimated *Carcinus maenas*.

For both species, in neuromuscular preparations of cold-acclimated crabs (and re-cold-acclimated, for *Carcinus maenas*) the amplitude of the EJPs decreased with the increase in temperature, with the maximum values occurring at 8-12°C (Figures 5.16-5.18 and 5.20-5.22).

Little difference was observed between the amplitudes of the single-pulse EJP (A_S) and the first pulse EJP (A_1) and between their dependence on temperature.

In preparations of warm-acclimated *Cancer pagurus*, A_S and A_1 increased with temperature, at a very low rate, with maximum values occurring at 18-22°C (Figures 5.16 and 5.17), while A_2 was nearly constant over the range of temperature investigated, with slightly higher values at 8-10°C and 22-24°C (Figure 5.18), due to increased facilitation, in particular at low temperatures (Figure 5.19).

Figure 5.16. The temperature dependence of the amplitude of the **single-pulse** (Asg) evoked excitatory junction potential (**EJP**) in closer muscle fibres from **8°C-acclimated** (*n*=3) and **22°C-acclimated** (*n*=3) *Cancer pagurus* (the amplitudes in mV were normalized against the values at 10°C in each curve and averaged; the first number after Asg in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature; the standard deviations are not shown)
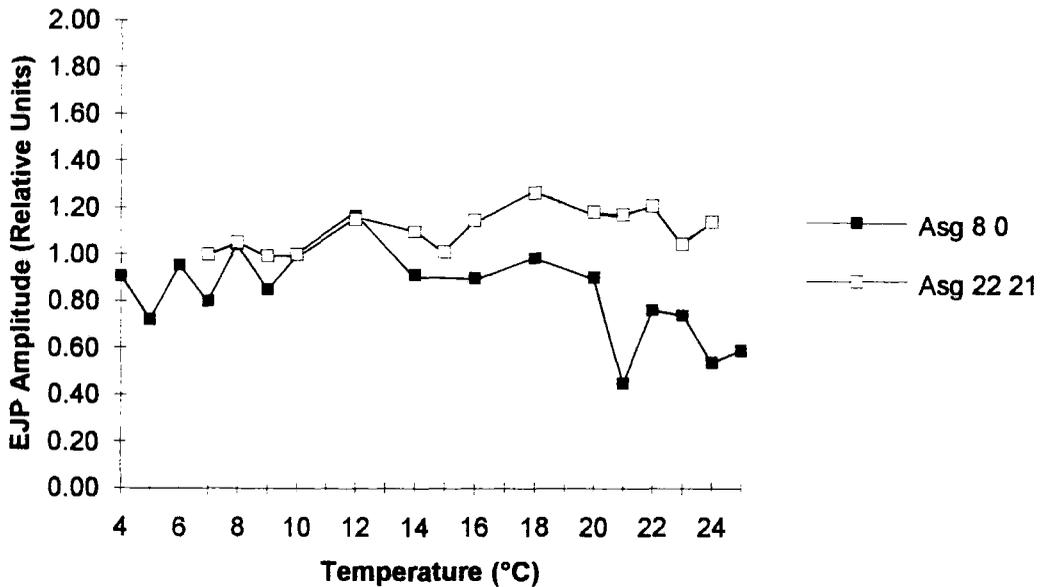
Figure 5.17. The temperature dependence of the amplitude of the excitatory junction potential (**EJP**) evoked by the **first pulse** (A1) in double-pulse stimulation mode in closer muscle fibres from **8°C-acclimated** (*n*=3) and **22°C-acclimated** (*n*=3) *Cancer pagurus* (the amplitudes in mV were normalized against the values at 10°C in each curve and averaged; the first number after A1 in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature; the standard deviations are not shown)
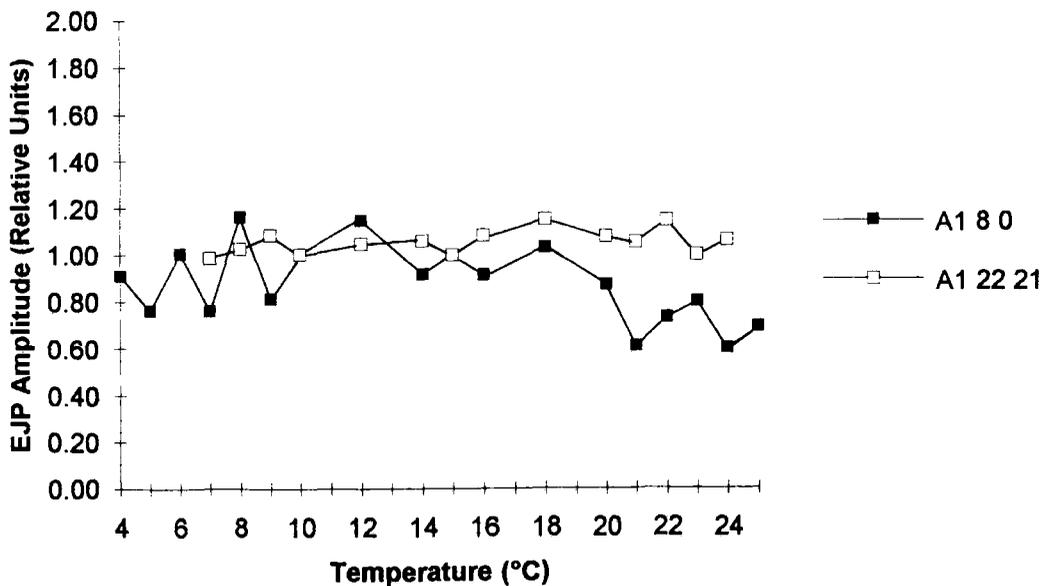
Figure 5.18. The temperature dependence of the amplitude of the excitatory junction potential (EJP) evoked by the **second pulse** (**A2** - **facilitated EJP**) in double-pulse stimulation mode in closer muscle fibres from **8°C-acclimated** (n=3) and **22°C-acclimated** (n=3) *Cancer pagurus* (the amplitudes in mV were normalized against the values at 10°C in each curve and averaged; the first number after A2 in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature; the standard deviations are not shown)

Figure 5.19. The temperature dependence of average **facilitation** (**Fac**) [Fac = (A2-A1)/A1, in double-pulse stimulation mode] in closer muscle fibres of **8°C-acclimated** (n=3) and **22°C-acclimated** (n=3) *Cancer pagurus* (the first number after **Fac** in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature; the standard deviations are not shown)

Figure 5.20. The temperature dependence of the amplitude of the **single-pulse** (Asg) evoked excitatory junction potential (**EJP**) in closer muscle fibres from **8°C-acclimated** (*n*=3), **22°C-acclimated** (*n*=6) and **8°C-reacclimated** from 22°C (*n*=3) *Carcinus maenas* (the amplitudes in mV were normalized against the values at 10°C in each curve and averaged; the first number after **Asg** in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature, d=down-shift in acclimation temperature; the standard deviations are not shown)
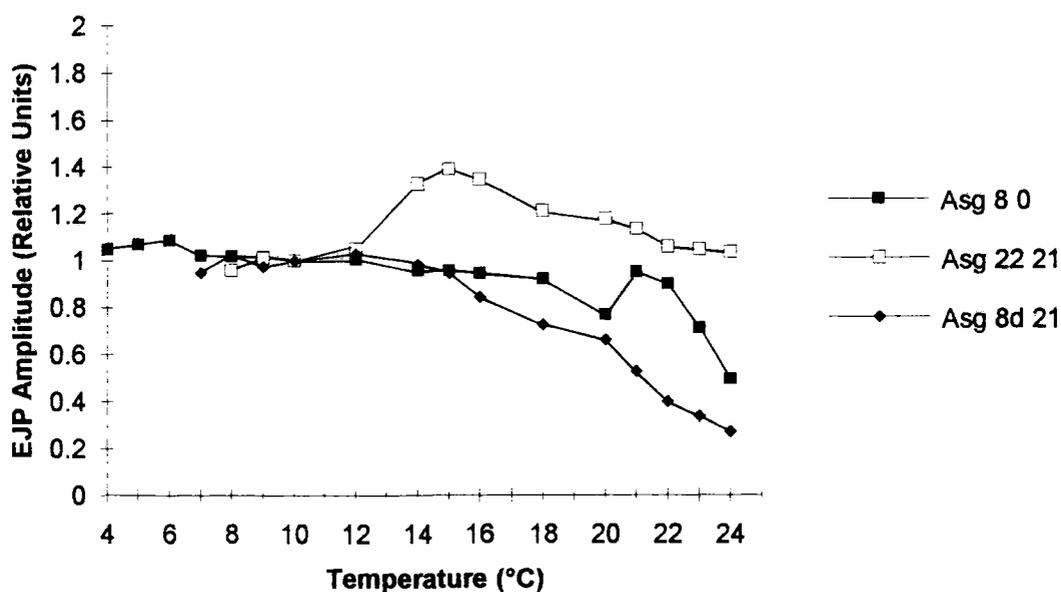
Figure 5.21. The temperature dependence of the amplitude of the excitatory junction potential (EJP) evoked by the **first pulse** (**A1**) in double-pulse stimulation mode in closer muscle fibres from **8°C-acclimated** (*n*=3), **22°C-acclimated** (*n*=6) and **8°C-reacclimated** from 22°C (*n*=3) *Carcinus maenas* (the amplitudes in mV were normalized against the values at 10°C in each curve and averaged; the first number after A1 in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature, d=down-shift in acclimation temperature; the standard deviations are not shown)
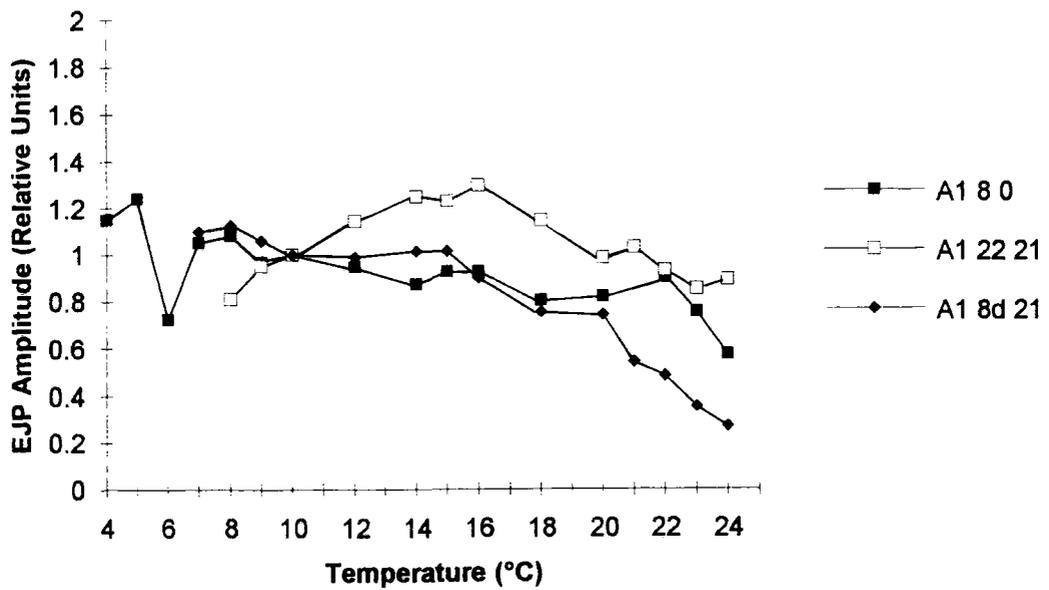
Figure 5.22. The temperature dependence of the amplitude of the excitatory junction potential (EJP) evoked by the **second pulse (A2 - facilitated EJP)** in double-pulse stimulation mode in closer muscle fibres from **8°C-acclimated** (*n*=3), **22°C-acclimated** (*n*=6) and **8°C-reacclimated** from 22°C (*n*=3) *Carcinus maenas* (the amplitudes in mV were normalized against the values at 10°C in each curve and averaged; the first number after A2 in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature, d=down-shift in acclimation temperature; the standard deviations are not shown)
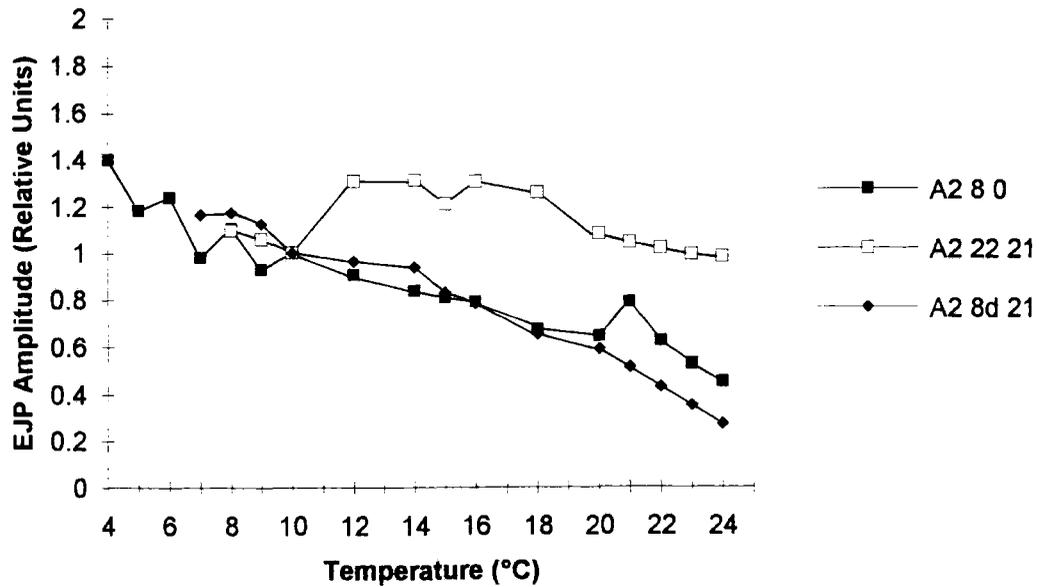
Figure 5.23. The temperature dependence of average **facilitation** (**Fac**) [Fac = (A2-A1)/A1, in double-pulse stimulation mode] in closer muscle fibres of **8°C-acclimated** (*n*=3), **22°C-acclimated** (*n*=6) and **8°C-reacclimated** from 22°C (*n*=3) *Carcinus maenas* (the first number after **Fac** in legend represents the acclimation temperature and the second number the number o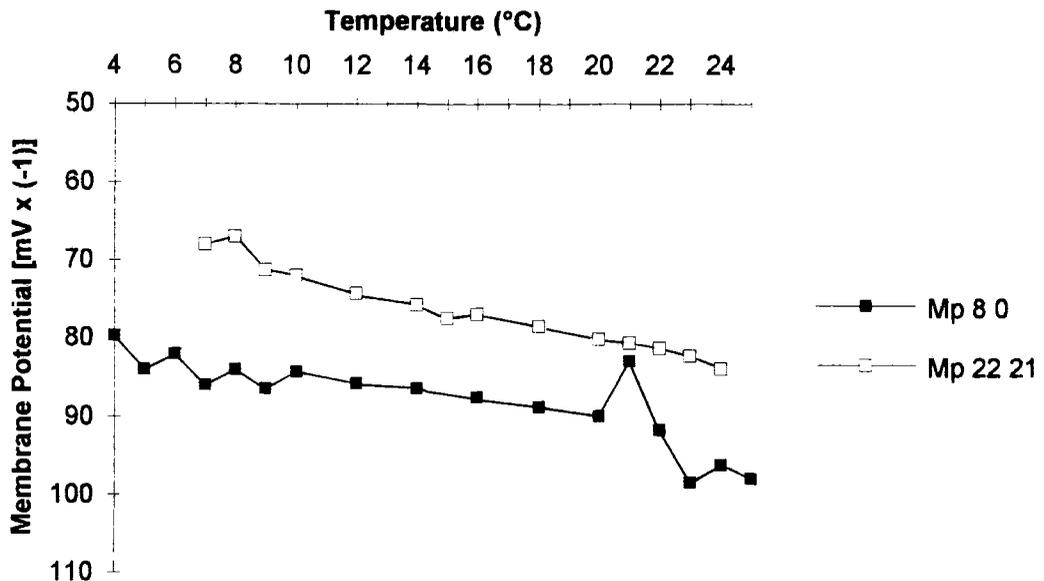f days the crabs were maintained at that temperature, d=down-shift in acclimation temperature; the standard deviations are not shown)
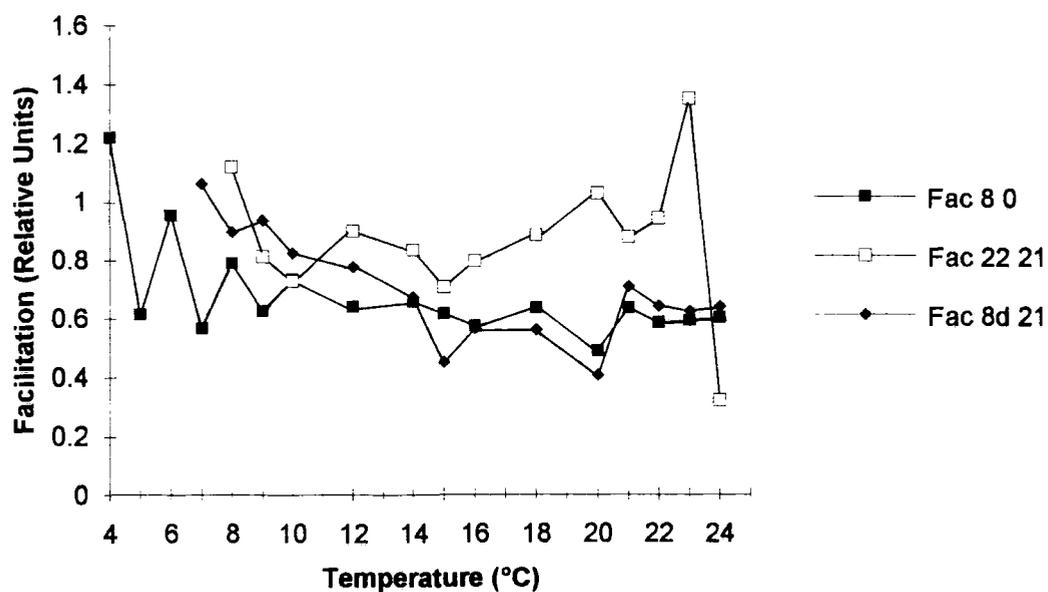
In warm-acclimated *Carcinus maenas*, A_s and A_1 increased with temperature from 8°C up to 14-16°C, where maximum values occurred, then decreased slightly from 16°C to 24°C (Figures 5.20 and 5.21); A_2 followed a similar pattern, but with maximum values occurring over a broader temperature range, from 12°C to 18°C (Figure 5.22), although facilitation was minimum at 14-16°C (Figure 5.23).

The plots of the temperature dependence of A_1 and A_2 at different moments of time during the warm-acclimation process are shown in Figures 5.24 and 5.25 for *Cancer pagurus* and in Figures 5.27.a and 5.28.a for *Carcinus maenas*. The variation in time of the ratio between the EJP amplitude at 22±1°C and at 8±1°C is shown in Figure 5.26 for *Cancer pagurus* and in Figure 5.29 for *Carcinus maenas*. Figures 5.27.b and 5.28.b show the plots of the temperature dependence of A_1 and A_2 at different moments of time during the cold-acclimation process, from 22°C to 8°C, for *Carcinus maenas*. The data for the single-pulse EJP are not shown as their plots were nearly identical to those of the first pulse EJP (A_1). Figures 5.30 and 5.31.a show the temperature dependence of the synaptic facilitation during warm-acclimation of *Cancer pagurus* and *Carcinus maenas*, respectively, and Figure 5.31.b shows the temperature dependence of the synaptic facilitation during re-cold-acclimation of *Carcinus maenas* from 22°C to 8°C.

In *Cancer pagurus*, after a week at 15°C, A_1 was relatively constant over the range 8-14°C, then decreased more rapidly than in preparations of 8°C-acclimated crabs (Figure 5.24). The amplitudes at warm temperatures increased considerably after the first week at 22°C, compared to those at 8-10°C, with maxima occurring at 18-20°C. After the second week at 22°C the amplitudes at 12-20°C were still higher than those at 10°C, and much higher than those at 8°C. At the end of the third week at 22°C the variation in A_1 with temperature was not as large as after the first and second week. A_1 increased with temperature from 8°C to 22-24°C at a low rate. The changes with time in the temperature-dependence of the amplitude of the second-pulse EJP (A_2) followed the same pattern described for A_1, however with less

Figure 5.24. The temperature dependence of the amplitude of the excitatory junction potential (**EJP**) evoked by the **first pulse** (**A1**), in double-pulse stimulation mode in closer muscle fibres from *Cancer pagurus* **during acclimation from 8°C to 22°C** (the amplitudes in mV were normalized against the values at 10°C in each curve and averaged; the first number after A1 in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature; A1 8 0 *n*=3, A1 15 7 *n*=4, A1 22 7 *n*=2, A1 22 14 *n*=1, A1 22 21 *n*=3; the standard deviations are not shown)
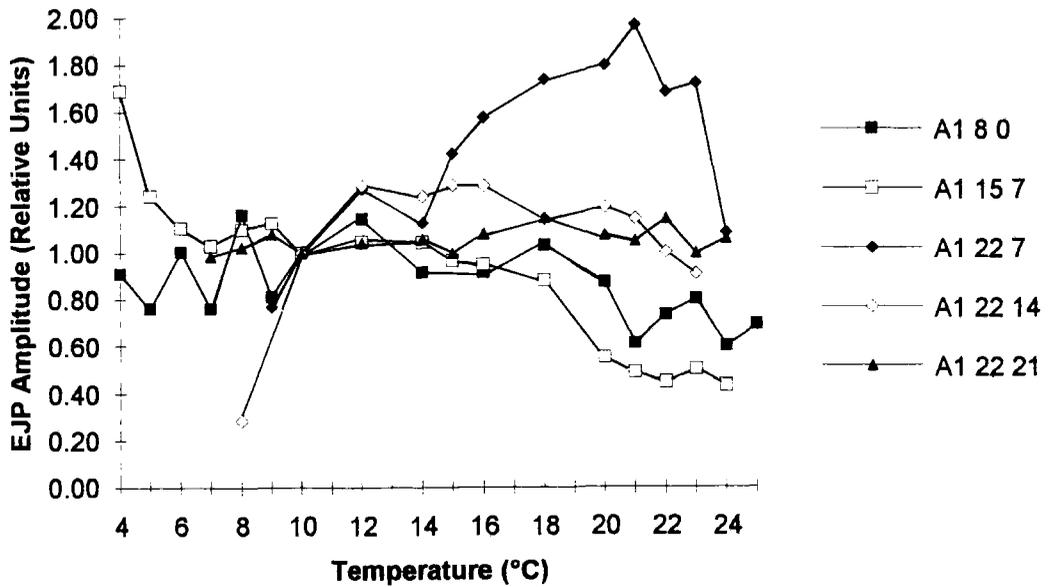
Figure 5.25. The temperature dependence of the amplitude of the excitatory junction potential (EJP) evoked by the **second pulse (A2 - facilitated EJP)** in double-pulse stimulation mode in closer muscle fibres from *Cancer pagurus* **during acclimation from 8°C to 22°C** (the amplitudes in mV were normalized against the values at 10°C in each curve and averaged; the first number after A2 in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature; A2 8 0 $n=3$, A2 15 7 $n=4$, A2 22 7 $n=2$, A2 22 14 $n=1$, A2 22 21 $n=3$; the standard deviations are not shown)
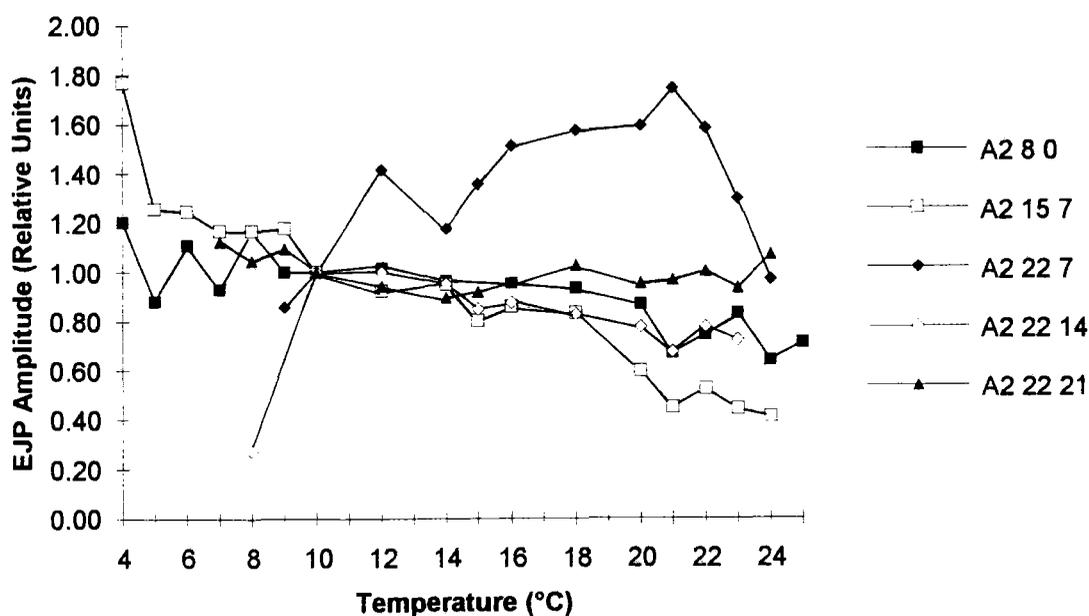
Figure 5.26. The **time-course** of the changes in the **ratio between the amplitudes measured at 22°C and at 8°C** for **EJPs** evoked by the first [A1(22°C)/A1(8°C)] and the second [A2(22°C)/A2(8°C)] pulse in double-pulse stimulation mode in closer muscle fibres of *Cancer pagurus* **during acclimation from 8°C to 22°C**
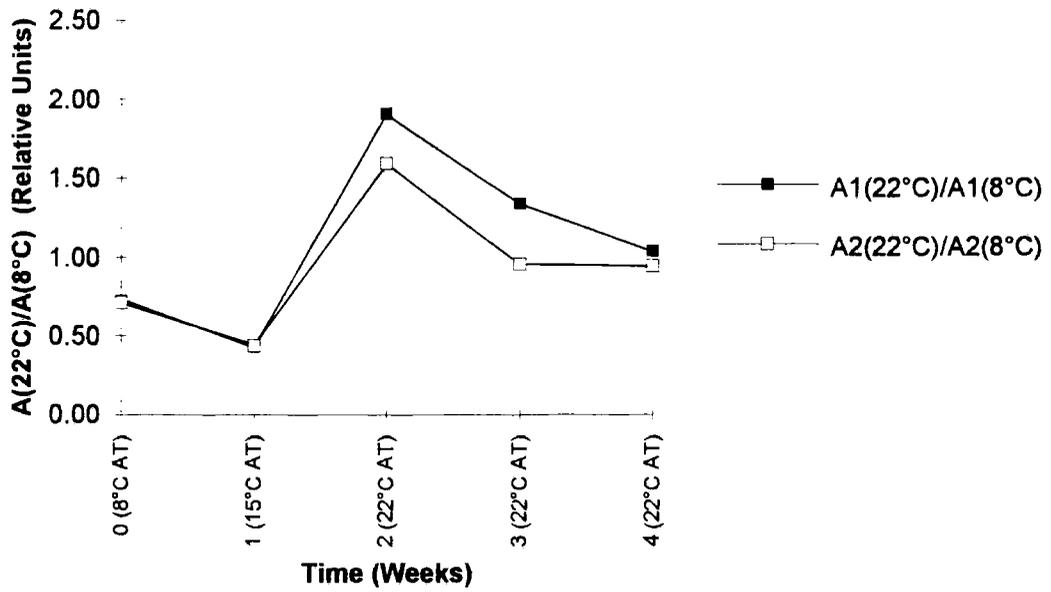
Figure 5.27. The temperature dependence of the amplitude of the excitatory junction potential (**EJP**) evoked by the **first pulse (A1)**, in double-pulse stimulation mode in closer muscle fibres from *Carcinus maenas* **(a) during acclimation from 8°C to 22°C** and **(b) during acclimation from 22°C to 8°C** (the amplitudes in mV were normalized against the values at 10°C in each curve and averaged; the first number after **A1** in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature, d=down-shift in acclimation temperature; A1 8 0 *n*=3, A1 15 4 *n*=2, A1 15 7 *n*=2, A1 22 7 *n*=3, A1 22 14 *n*=3, A1 22 21 *n*=6, A1 15d 4 *n*=2, A1 15d 7 *n*=2, A1 8d 7 *n*=1, A1 8d 14 *n*=2, A1 8d 21 *n*=3; the standard deviations are not shown)

(a)
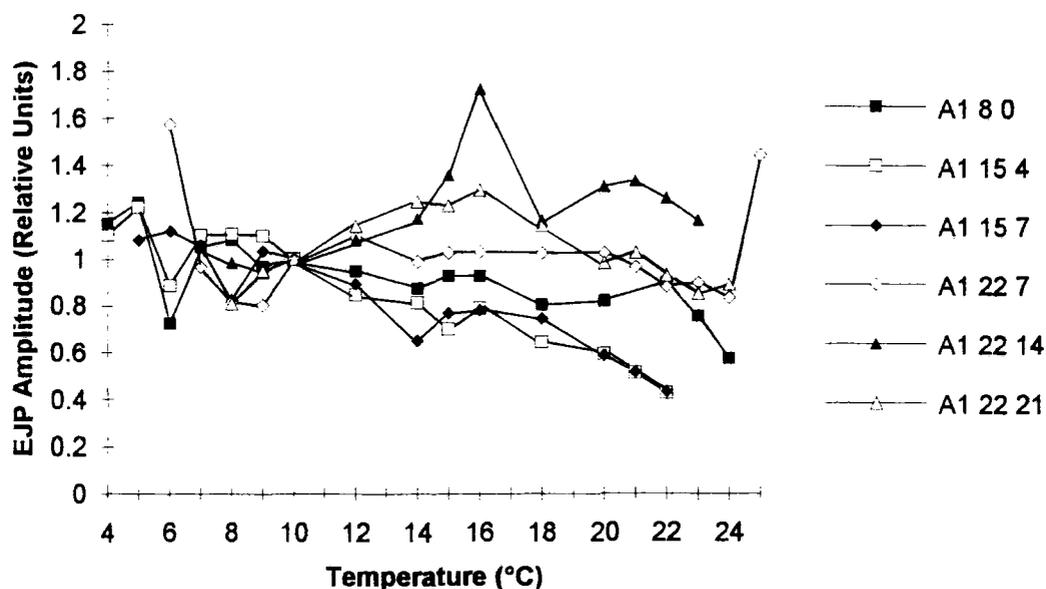
(b)
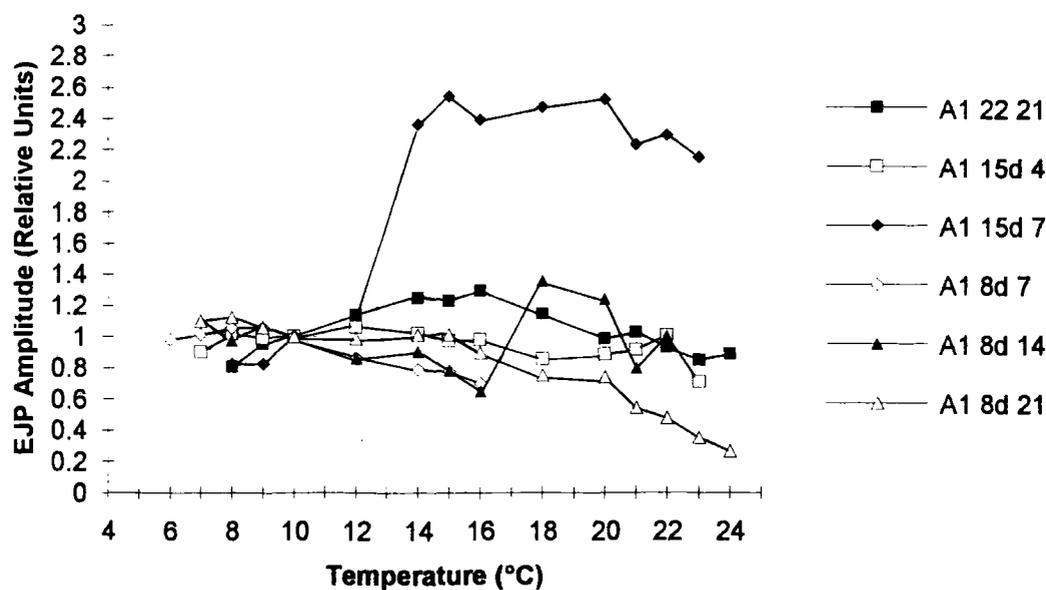
Figure 5.28. The temperature dependence of the amplitude of the excitatory junction potential (EJP) evoked by the **second pulse (A2 - facilitated EJP)** in double-pulse stimulation mode in closer muscle fibres from *Carcinus maenas* **(a) during acclimation from 8°C to 22°C and (b) during acclimation from 22°C to 8°C** (the amplitudes in mV were normalized against the values at 10°C in each curve and averaged; the first number after A2 in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature, d=down-shift in acclimation temperature; A2 8 0 $n=3$, A2 15 4 $n=2$, A2 15 7 $n=2$, A2 22 7 $n=3$, A2 22 14 $n=3$, A2 22 21 $n=6$, A2 15d 4 $n=2$, A2 15d 7 $n=2$, A2 8d 7 $n=1$, A2 8d 14 $n=2$, A2 8d 21 $n=3$; the standard deviations are not shown)

(a)
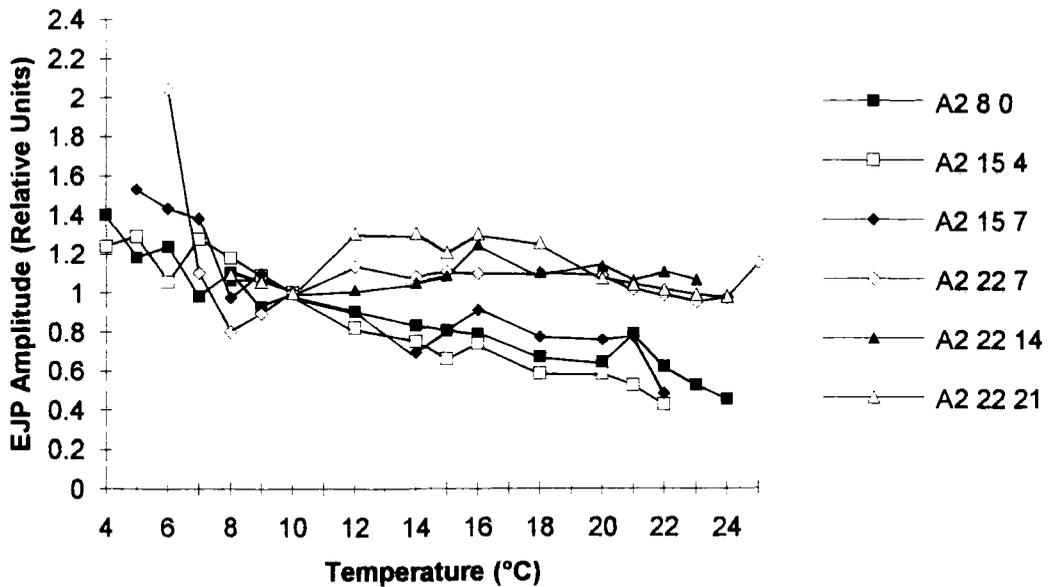
(b)
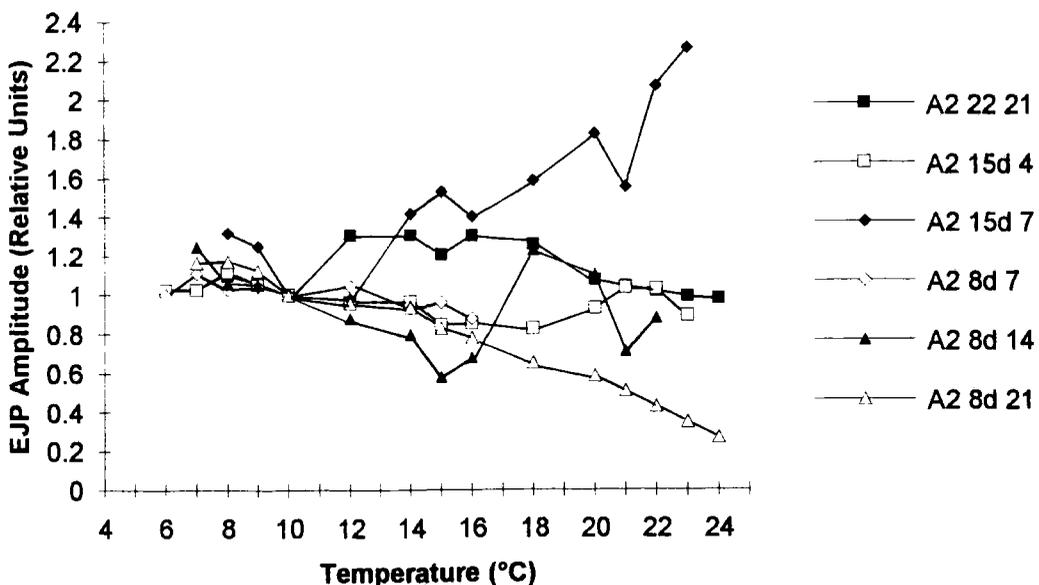
Figure 5.29. The **time-course** of the changes in the **ratio between the amplitudes measured at 22°C and at 8°C** for **EJPs** evoked by the first [A1(22°C)/A1(8°C)] and the second [A2(22°C)/A2(8°C)] pulse in double-pulse stimulation mode in closer muscle fibres of *Carcinus maenas* during **acclimation from 8°C to 22°C and from 22°C to 8°C** (the arrow marks the time of the down-shift in acclimation temperature)
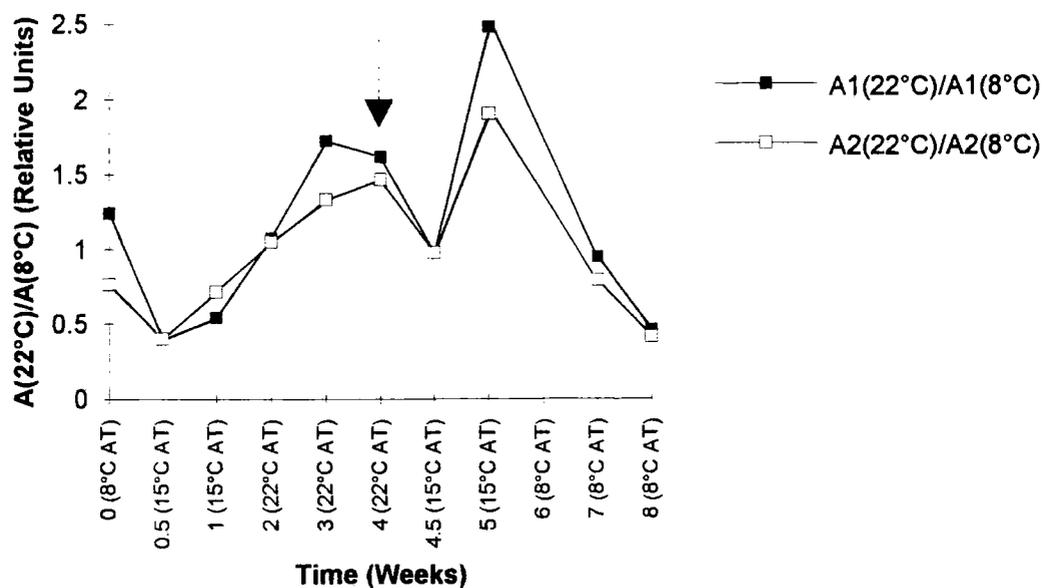
Figure 5.30. The temperature dependence of average **facilitation** (**Fac**) [Fac = (A2-A1)/A1, in double-pulse stimulation mode] in closer muscle fibres of *Cancer pagurus* **during acclimation from 8°C to 22°C** (the first number after **Fac** in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature; Fac 8 0 *n*=3, Fac 15 7 *n*=4, Fac 22 7 *n*=2, Fac 22 14 *n*=1, Fac 22 21 *n*=3; the standard deviations are not shown)
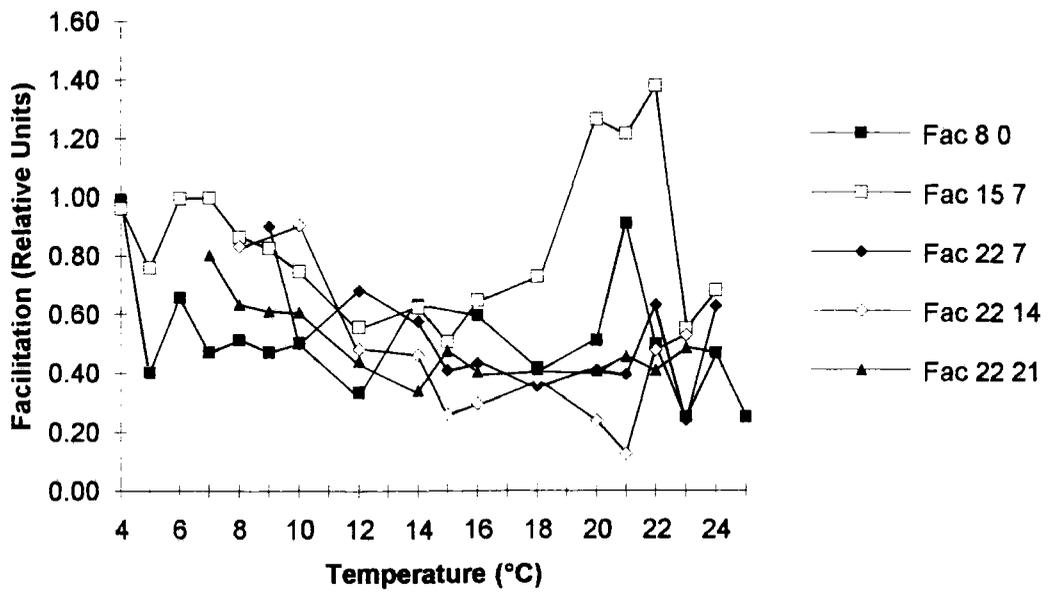
Figure 5.31. The temperature dependence of average **facilitation** (**Fac**) [Fac = (A2-A1)/A1, in double-pulse stimulation mode] in closer muscle fibres of *Carcinus maenas* (a) **during acclimation from 8°C to 22°C** and (b) **during acclimation from 22°C to 8°C** (the first number after **Fac** in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature, d=down-shift in acclimation temperature; Fac 8 0 *n*=3, Fac 15 4 *n*=2, Fac 15 7 *n*=2, Fac 22 7 *n*=3, Fac 22 14 *n*=3, Fac 22 21 *n*=6, Fac 15d 4 *n*=2, Fac 15d 7 *n*=2, Fac 8d 7 *n*=1, Fac 8d 14 *n*=2, Fac 8d 21 *n*=3; the standard deviations are not shown)

(a)

(b)
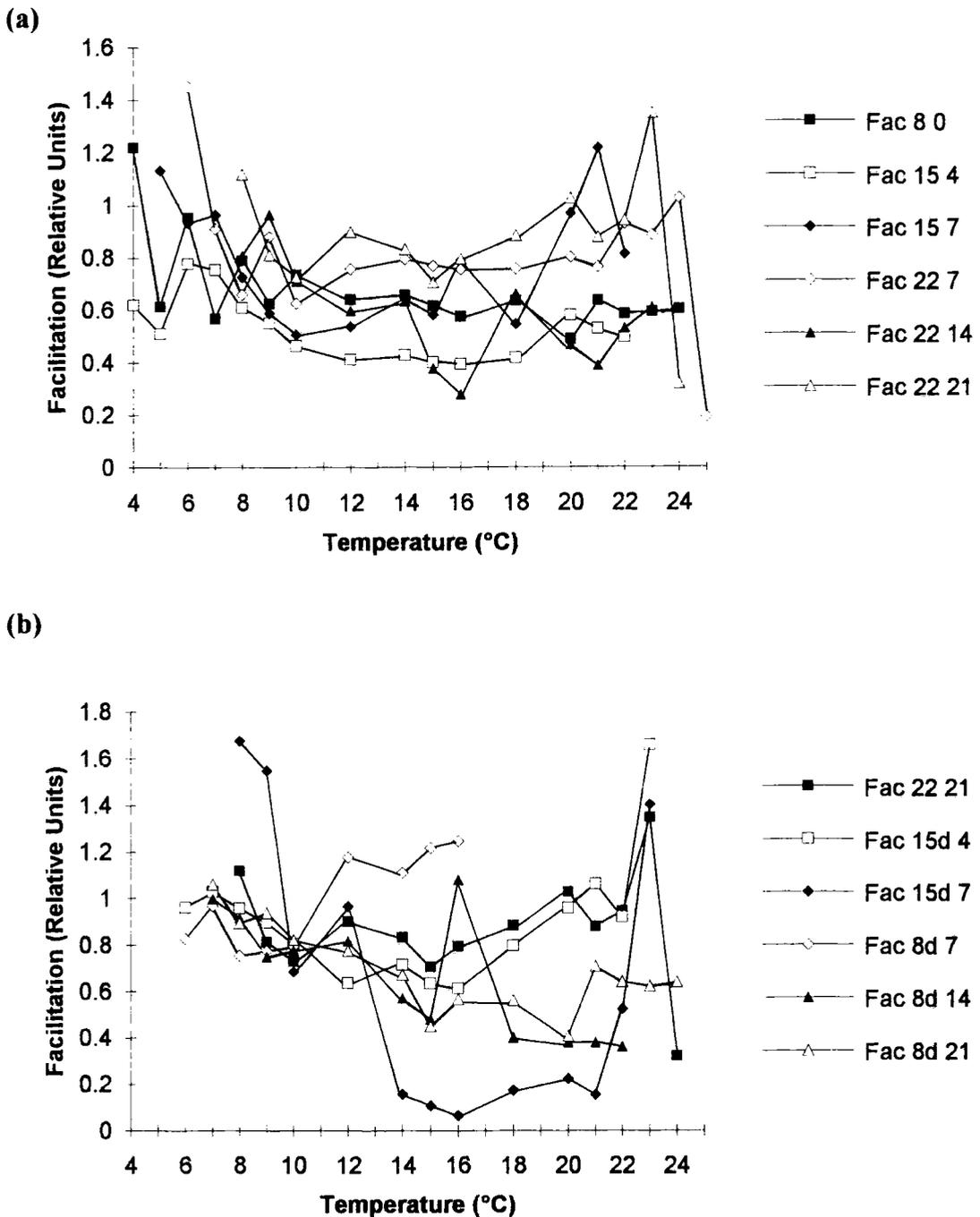
difference between the amplitudes at the cold and warm extremes (Figure 5.25), due to increased facilitation (Figure 5.30), in particular at cold temperatures.

Figure 5.26 shows that in *Cancer pagurus* neuromuscular preparations, the ratio between the EJP amplitude at warm temperatures (21-23°C) and at cold temperatures (8-10°C) increased during warm-acclimation, with a maximum after the first week at 22°C. After the second week at 22°C the A_1 ratio was still above 1 (higher values of A_1 at warm than at cold temperatures), and the A_2 ratio was close to 1, due to increased facilitation at cold temperatures. After 3 weeks at 22°C both A_1 and A_2 ratios were close to 1 (the EJPs had similar amplitudes at 22°C and at 8°C).

In *Carcinus maenas* in course of acclimation from 8°C to 22°C, the decrease in A_1 and A_2 with temperature was still observed after the first week at 15°C. The shape of the curves changed only after the first week at 22°C, when both A_1 and A_2 were fairly constant over the range 10-20°C and decreased slightly at temperatures above 20°C (Figures 5.27.a and 5.28.a). After the second and the third week at 22°C A_1 and A_2 displayed maxima at 15-16°C and the values at 20-22°C were higher than at 8-10°C. The changes in synaptic facilitation are shown in Figure 5.31.a. Facilitation at warm temperatures increased during the week at 15°C and the first week at 22°C, then the facilitation at cold temperatures increased as well. These changes correlated with the variation in time of the ratio between the amplitudes at warm temperatures and at cold temperatures (Figure 5.29), which initially decreased after transfer to 15°C, then gradually increased to values above 1 (larger amplitudes at 22°C than at 8°C temperatures) during the acclimation at 22°C.

During cold-acclimation of the 22°C-acclimated *Carcinus maenas* the amplitude and the facilitation curves followed the reverse changes (Figures 5.27.b, 5.28.b and 5.31.b), gradually returning to the typical shape for cold-acclimated crabs. The ratio between amplitudes at warm-and cold temperatures (Figure 5.29) was gradually reduced to values slightly lower than in crabs that were acclimated directly to cold temperatures.

5.3.4. Changes in Synaptic Facilitation During Temperature Acclimation

Figures 5.19 and 5.23 (referred to in section 5.3.3.) show the temperature dependence of synaptic facilitation in cold- and warm-acclimated *Cancer pagurus* and *Carcinus maenas*, respectively. In both species, the amount of facilitation increased towards the colder end of the temperature range investigated. In preparations of cold-acclimated crabs, this increase in facilitation was observed at temperatures below 7-8°C, and in those of warm-acclimated crabs at temperatures below 10-14°C. Therefore, warm acclimation shifted the ascendent portion of the facilitation vs temperature curves towards higher temperatures.

In preparations of cold-acclimated *Cancer pagurus* facilitation increased again at temperatures around 14-16°C, whereas in cold-acclimated *Carcinus maenas* facilitation remained low and fairly constant at temperatures above the acclimation temperature.

Preparations of warm-acclimated crabs also revealed a difference between the two species. In *Cancer pagurus* facilitation decreased from the low temperature end to temperatures around 12-14°C, then remained constant, while preparations of *Carcinus maenas* displayed minimum facilitation around 15°C, with another increase towards warmer temperatures, up to 20-23°C.

Figures 5.32 and 5.33 show the time course of the changes in facilitation at 8°C, 15°C and 22°C in *Cancer pagurus* and *Carcinus maenas*, respectively. Although in warm-acclimated crabs of both species facilitation at 8°C was slightly higher than at 22°C, in *Carcinus maenas* facilitation at 8°C increased more markedly during warm-acclimation than in *Cancer pagurus*. Also facilitation at 22°C was much higher in warm-acclimated *Carcinus maenas*, than in *Cancer pagurus*.

5.3.5. Latency of EJP

Figures 5.34 and 5.35 show the temperature dependence of the latency of the first pulse EJP for cold- and warm-acclimated *Cancer pagurus* and *Carcinus maenas*,

Figure 5.32. The **time-course** of the changes in **facilitation** (Fac) calculated at 8°C, 15°C and 22°C experimental temperatures (ET) in closer muscle fibres of *Cancer pagurus* during acclimation from 8°C to 22°C
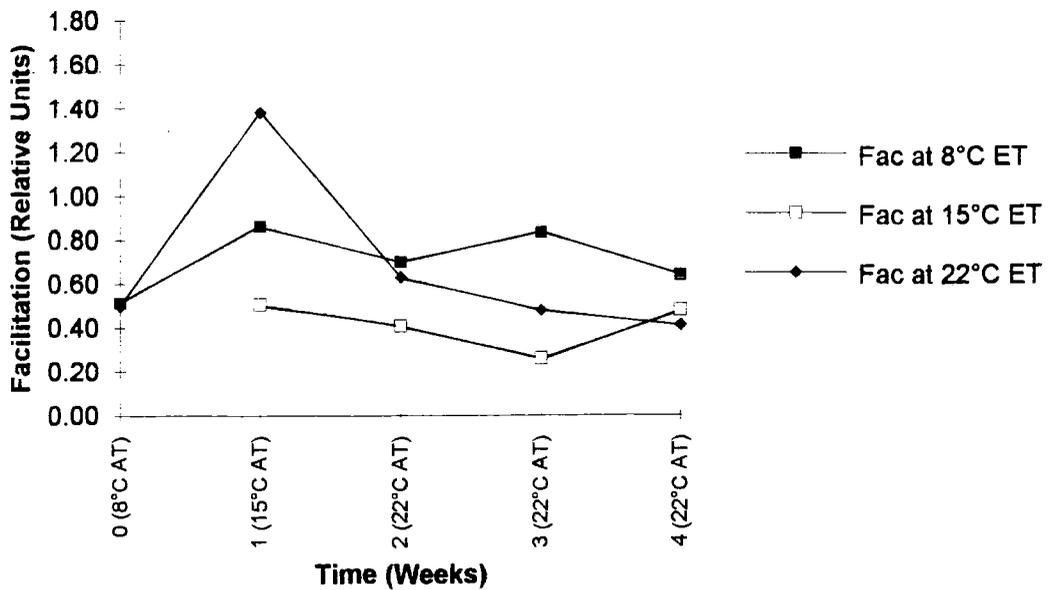
Figure 5.33. The **time-course** of the changes in **facilitation** (Fac) calculated at 8°C, 15°C and 22°C experimental temperatures (ET) in closer muscle fibres of *Carcinus maenas* during acclimation from 8°C to 22°C and from 22°C to 8°C (the arrow marks the time of the down-shift in acclimation temperature)
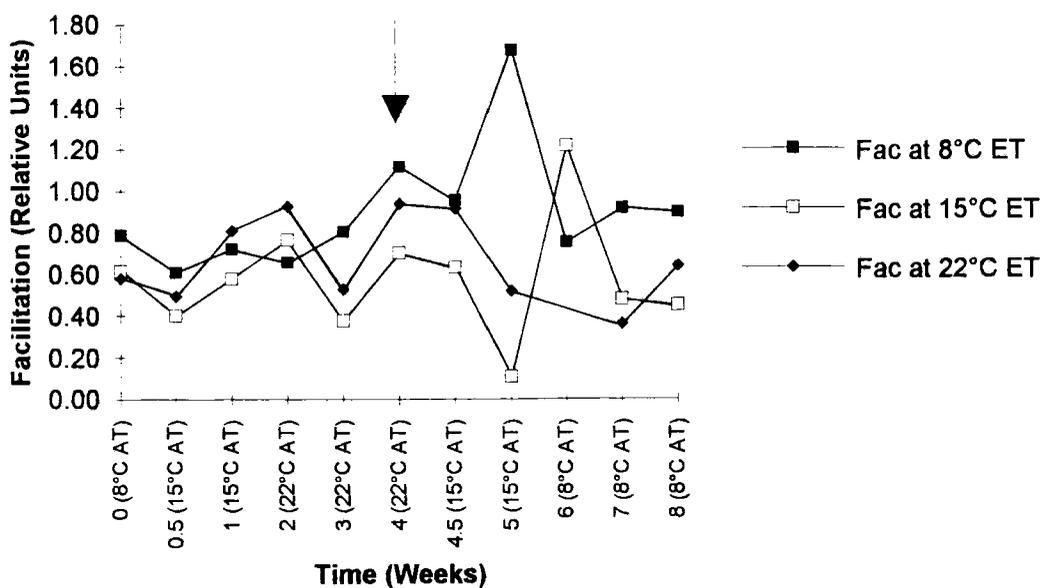
Figure 5.34. The temperature dependence of the average **latency** (**Lat**) of the excitatory junction potential evoked by the first pulse in the double-pulse stimulation mode in closer muscle fibres from **8°C-acclimated** (*n*=3) and **22°C-acclimated** (*n*=3) *Cancer pagurus* (the first number after **Lat** in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature; the standard deviations are not shown)
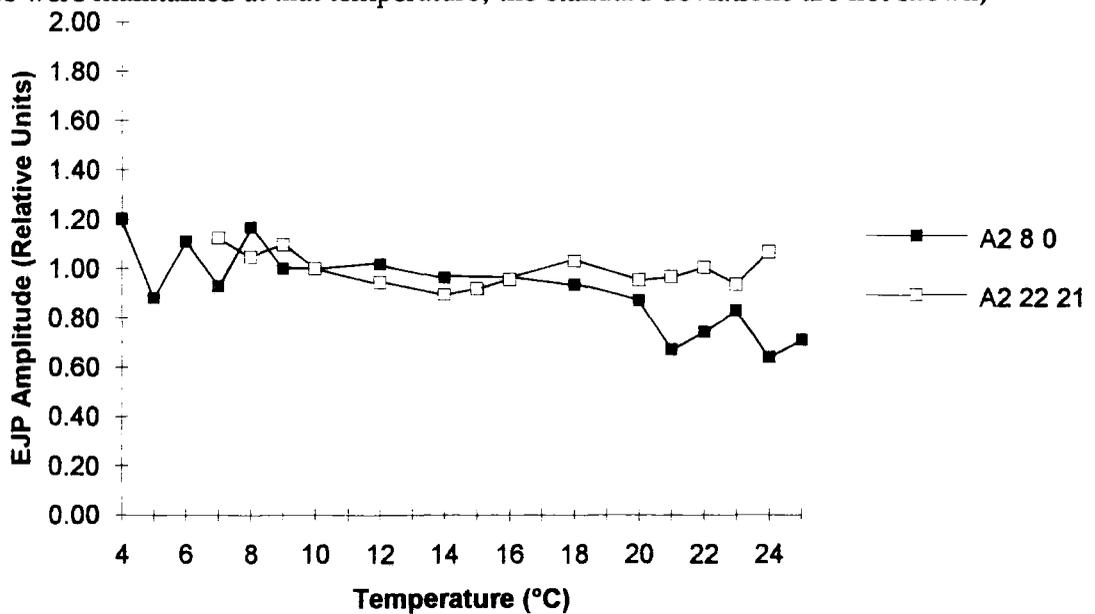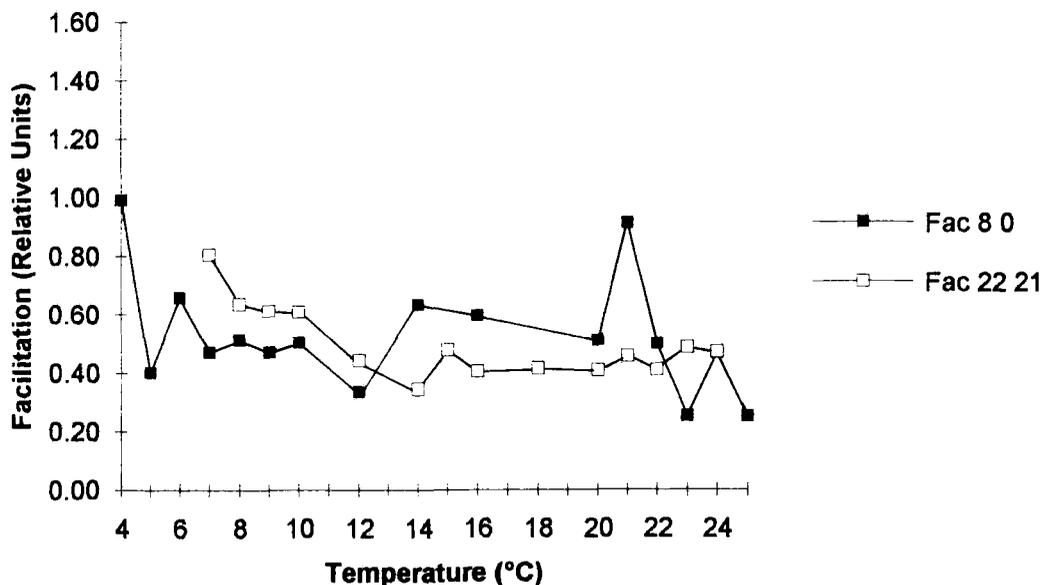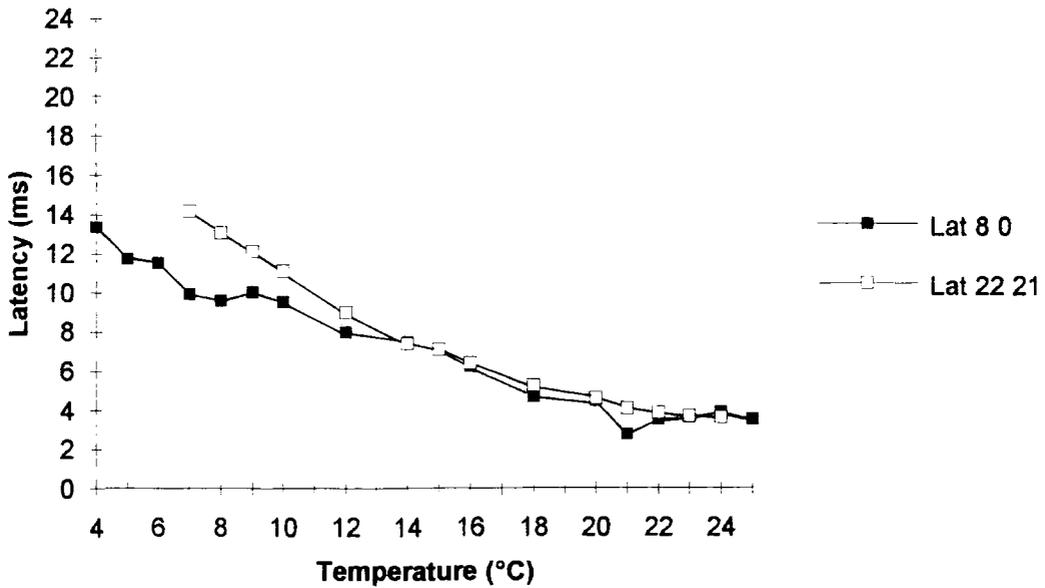

Figure 5.35. The temperature dependence of the average **latency** (**Lat**) of the excitatory junction potential evoked by the first pulse in the double-pulse stimulation mode in closer muscle fibres from **8°C-acclimated** (*n*=3), **22°C-acclimated** (*n*=6) and **8°C-reacclimated** from 22°C (*n*=3) *Carcinus maenas* (the first number after **Lat** in legend represents the acclimation temperature and the second number the number of days the crabs were maintained at that temperature, d=down-shift in acclimation temperature; the standard deviations are not shown)
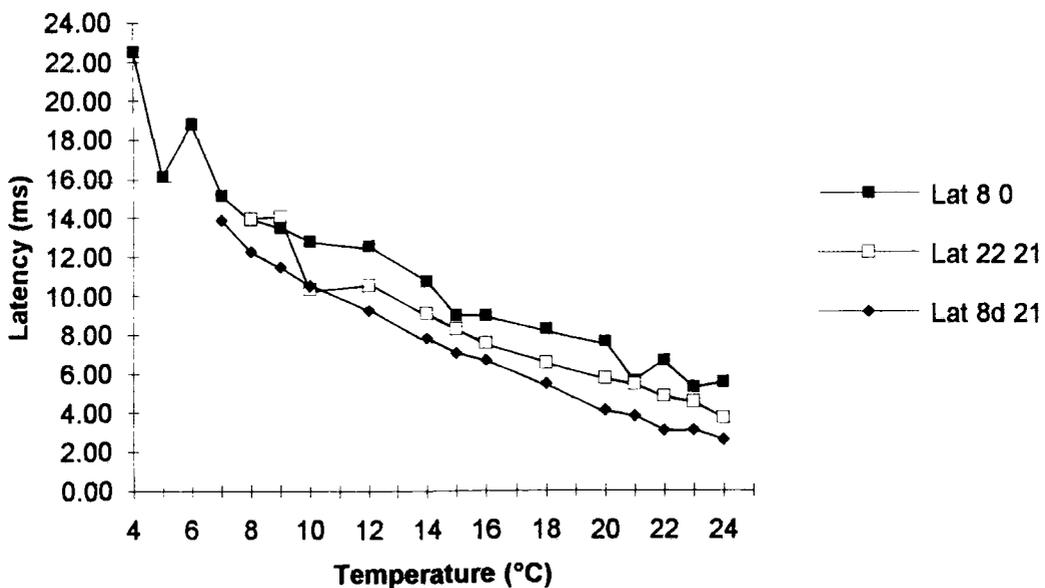
Table 5.4. The equations of the **exponential decay curves** fitted to the average plots of the **temperature dependence of the latency of the excitatory junction potential** evoked by the first pulse, in the double-pulse stimulation mode, in closer muscle fibres of cold- and warm-acclimated *Cancer pagurus* and *Carcinus maenas* (the *n* values represent the number of preparations averaged before the curve fitting)

Species	Acclimation Temperature	*n*	Equation of the Exponential Decay	Correlation Coefficient (R^2)
Cancer pagurus	8°C	3	$y = 17.5 \times 10^{-0.03x}$	0.94
	22°C	3	$y = 25.3 \times 10^{-0.04x}$	0.99
Carcinus maenas	8°C	3	$y = 25.2 \times 10^{-0.03x}$	0.96
	22°C (from 8°C)	6	$y = 25.82 \times 10^{-0.03x}$	0.98
	8°C (from 22°C)	3	$y = 28.31 \times 10^{-0.04x}$	0.99

respectively. The values of the latency of the single pulse EJP were not different from those of the first pulse EJP and are not shown.

The latency vs temperature plots fitted well exponential decay equations. No difference in the rates of decay was observed between cold- and warm-acclimated *Cancer pagurus*. In warm-acclimated *Carcinus maenas* the rate of decrease in latency with temperature was slightly faster than in cold-acclimated crabs. Table 5.4 lists the equations of the exponential decay fitted to the average latency versus temperature plots.

The significance of the absolute values of latency was not clear as these may be influenced by the exact location of the stimulating electrodes and insertion of the microelectrode, which varied from one preparation to another, therefore these were not considered.

5.4. Discussion

5.4.1. Conduction Velocity

The conduction velocity of axons from cold- and warm-acclimated *Cancer pagurus* and *Carcinus maenas* was affected by both measurement temperature and acclimation temperature. The conduction velocity (CV) increased linearly up to a certain temperature, above which it displayed a plateau, then decreased over 1-2°C just before the conduction blockage temperature. Most preparations of cold-acclimated *Cancer pagurus* did not display the plateau portion and the average curve showed a continuous linear increase up to the average conduction blockage temperature. Axons of cold-acclimated *Carcinus maenas* did display the plateau in CV at warmer temperatures and the short decreasing portion, characteristic for axons of warm-acclimated crabs. The flattening of the conduction velocity temperature-dependence curves suggested either the occurrence of a change in the processes involved in axonal conduction at warmer temperatures or, considering that axons

were maintained *in vitro* conditions, the existence of a limiting factor, such as oxygen availability.

Curves of a discontinuous shape were shown for the temperature dependence of conduction velocity in giant nerve of *Lumbricus terrestris* (Lagerspetz and Talo, 1967), carp nerve (Harper *et al.*, 1990), giant axons of squid (Easton and Swenberg, 1975) and lobster (Romey, Chicheportiche and Lazdunski, 1980), and in nerves of other species, reviewed by Macdonald (1994). In most of these cases, the curves displayed a break point at higher temperatures, beyond which the conduction velocity continued to increase, but at a slower rate. Some curves were described by two semilog-linear equations, others were multilinear or curvilinear even in semilog plots. The actual interpretation of these plots is difficult. Although discontinuity points are fairly obvious, often different types of curves, such as both linear and exponential, can be fitted to the same set of data with high correlation coefficients. Curvilinear or intersecting multilinear plots imply that the rate-limiting step changes with temperature (Macdonald, 1994).

The increase in conduction velocity with temperature is due to temperature-accelerated Na^+ channel activation and inactivation (Macdonald, 1994). The break points may reflect a conformational change in the ion channel protein complex, or may be a consequence of a lipid phase transition which could modify the functioning of the channels, or of a more subtle change in the lipid microenvironment of the channel protein (Harper *et al.*, 1990). The lipid phase transition is an unlikely explanation for the break points observed in our CV-temperature curves. No break points were found in the temperature-dependence of the fluidity of our crab muscle membranes (Chapter 3, this thesis) and neither in the neuronal membranes of crayfish (Kivivuori, Laine and Lagerspetz, 1984) or amphipods and isopods (Lahdes, Kivivuori and Lehti-Koivunen, 1993).

The conduction failure at high temperatures could be due to the failure of various components of the action potential, or to the rapid activation of the potassium conductance and inactivation of the sodium conductance (Chapman, 1967).

The brief decrease observed in conduction velocity over 1-2°C before the temperature of conduction blockade is difficult to explain, but it has been mentioned by other authors. Lagerspetz and Talo (1967) mentioned in their results description that at temperatures above 30°C the conduction velocity of some of the fibres measured decreased, and they also quote an early paper of Bullock (1945) who found an increase in the time of latency in the giant fibres just before the cessation of conduction.

Temperature acclimation induced a few significant changes in the temperature dependence of conduction velocity in the two species of crab investigated. Instead of finding a greater thermal tolerance in the axons of warm-acclimated crabs, we found the opposite. The average temperatures of conduction blockage in warm-acclimated crabs were 3-4°C lower than in cold-acclimated ones. In warm-acclimated crabs the CV did not increase continuously with temperature, but displayed a plateau around the acclimation temperature, at values much higher than the conduction velocity in cold-acclimated crabs at their respective acclimation temperature. This change in the temperature-dependence of CV at higher temperatures, in warm-acclimated crabs, could be interpreted as an adaptive phenomenon which would prevent excessively high conduction velocities at warmer temperatures. By analogy with the method of calculation of the homeoviscous response for shifts in membrane fluidity curves (Wodtke and Cossins, 1991) the efficacy of the acclimatory response (%AR) was calculated. In *Cancer pagurus* the difference between the CV at 22°C in axons from cold and warm-acclimated crabs was not significant, but the average CV for warm-acclimated axons was faster than for cold-acclimated axons, leading to a negative %AR of -23%. In *Carcinus maenas* the %AR was 38%, suggesting a partial acclimation of the processes involved in axonal conduction.

The review of Macdonald (1994) on the acclimation of conduction velocity to temperature and pressure mentions a number of studies which have investigated the effects of acclimation to low temperature of various species. Compensatory changes, offsetting the slowing effect of low temperature, have been demonstrated in

earthworm median and giant lateral fibres (Lagerspetz and Talo, 1967), the vagus nerve of the carp (Harper *et al.*, 1990), crayfish giant axons, the optic nerve of goldfish, and frog sciatic nerve (Macdonald, 1994). The data on conduction blockade were summarized by Macdonald (1990) and also showed a corresponding compensatory trend.

The higher thermal sensitivity of axons from warm-acclimated marine crabs, compared to cold-acclimated ones, demonstrated by our experiments, may be due to some degenerative changes that occur in the neuromuscular system of crabs maintained for three weeks at a constant temperature of 22°C. If such results could be expected for the stenothermal species, *Cancer pagurus*, they were rather surprising for the eurythermal species, *Carcinus maenas*. Another possible explanation is the experimental procedure used, in which the nerves from warm-acclimated crabs were first slowly cooled and then re-warmed and the measurements taken, meaning that the total time spent *in vitro* was approximately 30 minutes longer than for the nerves from cold-acclimated crabs (approximately 1.5 hours for warm-acclimated nerves, compared to 1 hour for cold-acclimated nerves), without perfusion or aeration. Easton and Swenberg (1980) observed an appreciable decrease in velocity with time in giant axons of squid, after more than 1-2 hours, even in conditions of continuous superfusion and aeration. If, as assumed above when discussing the shape of the conduction velocity vs. temperature curves, oxygen availability is a limiting factor at warmer temperatures, the effect would be more marked in axons from warm-acclimated crabs which were maintained for longer periods *in vitro*.

Lagerspetz and Talo (1967) found only slightly lower values of conduction velocity in axons of warm-acclimated *Lumbricus* than in axons of cold-acclimated animals, demonstrating only a slight compensatory acclimation, more evident at the low extreme of the range of temperature covered. Their results showed a continuous linear increase in CV with temperature up to 30°C in both cold- and warm-acclimated axons. Harper *et al.* (1990) found break points in their semilog-linear Arrhenius plots of conduction velocity in vagus nerve of cold and warm-acclimated carp, which were

shifted by acclimation temperature towards lower or higher temperatures, depending on the type of fibre measured. In unmyelinated fibres, the break temperatures were higher for warm-acclimated carp, whereas in myelinated fibres the break temperatures were lower for warm-acclimated nerves than for cold-acclimated nerves. The shift in the break point for unmyelinated fibres, from 17.4°C to 23.6°C, following a shift of 20°C in acclimation temperature, indicated approximately 31% compensation, whereas the overall compensation in conduction velocity was around 20% (Harper *et al.*, 1990). The rates of increase in conduction velocity with temperature in crab, earthworm and carp were similar in warm-acclimated axons, and faster in earthworm and carp than in crab in cold-acclimated axons (graphic comparisons, described in 5.3.1.).

Macdonald (1994) also reviewed two examples of an apparent inability to acclimate to low temperature: the tortoise *Testudo greca*, despite of the fact that it experiences a diurnal temperature range of 20°C, did not display any acclimatory changes in conduction velocity after acclimation to 5-7°C; also the Bullhead fish (*Ictalurus nebulosus*) did not develop any compensation during acclimation at 10°C, 20°C or 30°C in either peripheral nerves or the spinal cord. A degree of uncertainty existed about the latter example, as the preparations may have retained the anaesthetic applied to the whole fish.

The axonal conduction was more sensitive to temperature in *Cancer pagurus* than in *Carcinus maenas*, as the temperatures of conduction blockage and those of the break points in the velocity versus temperature curves in axons of *Cancer pagurus* were lower than in *Carcinus maenas*. This was correlated with the relative stenothermality of *Cancer pagurus* and the eurythermality of *Carcinus maenas*.

Examples of evolutionary adaptation of conduction velocity have been reviewed by Macdonald (1994). Temperature coefficients were found to be reduced in the cold-adapted nerves, and the curves of temperature-dependence of conduction velocity showed a partial compensation for conduction velocity being slowed by low temperature. Easton and Swenberg (1975) compared two species of squid living at

different ambient temperatures and related the differences observed in the temperature coefficients of conduction velocity to the difference in habitat temperature. Conduction was sustained at lower temperatures in the presumed cold-acclimatized species (*Loligo pealei*) than in the species living in a warmer habitat (*Loligo vulgaris*), but at higher temperatures the velocity was not so well sustained in the cold-acclimatized species. The slope of the log velocity versus temperature plots was steeper for the warm-acclimatized species *Loligo vulgaris* (Easton and Swenberg, 1975). In our experiment, we have also found a slightly steeper slope of the velocity versus temperature plot for warm-acclimated *Cancer pagurus*, than for the cold-acclimated crabs.

The observed changes in conduction velocity with experimental and acclimation temperature could be due to thermally-induced changes in various control mechanisms, as there are several factors involved in the adaptation or acclimation of conduction velocity to a given temperature (or range of temperatures), which include cable properties, such as cell size, membrane capacitance and cytoplasmic resistance, and molecular properties such as channel gating, and as a result conduction velocity lacks a single rate-limiting step (Macdonald, 1994).

5.4.2. Membrane Resting Potential

The resting membrane potential increased in absolute value (hyperpolarized) with temperature in the closer muscle of both species investigated. No discontinuities were observed in the temperature dependence of the membrane potential, which could be described as linear, with coefficients of correlation greater than 0.9 for most preparations.

These findings were in general agreement with other reports on the effect of temperature and temperature acclimation on the resting potential of crustacean membranes (Stephens, 1990), with the exception of the fact that the temperature dependence of the muscle membrane potential exhibited two linear components in crayfish (*Astacus leptodactylus*) (Harri and Florey, 1977, 1979), and two linear-

logarithmic components in crab (*Pachygrapsus crassipes*) (Stephens and Atwood, 1982). No discontinuity was observed in another species of crayfish (*Procambarus clarkii*) over the 14-39°C range (White, 1983). Similar temperature dependences were reported for the axonal resting membrane potential: two linear components in the medial giant axon of *Astacus astacus* (Kivivuori and Lagerspetz, 1982) and in the fast bender excitor axon of *Pachygrapsus crassipes* (Stephens, 1988), and two linear-logarithmic components in the slow bender excitor axon of *Pachygrapsus crassipes* (Stephens 1985a).

In all cases mentioned above, the component observed at cooler temperatures had a steeper slope and, in some cases, including our results (slopes ranging from 0.6 to 0.9 mV/°C), the change in membrane potential took place faster than predicted by the Nernst equation (0.2 mV/°C for a preparation with a -70 mV resting potential at 20°C, from White, 1983). Harry and Florey (1979) reported slope values of 0.34-2.0 mV/°C at lower temperatures and of 0.1-0.38 mV/°C at higher temperatures, above the discontinuity point, for crayfish muscle membranes. Florey and Hoyle (1976) suggested that the two linear or log-linear components can be explained in terms of the differential effects of temperature on the membrane conductance to potassium and chloride, relative to sodium. The experiments of White (1983) on crayfish neuromuscular preparations indicated that the faster rate of increase in membrane potential with temperature was due, at least in part, to heat-induced metabolic effects on both the Na^+-K^+ pump and on the Na^+/K^+ permeability ratio. The influence of the temperature effects on the Na^+ pump appeared to predominate at warmer temperatures, and the influence of the temperature effects on the ion permeability at lower temperatures (White, 1983). The shift of the discontinuity point in the temperature dependence of the resting membrane potential following temperature acclimation can be related to the temperature acclimation of the Na^+-K^+ pump function (Bowler, Gladwell and Duncan, 1973, Gladwell, Bowler and Duncan, 1975).

Temperature acclimation did not affect the rate of increase in membrane potential with temperature in *Carcinus maenas* and little compensation could be

observed in this species between cold- and warm-acclimated membranes. The curves for cold- and warm-acclimated membranes nearly overlapped, therefore warm-acclimated membranes were hyperpolarized compared to cold-acclimated membranes, when both were measured at their respective acclimation temperature. In *Cancer pagurus* warm-acclimated membranes had higher rates of increase in membrane potential with temperature than cold-acclimated membranes, but warm-acclimation depolarized the membranes, therefore the values of the resting membrane potential at their respective acclimation temperatures were very close, showing a great extent of compensation.

Temperature acclimation usually shifted the curves of membrane potential vs. temperature in a compensatory manner. Cold and warm-acclimated *Austropotamobius pallipes* had similar muscle fibre resting potentials when measured at the respective acclimation temperature (Gladwell, Bowler and Duncan, 1975). The muscle (Stephens and Atwood, 1982) and axonal (Kivivuori and Lagerspetz, 1982) membranes of warm-acclimated crayfish were depolarized in comparison with those of cold-acclimated animals, leading to a lower difference in the resting membrane potentials measured at the respective acclimation temperatures than that induced by the rapid changes in measurement temperature. The discontinuity points were also shifted in an acclimatory manner towards warmer temperatures by warm-acclimation (Stephens, 1990). The effects of warm-acclimation on *Cancer pagurus* membranes were consistent with these observations, but not those of warm-acclimation on *Carcinus maenas* membranes.

The time-course experiment suggested that the changes in membrane potential during warm or cold-acclimation and the re-equilibration of the membranes at the new temperature required approximately two weeks in both species.

One of the differences observed between the two species was that transfer to warmer temperatures depolarized the *Cancer pagurus* membranes and hyperpolarized the *Carcinus maenas* membranes, the other was in the extent of the acclimatory compensation. The hyperpolarization observed in *Carcinus maenas* may be due to the

thermodynamic effect of temperature on the processes involved in the generation and maintenance of the resting membrane potential. Gradual acclimation reduced the extent of the hyperpolarization to values close to those measured in cold-acclimated membranes at 22°C, with no apparent compensation if only the curves for fully acclimated crabs were compared. The equilibration of the membrane potential at higher excitability thresholds in warm-acclimated crabs, compared to cold-acclimated crabs, may be part of the strategy by which *Carcinus maenas*, as a eurythermal species normally exposed to large fluctuations in temperature, maintains balanced neuromuscular function at warmer temperatures. A possible explanation for the initial depolarization observed in *Cancer pagurus* membranes is that, following transfer to warmer temperatures, the mechanisms involved in the maintenance of the resting membrane potential were poorly functioning, and the membranes were depolarized in a similar manner with the events described by Gladwell, Bowler and Duncan (1975) in muscle membranes of crayfish during exposure to lethal high temperatures. Gradual adjustment to the new acclimation temperature repolarized the membranes to values close to those measured for membranes of cold-acclimated *Cancer pagurus* at 8°C, showing near perfect compensation.

A study of Blundon (1989) on a eurythermal (*Callinectes sapidus*, blue crab) and a warm water stenothermal (*Menippe mercenaria*, stone crab) species of marine crab showed that muscle fibres from the warm-acclimated (30°C) eurythermal species were depolarized compared to membranes of cold-acclimated (8°C) crabs, and although the membrane potential did not vary in a continuous manner with experimental temperature, the values measured at the respective acclimation temperatures were not significantly different. Membrane potential increased with temperature in the cold-acclimated stenothermal species (slope -0.76 mV/°C), but no data were shown for warm-acclimated stone crab, only for laboratory cooled crabs, which were considered to be incompletely cold-acclimated (Blundon, 1989).

5.4.3. Excitatory Junction Potentials

The amplitudes of EJPs (single pulse or first and second pulse EJPs in paired-pulse stimulation) in neuromuscular preparations of cold-acclimated crabs were maximum at low temperatures and decreased with increasing temperature. In many crustacean neuromuscular preparations the amplitude and the time course of the excitatory junctional potentials (EJP) evoked by nerve stimulation were maximum at the acclimation temperature and decreased with the increase in temperature; also the tension produced in muscle declined with temperature (Harry and Florey, 1977, Stephens and Atwood, 1982, White, 1983). These effects could be explained on the basis of changes in the membrane conductance (Fischer and Florey, 1981) and of a decline in neuromuscular efficacy with temperature. The temperature-dependence curves shown in these papers were similar to the curves we plotted for warm-acclimated preparations: an initial increase up to temperatures around the acclimation temperature, followed by a decrease at temperatures above the acclimation temperature. Stephens (1990) reviewed the explanations proposed for the observed shapes of temperature-dependence curves: the decline in EJP amplitude at cold temperatures may be due to conduction block at certain nerve branches (Hatt and Smith, 1975) or to cold-induced decreases in the quantal content (White, 1983), perhaps produced by alterations in the amount of calcium entering the terminal (Charlton and Atwood, 1979), although no acclimation of the Ca^{2+} channels function was found in fish retinal cells (Cunningham and Hyde, 1995). The decline in EJP amplitude at warm temperatures may be due to changes in membrane conductance and a decrease in quantal content (White, 1983).

Warm-acclimation changed the shape of the temperature-dependence curves of the single-pulse and first-pulse EJPs and shifted the range of maximum amplitudes from 8-12°C to 18-22°C for *Cancer pagurus*, and to 14-18°C for *Carcinus maenas*. Stephens and Atwood (1982) found similar shapes for both cold- and warm-acclimated preparations, with maxima around their respective acclimation temperatures, thus acclimation shifted the temperatures at which maximum amplitudes

occurred in an adaptive manner. Harri and Florey (1979) showed that the amplitude of the unfacilitated slow EJPs in cold-acclimated crabs was relatively independent of temperature up to 28°C, then declined sharply at higher temperatures, while in warm-acclimated animals it had the characteristic increase, followed by a decline, with the peak around the acclimation temperature. The fast EJPs were much larger than the slow ones, and those of cold-acclimated preparations were generally larger than those of warm-acclimated preparations and displayed maxima at intermediate temperatures, higher than the acclimation temperature (Harri and Florey, 1979).

The difference in range of maximum amplitudes between warm-acclimated *Cancer* and *Carcinus* was due to an overall poorer neuromuscular function in warm-acclimated *Cancer pagurus*, and not to better acclimatory compensation, as it may appear. The data were averaged after normalization to the value at 10°C. Individual preparations of warm-acclimated *Cancer* exhibited a small increase in EJP amplitudes towards warmer temperatures, but the absolute values of amplitudes were low. At the same time, in *Carcinus* the amplitudes at 22°C were also relatively higher than those measured at 8-10°C, but those measured at temperatures around 15°C were much higher, in absolute values and obviously in relative proportion, compared to those at both the warm and the low extremes of the temperature range.

In cold-acclimated crabs of both species, the amplitude of the second-pulse EJP decreased with increasing temperature and only low facilitation could be observed at warmer temperatures. In warm-acclimated *Cancer pagurus* the amplitude of the second pulse EJP was little influenced by temperature, due to increased facilitation at the low and high extremes of the temperature range investigated. In warm-acclimated *Carcinus maenas* the amplitude of the second pulse EJP was also relatively independent of temperature from 12°C to 18°C, but had lower values at the low and high extremes, despite increased facilitation.

Our results for the second-pulse EJP and facilitation were different in a few respects from those of Stephens and Atwood (1982) and resembled more those described by Harri and Florey (1979) for the slow EJPs. Stephens and Atwood (1982)

found that cold-acclimated preparations had minimum facilitation around their acclimation temperature and facilitation increased at temperatures below and above this point, more marked at warmer temperatures, whereas in our cold-acclimated preparations facilitation was high at temperatures below the acclimation temperature, but at temperatures above the acclimation temperature facilitation became fairly independent of temperature in *Cancer pagurus* and decreased with increasing temperature in *Carcinus maenas*, similar to the results reported by Harri and Florey (1979) for slow EJPs. In the warm-acclimated preparations described by Stephens and Atwood (1982) minimum facilitation occurred at temperatures few degrees lower than the acclimation temperature and again the increase seemed to be more rapid towards warmer temperatures than towards low temperatures. Our facilitation curves for warm-acclimated preparations had the 'U'-shape shown by Stephens and Atwood (1982), but the increase seemed to be more rapid and greater towards low temperatures. Also the temperature below which facilitation increased (at the cold end of the temperature range) was higher in preparations of warm-acclimated crabs, suggesting a compensatory shift in facilitation at low temperatures with temperature acclimation. Harri and Florey (1979) found little variation with temperature in the facilitation of both slow and fast EJPs in warm-acclimated preparations.

The increase in facilitation at temperatures outside a narrow range around the acclimation temperature, at which the EJP amplitude and time-course decline, was interpreted as a mechanism through which the nerve-muscle system is capable of maintaining its functional integrity over a wider thermal range (Stephens, 1990). Neither of the species used for our experiments displayed a great degree of facilitation at warm temperatures in cold-acclimated animals. This was not surprising for *Cancer pagurus*, because individuals of this species are not very likely to encounter temperatures above 18-20°C in their natural environment, therefore it would not have evolved this compensatory mechanism for reduced neuromuscular efficacy at warm temperatures, but in *Carcinus maenas* we would have expected higher facilitation at warm temperatures in cold-acclimated individuals. Facilitation appeared to play an

important role in the sustainment of function in warm-acclimated animals, in particular in *Carcinus maenas* which had on average higher values of facilitation than warm-acclimated *Cancer pagurus* and also than cold-acclimated *Carcinus*.

The correlation between the temperatures of maximum EJP amplitude and minimum facilitation suggested that warm-acclimated *Carcinus maenas* displays optimal neuro-muscular function at temperatures around 15°C, which would mean an acclimatory response of about 50%. It was difficult to define a temperature of optimal function for warm-acclimated *Cancer pagurus* in the same way, because these did not display a clear maximum in EJP amplitude.

Rathmayer and Hammelsbeck (1985) showed that the crab closer muscle contains four distinct fibre types, which differ in the amount, time course and frequency dependence of facilitation of both slow and fast EJPs. We have not clearly established the type of fibre measured, but the basic shapes of the EJPs recorded suggest a predominance of types I and IV, as classified and characterized by Maier, Pette and Rathmayer (1986) and Rathmayer and Hammelsbeck (1985). These authors have not investigated if the fibre types differ in respect with the temperature dependence of their functional parameters.

The dependence of facilitation on the inter-pulse interval in *Carcinus* and *Cancer* closer muscle fibres has been studied in our laboratory (Pearson, personal communication) and the results were similar to those of Stephens (1990) who showed that in muscle fibres with small amplitude EJPs, the amount of facilitation decays as the time interval between the EJPs is increased and the decay can be divided into two logarithmic-linear components, the slopes and the intersection point of which depend on the temperature of measurement. These two components of decay have been explained in terms of a decline in the probability of transmitter release following stimulation, possibly related to two different processes for the removal of calcium (Kita, Narita and Van der Kloot, 1980).

The latency of EJPs decreased with temperature in an exponential manner and was little affected by the acclimation temperature. Similarly, Tiiska and Lagerspetz

(1994) did not find any difference between the duration and temperature dependence of the synaptic delay of the end plate potential in sartorius muscle of 4°C and 24°C-acclimated frogs.

The time-course experiment suggested that the neuromuscular function was acclimated at the new temperature after approximately two weeks. The relative changes observed after the third week at 22°C could be interpreted as a decline in the neuromuscular transmission, due to prolonged exposure to a high constant temperature. At this stage, when only one experiment was carried out, involving only a small number of preparations, and when there are no other similar reports to compare our results with, these were difficult to interpret in more detail or even be speculated upon. The small number of individual preparations measured and the variability observed between these preparations did not allow a proper statistical analysis of the data obtained and suggested that this type of experiment should be carried out on tagged crabs, in order to avoid the problem of individual variability, and on a larger number of animals. An initial attempt of tagging the crabs was made, but it failed due to casualties occurred throughout the experiment.

Although the stenothermal crabs *Cancer pagurus* were able to acclimate to 22°C and maintain the neuromuscular function at this warm temperature, on overall, considering all the experiments presented in this chapter, they appeared to be more thermally sensitive than the eurythermal species *Carcinus maenas*. *Cancer pagurus* failed to go through the reverse acclimation process, from 22°C to 8°C. Blundon (1989) has tested the same hypothesis, whether animals that frequently experience large temperature fluctuations will have a greater ability to cope with such fluctuations than animals living in a more constant thermal environment, and found that when warm-acclimatized (30°C, summer temperature) eurythermal crabs (blue crab, *Callinectes sapidus*) were transferred directly to 8°C, all survived and after four weeks generated similar muscle stress *in vivo*, and had similar *in vitro* neuromuscular properties to crabs maintained for two weeks at constant 30°C. At the same time,

warm-acclimatized stenothermal crabs (stone crabs, *Menippe mercenaria*) did not survive a direct transfer to 8°C, and even after a gradual transfer over three weeks from 30°C to 8°C, and another four weeks at 8°C had lower *in vivo* and *in vitro* neuromuscular performance, compared to crabs of the same species maintained at constant 30°C. Only a natural cooling of the stenothermal crabs, over six months (from summer to winter) in outdoor tanks yielded animals with levels of neuromuscular function similar to those of the summer warm-acclimatized and two weeks warm-acclimated crabs (Blundon, 1989).

Chapter 6: Changes in the Thermal Tolerance of Marine Crabs with Temperature Acclimation

6.1. Introduction

The importance of studies on the thermal tolerance ranges of various species has been emphasized by the predicted global warming (Logue, Tiku and Cossins, 1995) and also by the increasing use of fresh or marine water for the cooling systems of power-generating stations, the outlets of which cause significant rises in water temperature in the surrounding areas (Chung and Strawn, 1984). These temperature changes may adversely affect whole communities. Ecosystems particularly at risk are those with a long history of stable, cold environmental conditions, such as the Antarctic ocean or the Baltic sea (Lahdes, Kivivuori and Lehti-Koivunen, 1993); but animals living at temperatures close to their upper thermal limit, such as the tropical species, may also be susceptible (Prosser and Heath, 1991). The elimination of more thermally sensitive species will clearly disturb the delicate balance in food webs (Bamber, 1990).

The range of tolerated temperatures varies from species to species, and from population to population, and is largely genetically fixed as a consequence of the evolutionary thermal experience of the species or population, and is therefore an adaptive feature. On a shorter-term, the thermal tolerance also depends on the thermal experience within the life of the group of individuals, which is determined by their geographic location, type of habitat, age and season (Cossins and Bowler, 1987, Prosser and Heath, 1991). Such thermal experience of a species or population includes various features of the thermal regime, such as maximum and minimum temperature experienced, mean temperature and patterns and rates of temperature fluctuation on a daily, seasonal and annual basis and is an acclimatization feature (Cossins and Bowler, 1987). Temperature acclimatizations and acclimations also influence the thermal tolerance of a group of animals in an adaptive manner (Prosser and Heath, 1991).

Methods have been developed and widely used for determining the lethal limits and the thermal tolerance ranges for different groups of animals (review in Cossins and Bowler, 1987). One approach, described by Precht (1973) as Method 1 for measuring the thermal resistance of a group of individuals, taken from the field (acclimatized), or acclimated to a given temperature, is to subject them to a given set of thermal conditions and monitor their percentage survival as a function of temperature and exposure time. Three different such methods have been applied: a) the time for 50% mortality at a given exposure temperature is recorded, or b) the temperature at which 50% of the animals survive for a given exposure time is determined, and finally c) where the percentage survival at a specified temperature and exposure time is determined (%S method). In practice, for Methods a and b, the percentage survival is plotted as a function of time or temperature, respectively, then the time or temperature for 50% mortality, termed median lethal dose (LD_{50}), is estimated graphically (Cossins and Bowler, 1987). A major disadvantage of this method is that animals may suffer from the initial shock of the sudden exposure to a high or low temperature.

An alternative method, described as Method 2 by Precht (1973), involves a gradual heating or cooling of the animals, at a fixed and usually rapid rate until certain symptoms of functional disturbance appear. This is the basis for the 'critical thermal maximum' (CTMax) concept which was originally introduced by Cowles and Bogert (1944) and Lowe and Vance (1955), who worked on species of reptiles, and defined the CTMax as the 'thermal point at which locomotory activity becomes disorganised and the animal loses its ability to escape from conditions that will promptly lead to its death' (from Lagerspetz and Bowler, 1993). This method has been redefined by Hutchison (1961) and more recently reviewed by Paladino et al. (1980) and Ushakov and Pashkova (1984) who showed that the method can be used repeatedly on the same individuals, provided the rate of change in temperature during the experiment is fast enough not to allow changes in the thermal resistance of the animals to occur as a consequence of additional acclimation.

An important problem, related to both types of method described above, is the establishment of a suitable criterion of thermal death (Cossins and Bowler, 1987). The clearest procedure for the first approach is to expose samples of animals to a lethal temperature for a specific period and then to return them to their holding temperature and count as dead those that fail to recover after a given time, but this has the disadvantage of requiring large numbers of animals, a separate group for each combination of time and temperature. Another possibility is to use a reversible symptom of thermal death, such as the loss of righting response, loss of equilibrium or the cessation of respiratory movements used for the determination of the critical thermal maxima or minima (Lagerspetz and Bowler, 1993, Lahdes, Kivivuori and Lehti-Koivunen, 1993, Kivivuori and Lagerspetz, 1990, Buchanan, Stewart and Davies, 1988, Layne, Claussen and Manis, 1987), but it would be necessary to establish the relationship between the chosen criterion of thermal death and true death, and these may vary from species to species and from one group of animals to another (Cossins and Bowler, 1987).

Another problem is the actual ecological significance of the estimated median lethal doses and the CTMax and CTMin values. Following the original definition for reptiles (Cowles and Bogert, 1944, Hutchison, 1961), the critical thermal maximum was more generally defined as the upper temperature at which the coordinated movements of the animal will disappear (Claussen, 1980). The righting reflex is an example of coordinated movement for crustaceans, which is also easy to test simultaneously on several individuals. The loss of this reflex during gradual heating or cooling of the animals is probably the most common criterion used for the determination of critical thermal limits of crustacean species (Claussen, 1980). It seems to be an ecologically valid criterion for a thermally induced perturbation of the nervous system, due to an instantaneous reversible effect on some specific parts of the sensomotor nervous pathway mediating this behaviour. In *Asellus aquaticus* the temperature at which this reflex was lost was only approximately 0.5°C above the temperature of spontaneous loss of the precopulatory grasp reflex in males

(Lagerspetz and Bowler, 1993). On the other hand, heat death is a time dependent process at high temperatures and is irreversible. It is, in fact, a different measure, determined by the loss of irritability or respiratory movements, most likely due to irreversible thermal injury to cell membranes (Bowler and Manning, 1994). The determination of the temperatures at which the first signs of heat or chill distress appear (CTMax and CTMin, respectively) is considered to be more useful for ecologists and physiologists when trying to assess the impact an alteration in environmental temperature might have on a population or a species, as these occur at temperatures which the animals are likely to meet in nature (Lagerspetz and Bowler, 1993, Layne, Claussen and Manis, 1987, Maness and Hutchison, 1980).

There have been attempts to compare the lethal limits and the critical thermal maxima temperatures in the same study (Claussen, 1980, Buchanan, Stewart and Davies, 1988, Kivivuori and Lagerspetz, 1990, Lagerspetz and Bowler, 1993, Lahdes, Kivivuori and Lehti-Koivunen, 1993). The difference between the CTMax and the LD_{50} values depended on the method and thermal injury criteria used. Claussen (1980) found that in crayfish the increases in LD_{50} and in CTMax during warm acclimation had similar time-courses, but Lagerspetz and Bowler (1993) who paired the changes in CTMax and LD_{50} in each individual from a group of fully acclimated *Asellus* did not find any correlation between the magnitude of the change in CTMax and that in LD_{50}. They suggested that the mean CTMax and LD_{50} of a group may increase or decrease concomitantly during warm or cold acclimation, respectively, but the absolute values of these two thermal tolerance indices may be independent in a fully acclimated individual.

Ectothermic animals have developed a variety of strategies to cope with the fluctuations in environmental temperature (Prosser, 1973). The amount of stress a given temperature imposes on an ectotherm will depend on the absolute value of the temperature, the rate of temperature change and the duration of exposure to the altered thermal conditions. The response to such thermal stress also depends on the thermal history of the animals investigated. These factors will determine the intrinsic

ability of an ectotherm to respond and will also influence the type of response made by the animals subjected to the stress (Cossins and Bowler, 1987). These aspects have to be considered when interpreting and comparing the tolerance ranges reported by different authors. A very frequent situation is that direct comparison with results from other research groups is not possible because of differences in the experimental parameters used and in the experimental design, which may influence the final results. A few recent papers have pointed out the necessity to establish a set of standardized methods in this research field (Lahdes, 1995, Lahdes, Kivivuori and Lehti-Koivunen, 1993) and recommended the methods leading to the plotting of the tolerance polygons (Brett, 1958), which would give a complete picture of the thermal resistance of a given sample of animals.

The construction of a tolerance polygon for a given sample of a population or a species, described by Cossins and Bowler (1987), involves the determination of the upper and lower incipient lethal temperatures of those animals at different acclimation temperatures. The term 'upper incipient lethal temperature' was defined by Fry (1971) as the lowest median lethal temperature (LD_{50}), based on the observation that the LD_{50} decreases as the exposure time increases, until it reaches a plateau (Figure 6.1.a). The significance of this lowest LD_{50}, or upper incipient lethal temperature (ILT) is that this is the critical temperature above which 50% of the sample is killed in a discrete period (zone of resistance), and below which 50% mortality does not occur even for extended exposure periods (zone of tolerance). A lower ILT was defined in the same way for cold exposure experiments. The upper and lower ILT are influenced by the acclimation temperature, up to an ultimate upper ILT, where the upper ILT coincides with the acclimation temperature, and a correspondent ultimate lower ILT. These two points, together with the plots of the upper and lower ILT as a function of acclimation temperature will define the tolerance polygon for a sample of a population (Figure 6.1.b). The polygon represents the zone of tolerance, or the combinations of acclimation and lethal temperatures that 50% of the sample can withstand for an

Figure 6.1. (a) The effect of exposure time on the median lethal temperature (LD_{50}) as established from dosage mortality curves. The minimal LD_{50} represents the 'incipient lethal temperature' which separates the zones of tolerance and resistance (From Cossins and Bowler, 1987, data from Doudoroff, 1945);
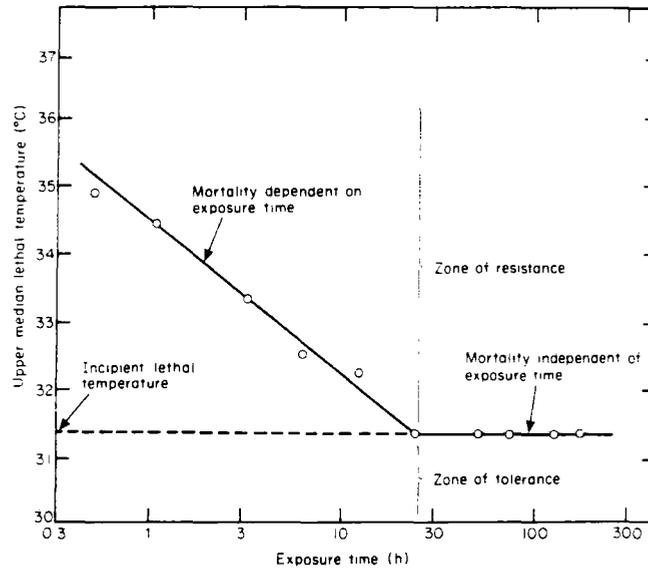
(b) The effect of acclimation temperature on the upper and lower incipient lethal temperatures (ILT) of the marine fish *Spheroides maculatus*. Construction of the 'tolerance polygon'. The area within the polygon represents the combination of test and acclimation temperature that is survived by at least 50% of the sample (From Cossins and Bowler, 1987, after Hoff and Westman, 1966)
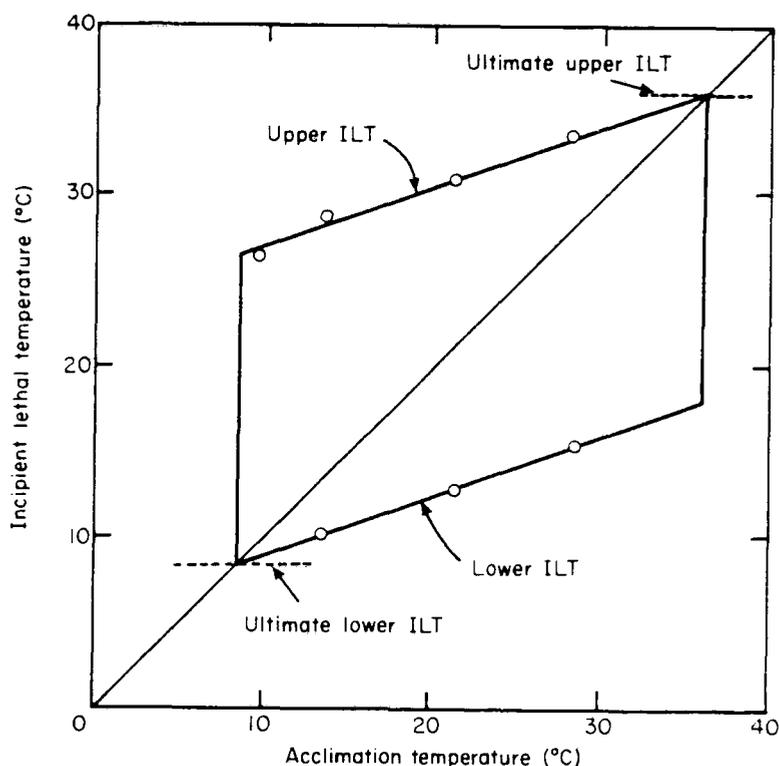
Figure 6.2. The tolerance polygon for the sockeye salmon (*Onchorhynchus nerka*) together with the more limited temperature zones within which activity and reproduction can take place (from Cossins and Bowler, 1987, after Brett, 1958)
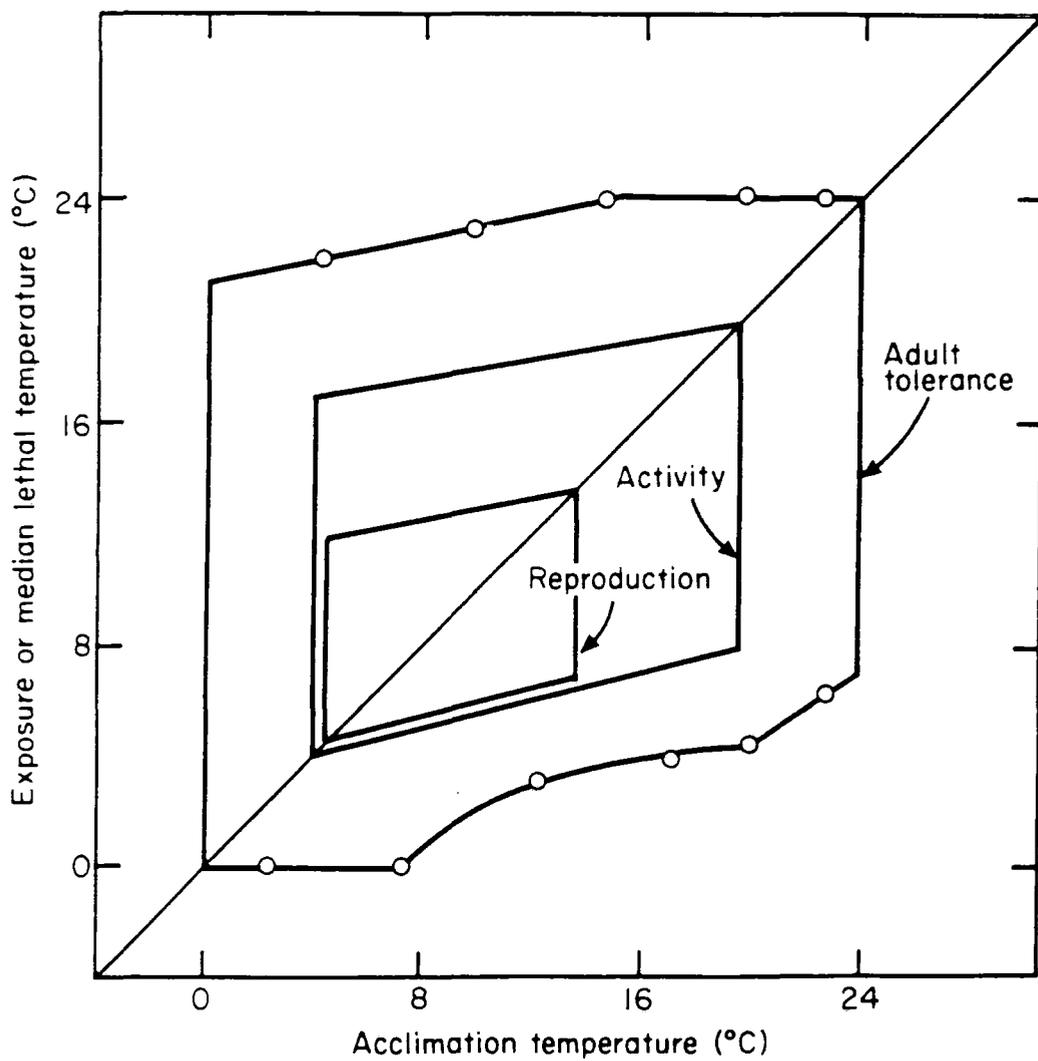
indefinite period. The 'ultimate incipient lethal temperature' probably corresponds to the CTMax measured by Cowles and Bogert (1944) (Cossins and Bowler, 1987).

Inside the polygon for survival, smaller polygons for vital functions such as activity and reproduction can be plotted (Brett, 1958) (Figure 6.2). These significantly illustrate the greater thermal sensitivity of these functions, compared to that of adult survival. The geographical distribution of many species correlates more closely with the temperatures that occur during the breeding season, probably because gametogenesis and embryogenesis are generally the least-tolerant stages of the life cycle (Cossins and Bowler, 1987). Successive development or growth stages may have tolerance zones of different areas. Baroudy and Elliott (1994) determined the tolerance polygons for alevins, fry and parr of Arctic charr (*Salvelinus alpinus*) and found that their areas increased in the order alevins < fry < parr, with the alevins being less tolerant than fry and parr only at lower acclimation temperatures, but not at higher acclimation temperatures.

The area covered by the tolerance polygons of stenothermal species is much lower than for eurythermal species, showing that the extent to which acclimation can extend the thermal tolerance range of a species is related to the lifestyle of the species and also to the seasonal range of temperatures that it usually encounters. However, the ultimate ILT do not represent genetically fixed limits of thermal resistance for the species as a whole, since tolerance polygons may be altered to some extent by other factors than temperature, such as season and photoperiod and, for aquatic organisms, oxygen tension and salinity (Cossins and Bowler, 1987).

Tolerance to one environmental variable may be reduced due to a synergistic effect from unfavourable conditions involving another variable (Vernberg and Vernberg, 1981, McLeese, 1956). A recent study on two populations of the longwrist hermit crab (Young, 1991) showed that a southern population had narrower tolerance ranges to temperature and salinity than a northern population, due to more intense thermal stress during summer, while the northern population lived in favourable thermal conditions throughout the year.

For the purpose of the work presented in this thesis, the critical thermal maximum method was chosen as an ecologically significant criterion for comparing the thermal tolerance of the relatively stenothermal *Cancer pagurus* and the more eurythermal *Carcinus maenas* crabs. The absolute values of the CTMax were also used as a measure of heat resistance at whole organism level, to be compared to the heat resistance of isolated nerve function, detailed in Chapter 5. The only variables used were the acclimation temperature and the season. The effects of other factors were not investigated, but data available on crustacean species are reviewed in the discussion of this chapter.

6.2. Materials and Methods

6.2.1. Animals

Crabs of both species (intermoult stage) were acclimated at 8°C or at 22°C for three weeks. Acclimation at 22°C involved one week at 15°C, prior to transfer for three weeks at 22°C. Animals of similar size were selected: 8-10 cm carapace width for *Cancer pagurus* and 6-7 cm carapace width for *Carcinus maenas*. They were maintained in aerated filtered sea water, in plastic tanks and fed (fish, commercial source) once a week. The photoperiod was 12 hours light-12 hours dark.

6.2.2. Critical Thermal Maxima

Crabs from the same lot were measured first time at the end of the 3 weeks of cold-acclimation at 8°C, then at the end of the 3 weeks at 22°C. One experiment used crabs caught in July 1994 and a second experiment used crabs caught in November 1994. During the second experiment, a few crabs of each species were also measured at the end of the week at 15°C.

The measurements were carried out in a stainless steel bath with the bottom covered in gravel and stones. The sea water in the bath was directly heated by a a temperature control unit Tempunit TU-16D (Techne), linked to a temperature

programmer unit TP-16 (Techne) at a rate of 0.2°C/minute, starting from the acclimation temperature of each group investigated. Rapid mixing of the water in the bath, to avoid temperature gradients, was ensured by the propeller of the heater and several air diffusers. The temperature of the bath was monitored by a thermocouple placed near the crabs and connected to a digital thermometer. Before starting the heating programme the crabs (4-5 per run) were allowed to settle in the new environment for about 10 minutes after transfer from the acclimation tanks. During the heating, the righting reflex was checked every 2°C for lower temperatures and every 0.5°C for higher temperatures (above 22°C for cold-acclimated and above 27°C for warm-acclimated *Cancer pagurus*; above 28°C for cold-acclimated and above 32°C for warm-acclimated *Carcinus maenas*, values which were established by preliminary experiments). The crabs were gently turned upside-down, using a smooth wooden spatula, and allowed maximum 5 minutes for righting. If the development of the reflex took longer than 1 minute, the temperature recorded was the temperature at the moment of righting. When a crab failed to right itself within 5 minutes, then the last temperature at which it was able to develop the righting reflex was recorded as the CTMax.

The rate of increase in temperature was chosen after preliminary experiments which used some of the largest *Cancer pagurus* crabs (from the selected lot) as a "control" to check if the internal temperature of the crab near its central nervous system increases at the same rate with the temperature of the water in the bath and reaches the same values. For this purpose, a small hole (1 mm diameter) was carefully drilled in the carapace, near the eyes, a thermocouple was inserted under the carapace and the hole was sealed well with Blu-Tack and with water resistant adhesive tape. The water in the bath was heated at two rates: 0.2°C/minute or 0.5°C/minute, and the temperatures of the crab, the water in the bath near the crab and the reading on the digital thermometer of the temperature control unit were recorded and plotted (Figure 6.3), showing that the internal temperature of the crab increased along with that of the water in the bath.

Figure 6.3. The correspondence between the internal temperature of a crab (**crab**), the temperature of the water in which the crab is immersed (**water**) and the programmed temperature (**heater**) during heating at 0.2°C/minute (**rate 1**) and at 0.5°C/minute (**rate 2**)
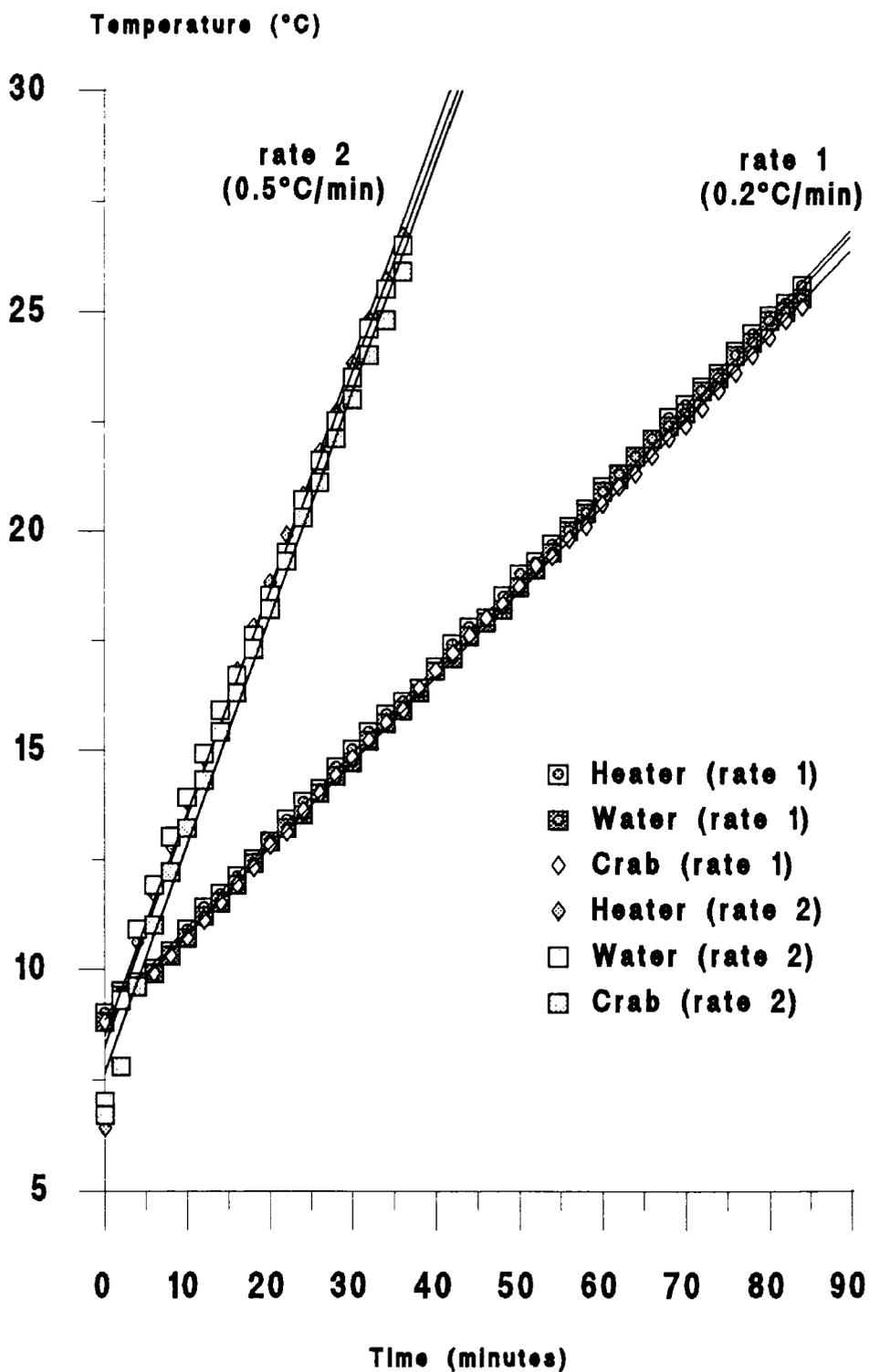
Crabs from both species were tested in their tanks for the times required to right themselves at their acclimation temperature. These ranged from under 30 seconds for *Carcinus maenas* up to 2-3 minutes for *Cancer pagurus*, when the defence position (crossed claws and tightly flexed legs) was induced in the individuals of the latter species by the turning procedure itself. Although the internal temperature of the crab followed close the temperature of the water even for the faster heating rate, it was decided that a rate of 0.2°C/minute was more suitable, allowing sufficient time for righting for both species within small changes in temperature. Heating rates varying from 0.2°C/minute to 1.4°C/minute have been used by other authors for similar experiments on crustacean species (Lahdes, Kivivuori and Lehti-Koivunen, 1993, Lagerspetz and Bowler, 1993, Buchanan, Stewart and Davies, 1988, Claussen, 1980).

6.2.3. Statistical Analysis

The groups of data were compared using the T-test or the oneway analysis of variance method, combined with the 'least significant difference' range test, when more than two groups of data were compared (SPSS for Windows). The level of significance used was $p < 0.05$. Significantly different groups were labelled with different letters, in increasing order of means: a < b < c.

6.3. Results

The average values of the CTMax of cold- and warm-acclimated *Cancer pagurus* and *Carcinus maenas* are shown in Table 6.1. Temperature acclimation significantly increased the CTMax of both species. In *Cancer pagurus* there was an increase of approximately 6°C, from a CTMax of 24.8±1.6°C in cold-acclimated crabs, to 31.2±1.2°C in warm-acclimated crabs. The increase was lower in *Carcinus maenas*, of approximately 3°C, from 32.3±1.1°C in cold-acclimated crabs, to 35.5±0.8°C in warm-acclimated *Carcinus*.

Table 6.1. The average values of the critical thermal maxima (**CTMax**) of cold- and warm-acclimated *Cancer pagurus* and *Carcinus maenas* (means±S.D.; n = number of crabs measured; significantly different groups, at p≤0.05, are labelled with different letters; group means increase in the order **a<b<c**)

Species	Acclimation Temperature	CTMax (°C)	n	Statistical Analysis
Cancer pagurus	8°C	24.79±1.62	17	a
	15°C	27.73±0.49	4	b
	22°C	31.18±1.19	12	c
Carcinus maenas	8°C	32.32±1.12	13	a
	15°C	34.16±0.26	4	b
	22°C	35.52±0.76	18	c

Table 6.2. The **'acclimation response ratios'** (**ARR** = ΔCTMax/ΔAT) calculated for 22°C-acclimated *Cancer pagurus* and *Carcinus maenas* and for crabs that were kept for one week at 15°C, compared to 8°C-acclimated crabs, using average CTMax values (**AT** = acclimation temperature; **CTMax** = critical thermal maximum)

Species	Δ AT	Δ CTMax (°C)	ARR
Cancer pagurus	14°C (8-22°C)	6.39	0.46
	7°C (8-15°C)	2.94	0.42
Carcinus maenas	14°C (8-22°C)	3.2	0.23
	7°C (8-15°C)	1.84	0.26

The measurements after 7 days at 15°C (carried out only in the autumn experiment on a few crabs from each species) showed that a period of only one week at 15°C was sufficient to induce a significant increase in the CTMax of both species.

The most interesting finding was that the absolute values of the CTMax of *Cancer pagurus* were significantly lower than those of *Carcinus maenas*, at all acclimation temperatures (Figure 6.4).

The acclimation response ratio (ARR), defined by Claussen (1977) as the ratio between the change in thermal tolerance and the total change in acclimation temperature (or field temperature, when seasonal or geographical differences are sought) was used to compare the intensity of the response in the two species. This ratio describes the change in heat tolerance per degree change in acclimation temperature. The ARR values were calculated for 22°C-acclimated crabs and for crabs kept for 1 week at 15°C relative to 8°C-acclimated crabs and are shown in Table 6.2. The ARR values for *Cancer pagurus* were 2-fold greater than those for *Carcinus maenas*. The ARR values after one week at 15°C were similar to those for the fully acclimated crabs (Table 6.2).

Data from the summer and autumn experiments were compared using the T-test and no significant seasonal difference was found. The data were pooled and the *n* values given in Table 6.1 represent the total number of crabs measured.

6.4. Discussion

Warm-acclimation significantly increased the CTMax of both *Cancer pagurus* and *Carcinus maenas*, in comparison with the values determined for cold-acclimated crabs. Changes in thermal tolerance with acclimation temperature have been reported for many other species, including the planarians *Dugesia tigrina* and *D. dorotocephala* (Claussen and Walters, 1982), the amphipod *Paramelita nigroculus* (Buchanan, Stewart and Davies, 1988), the lobster *Homarus americanus* (McLeese, 1956), the brine shrimp *Artemia* (Miller and McLennan, 1988), the crab *Pagurus*

Figure 6.4. Comparison between the values (means±S.D.) of the critical thermal maximum (CTMax) of *Cancer pagurus* (Cp) and *Carcinus maenas* (Cm) at different acclimation temperatures and their acclimation response ratios (ARR); ARR = ΔCTMax/ΔAT, in this case it represents the slope of the linear fit to the increase in CTMax with the acclimation temperature (AT), (Cp 8°AT n=17, 15°C AT n=4, 22°C AT n=12), (Cm 8°AT n=13, 15°C AT n=4, 22°C AT n=18)
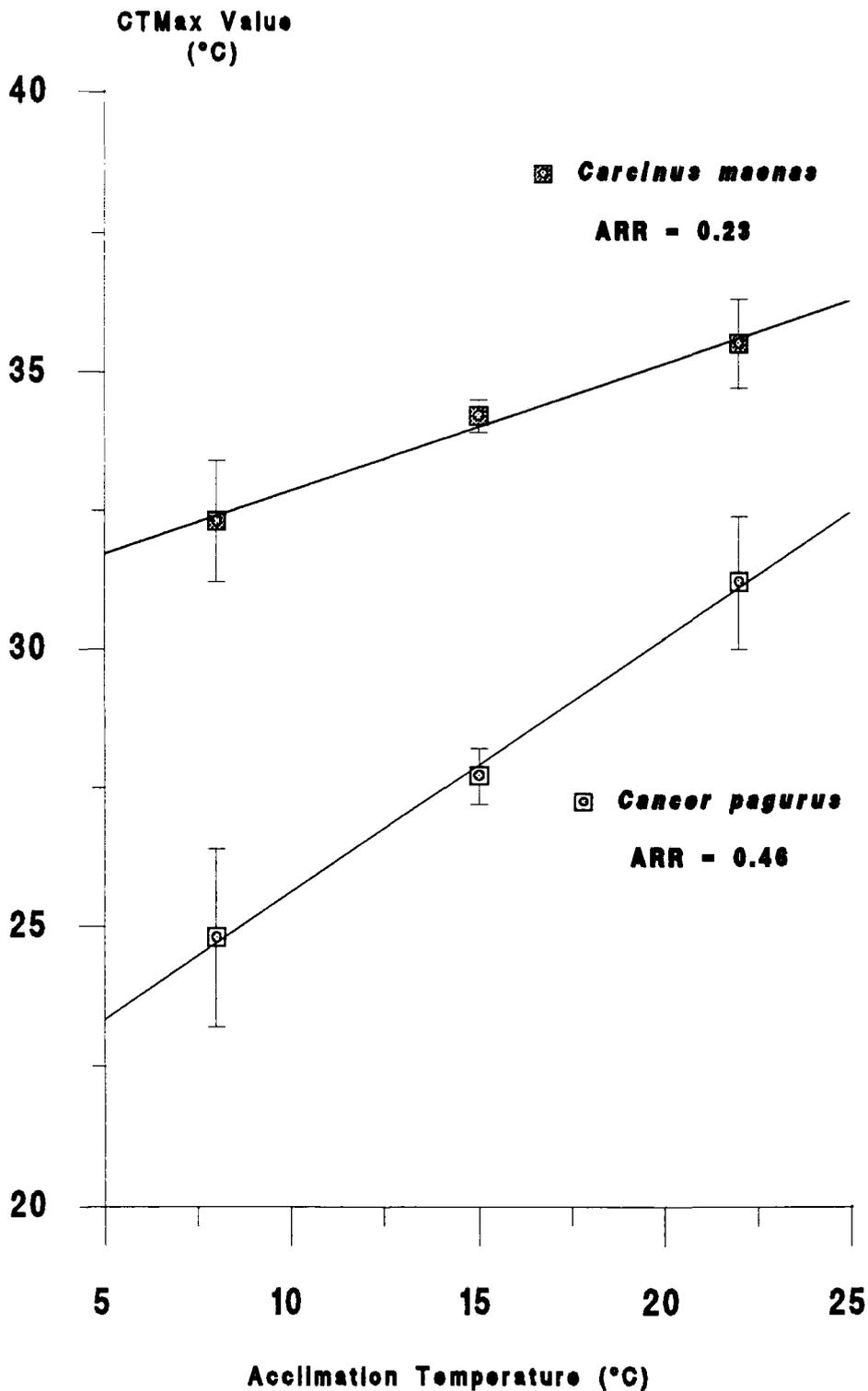
longicarpus (Young, 1991) and various species of crayfish, such as *Asellus aquaticus* (Lagerspetz and Bowler, 1993), *Orconectes rusticus* (Claussen, 1980, Layne, Manis and Claussen, 1985, Layne, Claussen and Manis, 1987), *O. virilis* (Claussen, 1980), and *Austropotamobius pallipes* (Bowler, Gladwell and Duncan, 1973).

Several of these studies have also investigated the time-course of the gain in thermal tolerance during warm-acclimation and its loss during cold-acclimation (Claussen, 1980, Layne, Manis and Claussen, 1985, Buchanan, Stewart and Davies, 1988, Lagerspetz and Bowler, 1993) and demonstrated that, although the rate of cold-acclimation is slower than that of warm-acclimation, both processes have relatively rapid time courses. For example, in crayfish the half-acclimation times (the time required for the development of 50% of the acclimatory response) for warm-acclimation of CTMax were shorter than one day (Claussen, 1980) suggesting that this species is able to respond rapidly to changes in environmental temperature. In our experiments, one week at 15°C was sufficient to induce a significant increase in the CTMax of both *Cancer pagurus* and *Carcinus maenas*. Although we did not check that the CTMax was stabilized after only a week (because all crabs were transferred to 22°C), this suggested that the thermal acclimation of this feature has a rapid time-course in these species, as well. This observation is supported by the comparison of the 'acclimation response ratio' (ARR) values for the 8°C to 15°C transfer with those for the 8°C to 22°C transfer (Table 6.2), which showed that the magnitude of the response after one week at 15°C was similar to that of the response after three more weeks at 22°C. These ARR values were calculated according to Claussen (1977) in order to compare the magnitude of the acclimatory response between the two species of crab studied and also to compare our results with those obtained for other species.

The absolute values of the CTMax of *Cancer pagurus* were significantly lower than those of *Carcinus maenas* at all acclimation temperatures demonstrating that *Cancer pagurus* is more thermally sensitive than *Carcinus*. The extent of the difference was such as the CTMax of warm-acclimated *Cancer pagurus* (31.2±1.2°C) was only as high as the CTMax of cold-acclimated *Carcinus maenas* (32.3±1.1°C).

The thermal tolerance of ectotherms appears to be correlated with the temperature range to which the organisms are exposed in their natural habitat (Newell, 1979). *Cancer pagurus* is a relatively stenothermal crab, with a rhythmical behaviour pattern which allows individuals to use predominantly the optimal phases of the tidal cycle for vital activities and is, therefore, less likely to be subjected to large fluctuations in temperature. *Carcinus maenas* is a more eurythermal crab which often stays on shore, behind the tide, in conditions where surface warming may raise the environmental temperature to much higher values (Newell, 1979) which they cope with through evaporative cooling. Ashanullah and Newell (1977) showed that *Carcinus* survives higher temperatures in air than under conditions where evaporation is prevented. In their experiments, *Carcinus maenas* acclimated to 10°C were placed in tilted tanks, with one end covered in water and the other exposed to air. The water in the tank was heated and it was found that at 15°C and 20°C none of the crabs crawled out of the water, whereas at 30°C all emerged after 25 minutes, at 32°C 89% of the crabs emerged within 20 minutes and at 34°C the crabs entered heat-coma and were unable to crawl out into air (Ashanullah and Newell, 1977). The average value of 32.3°C we found for the CTMax of *Carcinus* acclimated to 8°C seemed to correlate well with the heat-coma temperature of 34°C observed by Ashanullah and Newell (1977) for 10°C-acclimated *Carcinus*. It would have been useful to have a clearer picture of the temperature regimes to which *Cancer pagurus* and *Carcinus maenas* are subjected in natural conditions, maybe from field observations and temperature logging at a few subtidal and intertidal sites, but such data were not available.

Other studies on species living at different latitudes (Lahdes, 1995, Lahdes, Kivivuori and Lehti-Koivunen, 1993, Kivivuori and Lagerspetz, 1990) or on populations of the same species living at different latitudes (Young, 1991) supported the correlation between CTMax or other indices of thermal tolerance and the temperature range of their habitat. Antarctic species, such as the amphipod *Orchomene plebs* (CTMax 8-10°C, LT_{50} 19.5°C, Lahdes, Kivivuori and Lehti-

Koivunen, 1993), and the copepods *Calanoides acutus* and *Calanus propinquus* (short-term exposure LT_{50} 16-18°C, 12 hours exposure LT_{50} 14.3°C, Lahdes, 1995) had lower thermal tolerances than the Arctic and Baltic isopods *Saduria entomon* (CTMax 26°C, LT_{50} 31°C, Kivivuori and Lagerspetz, 1990, and LT_{50} 26°C, Percy, 1985) and than other eurythermic zooplanktonic species, such as the copepod *Eurythemora affinis* (CTMax 35°C, Bradley, 1976) and *Daphnia sp.* (short-term exposure LT_{50} near 40°C, MacIsaac, Herbert and Schwartz, 1985). A northern population of *Pagurus longicarpus* had wider tolerance ranges to temperature and salinity, and better survival under optimum conditions than a southern population of the same species (Young, 1991), although both populations had optimal temperatures around 16°C. The difference was assumed to be related to the fact that the northern crabs live in favourable temperature conditions (the range of favourable temperatures was defined as the optimal temperature ± 10°C) throughout the year, whereas the southern crabs are exposed to much warmer temperatures in summer, stress which would reduce their tolerance to experimental conditions (Young, 1991). In the same experiment, the acclimation temperature also influenced the tolerance range: in the northern population, the cold-acclimated crabs had wider tolerance ranges than the warm-acclimated ones, while in the southern population, the opposite was observed, i.e. the warm-acclimated crabs had wider tolerance ranges than the cold-acclimated ones, as expected. An exception was the comparison of a northern (*Orconectes virilis*) and a southern (*Orconectes rusticus*) species of crayfish. Claussen (1980) failed to find a major difference in the ability to warm-acclimate of the two species, or in the CTMax of the warm-acclimated crayfish.

A notable finding in our experiments was that the magnitude of the response to warm-acclimation was greater in *Cancer pagurus* than in *Carcinus maenas*. Although the absolute values of the indices for thermal tolerance have been correlated with distribution and habitat selection, the ecological correlates of thermal acclimation rates and magnitudes are still unelucidated (Claussen, 1980). One might expect to find rapid acclimation rates and large changes in the CTMax or CTMin of organisms

which are chronically exposed to large fluctuations in ambient temperature (Claussen, 1980). The observations of Kontogiannis (1973) suggested that tide pool organisms may fit this pattern, but our results do not support this idea.

A few speculative explanations can be proposed for the greater increase in the CTMax of *Cancer pagurus* following warm-acclimation (ΔCTMax) compared to *Carcinus maenas*. The most likely reason is that an acclimation temperature of 22°C, which is only 3-4°C lower than the CTMax of cold-acclimated *Cancer*, was perceived as a more threatening thermal challenge by individuals of this species and they developed the maximum response (an increase of 6-7°C in their CTMax), within their genetic limits. At the same time, a temperature of 22°C is approximately 10°C lower than the CTMax of cold-acclimated *Carcinus* and probably was not sensed as a threat to their vitality, therefore a less intense response (an increase of 3-4°C in their CTMax) was made to adjust to the new acclimation temperature.

Another explanation is related to the effect that a prolonged exposure to a constant temperature might have upon the thermal tolerance of individuals which are normally exposed to rather large fluctuations in temperature in their natural environment. *Cancer pagurus* will develop a maximum response to the new thermal conditions, as they normally live in relatively constant conditions of temperature, whereas *Carcinus* will develop only a minimum necessary response, as a constant temperature regime is perceived as 'unnatural' and may not trigger all the mechanisms for increasing their thermal tolerance.

Data from literature showed that the seasonal increases observed in the CTMax of the crayfish *Orconectes rusticus* (Mundahl, 1989, Layne, Claussen and Manis, 1987) had calculated ARRs in the range of 0.3-0.5, while those induced by laboratory acclimation at winter and summer temperatures of animals caught at the same time (Layne, Claussen and Manis, 1987, Claussen, 1980, Spoor, 1955) were lower, in the range of 0.1-0.25, also common for other laboratory acclimated species (Bowler, Gladwell and Duncan, 1973, Claussen, 1980). Also a diel cycle in CTMax was observed only in field acclimatized crayfish (Mundahl, 1989) and not in

laboratory acclimated individuals (Layne, Claussen and Manis, 1987). This difference in ARRs between field-acclimatized and laboratory-acclimated crayfish suggested that individuals acclimated to constant temperatures in the laboratory may lose part of their capacity for adjustment of their thermal tolerance compared to individuals acclimatized to natural temperature changes (Mundahl, 1989), phenomenon which is probably related to the absence of fluctuations in other environmental parameters, such as photoperiod, as well as in temperature.

Maness and Hutchison (1980) discussed the role of heat hardening, described as a transitory increase in heat tolerance induced by a sublethal exposure to lethal high temperatures, in the acute adjustment of the thermal tolerance of vertebrate ectotherms. They quoted several papers which demonstrated that acclimation to cyclical temperature regimes increased the rate and scope of thermal acclimation in fish, and showed that only the cyclical regimes with sublethal exposures to lethal or supralethal peak temperatures produced tolerances comparable to those observed in the field (for example Otto, 1974). Species from habitats with extreme fluctuations in diurnal temperatures may utilize heat hardening to survive lethal environmental temperatures and expand their thermal range of activity (Maness and Hutchison, 1980). These facts bring the discussion back to one of the initial questions, of the physiological causes of the differences between stenothermal and eurythermal organisms, which are still poorly understood.

The CTMax values reported for other warm-acclimated eurythermal species were usually above 35°C, such as 39°C for 25°C-acclimated planarians (Claussen and Walters, 1982), and 38.5°C and about 41.5°C for 25°C- and 35°C-acclimated crayfish *Orconectes rusticus*, respectively (Claussen, 1980). In the latter paper (Claussen, 1980) the acclimation periods used were shorter than those we used (6-8 days at 25°C and 5-7 days at 35°C) but the ARR (0.29) was close to that calculated for *Carcinus maenas* (0.23). *Austropotamobius pallipes* acclimated to 25°C was reported to be able to tolerate at least brief exposures to 37°C (Bowler, Gladwell and Duncan, 1973).

Studies on marine animals taken directly from their natural conditions (non-acclimated), using methods comparable to the CTM approach, found thermal tolerances ranging from 28.5°C to 40°C for barnacles (Ritz and Foster, 1968) and from 22°C to 37.2°C for various crustacean species (Huntsman and Sparks, 1924).

These observations, on laboratory acclimated and naturally acclimatized animals, suggested that the CTMax values determined for *Cancer pagurus* and *Carcinus maenas* were within the range found for temperate marine crustaceans, and the lower ARR calculated for *Carcinus maenas*, compared to *Cancer pagurus*, was due mainly to an elevated heat tolerance of the cold-acclimated *Carcinus*, which is in agreement with the eurythermy of the species. Also the large increase in the CTMax of *Cancer pagurus* with warm acclimation suggested that this species, although more thermally sensitive than a eurythermal species from the same geographical location, has greater adaptive capacity than presumed. Several studies on cold-adapted stenotherms, such as certain species of Arctic and Antarctic crustaceans have suggested that the ability of these species to withstand warmer temperatures is actually greater than suggested by earlier studies (Lahdes, 1995, Lahdes, Kivivuori and Lehti-Koivunen, 1993, Hirche, 1984).

Ushakov and Pashkova (1972, 1984) showed that individuals with a lower initial steady state level of heat resistance will develop greater changes to a new steady state level following thermal acclimation than individuals with a higher initial level of heat resistance, but this referred mainly to individual variability within a sample of an *Asellus aquaticus* population and it may not be valid for different species. In the same species (*Asellus aquaticus*) Lagerspetz and Bowler (1993) did not find a significant correlation between the initial values of CTMax and their maximal increase following warm-acclimation, but the methods used were different.

Our results did not reveal any seasonal differences between summer and autumn. Layne, Manis and Claussen (1985) and Layne, Claussen and Manis (1987) showed that the crayfish *Orconectes rusticus* acclimated in summer had higher

CTMax than animals acclimated in winter, and also the time-course of the changes was more rapid in summer than in winter (Layne, Manis and Claussen, 1985) but the scope for acclimation (ARR) was greater in winter than in summer (Layne, Claussen and Manis, 1987). However, the CTMax values for summer and autumn were not significantly different, either (Layne, Claussen and Manis, 1987). Similar seasonal differences were reported by Maness and Hutchison (1980) for aquatic vertebrate ectotherms and were interpreted as an adaptive response to higher summer temperatures. Lagerspetz and Bowler (1993) found no seasonal dependence of CTMax in *Asellus*, but their experiments were carried out on animals caught in either March, May, August or September, therefore did not include a winter time sampling.

Other factors which may influence the thermal tolerance of organisms, which we did not study, but have been considered by other authors are the sex and size (Lagerspetz and Bowler, 1993, Mundahl, 1989, Claussen, 1980), feeding status (Buchanan, Stewart and Davies, 1988) and acclimation in isolated state or in groups (Lagerspetz and Bowler, 1993) of the animals investigated. Neither of these papers, all on crustacean species, found any consistent and/or significant effect for the factors listed above. Several other papers confirm these observations, but a few exceptions have also been reported (reviews in Precht, 1973, Claussen 1980, Buchanan, Stewart and Davies, 1988). Our study, and the information quoted from other papers in this discussion referred to general thermal tolerance of adults and its modulation following thermal acclimation. Complex vital processes, such as growth and reproduction, or development of early life stages have different thermal limits, and take place over narrower ranges of temperature than those of adult survival (Brett, 1958, Precht, 1973, Cossins and Bowler, 1987).

Chapter 7. General Discussion and Conclusions

The aims of the work presented in this thesis were a) to identify the effects of temperature acclimation at cellular, functional (physiological) and whole organism level in a stenothermal and a eurythermal crab; b) to look for correlations between the temperature acclimation-induced changes, if any, at each of these levels; c) to compare the responses in the stenothermal and eurythermal species; and d) to assess the seasonal dependence of the acclimatory responses.

A combination of two co-existing species of decapod crustaceans, with different thermal ecology, was considered to be an interesting model of study for comparative purposes. The two species of marine crustacean chosen were the relatively stenothermal *Cancer pagurus* and the eurythermal *Carcinus maenas*.

Many species of marine and freshwater invertebrates are of commercial interest, including the edible crab *Cancer pagurus* chosen for this study, and/or are important links in food webs, therefore it is important to investigate their abilities to acclimate and perform vital functions at warmer temperatures, in the current context of predicted global warming and increased awareness of thermal pollution of marine and fresh waters.

Of the various cellular aspects that can be influenced by temperature acclimation, we chose to investigate the lipid composition and the biophysical state of plasma membranes. Several papers and reviews (Cossins and Raynard, 1987, Cossins and Bowler, 1987, Hazel, 1989, Hazel and Williams, 1990), including some recent ones published after the design of this research project (Cossins, 1994, Farkas *et al.*, 1994, Hazel, 1995), have pointed out that the relationship between membrane lipid composition and membrane biophysical state is not at all a simple one. Invertebrates have been little studied in these respects (Pruitt, 1990) and the preparations analysed were mostly whole animal or tissue extracts (Chapelle, 1978, Farkas and Nevenzel,

1981, Pruitt, 1988, Farkas *et al.*, 1994), not specific membrane fractions, of known purity.

For this purpose, the first step was to develop a rapid method for the isolation and purification of a plasma membrane-rich fraction from crab leg muscle tissue, which was described in Chapter 2 and has been published (Cuculescu and Bowler, 1993). According to the specific activity of two plasma membrane marker-enzymes, (Na^+-K^+) ATPase and Alkaline Phosphodiesterase I, the purified fraction was approximately 13-fold enriched in plasma membranes, with 16-18% recovery of the total activity in the crude homogenate, and the method developed was rapid and easy to carry out as a routine preparation procedure.

7.1. Correlations between Changes in Plasma Membrane Lipid Composition and Fluidity

The results of the membrane fluidity measurements using the steady-state fluorescence polarization method are presented in Chapter 3. The total phospholipid and cholesterol concentrations and the fatty acid profiles of PC, PE and total phospholipid fraction from membranes of cold and warm-acclimated crabs are shown in Chapter 4. Part of this work (a comparison between the 5°C, 8°C and 22°C-acclimated crabs, in spring and autumn) has been published (Cuculescu, Hyde and Bowler, 1995).

No clear correlation was observed between the changes in membrane fluidity and those in the unsaturation of membrane phospholipids. The results were both interesting and intriguing, and the possible interpretations were complicated by the rather strong influence of the background seasonal acclimatization. In each season the membrane fluidity measurements were carried out on aliquots from the same membrane preparations that were analysed for lipid composition (total phospholipid, cholesterol and PC, PE and TPL fatty acids). More subtle correlations might be

revealed by a multivariate statistical analysis of all the data, grouped by individual preparations.

One conclusion was that the type of acclimatory response depended on the absolute value of the acclimation temperature: acclimation to 15°C induced a significant increase in the S/U ratio of membrane PC and PE, compared to 5°C and 8°C-acclimated crabs, but no change in membrane order at DPH level, whereas acclimation to 22°C induced no significant increase in the S/U ratio of membrane PC and PE, compared to cold-acclimated crabs, but induced an increase in membrane order at DPH level.

A positive correlation was observed between the temperature acclimation-induced changes in plasma membrane fluidity and the Ch/PL molar ratio, in some cases. In both species, in autumn only, the lower fluidity of membranes from 22°C-acclimated crabs was paralleled by increased Ch/PL ratios, due to modulations of the total phospholipid levels in both species, and to an increase in the levels of cholesterol with acclimation temperature only in *Carcinus maenas*. Also in autumn, the 5°C and 15°C-acclimated crabs which had more ordered plasma membranes at TMA-DPH level (in both species) than the 8°C-acclimated crabs, had significantly higher levels of total phospholipid than the 8°C-acclimated crabs, both observations suggesting a tighter packing of the headgroups of phospholipid molecules in the bilayer. However, the possible interpretation that the membrane order at TMA-DPH level would be related to the level of total membrane phospholipid is speculative. For example, in the spring experiment, both total phospholipid and cholesterol were lower in the 22°C-acclimated crabs than in the 8°C-acclimated ones and no differences in order were found at TMA-DPH level. Probably for this type of interpretation it would have been more useful to express the concentrations of total phospholipid and cholesterol per mg membrane protein instead of g fresh weight of muscle tissue from which the membranes were isolated.

Roche and Pérès (1984) showed that the muscle tissue of sea dace did not vary its water content neither with acclimation temperature, nor with season. This

observation was considered important in relation to the way we expressed our total phospholipid and cholesterol data (mg per g fresh weight of muscle used for membrane preparation), as the possible variations in water content raised a question mark upon the validity of the comparison of data for different groups.

An intriguing aspect of our results was that we did not find the expected correlation between the observed changes in membrane fluidity, at DPH level, and the changes in fatty acid composition. The data on the fluidity at TMA-DPH level, from our work or available from other papers, together with the controversy on the place of incorporation of this probe into membranes (see Chapter 3) are not yet sufficient to develop an explanation on their basis.

The existing evidence is controversial. There are many examples of cold adaptation or acclimation associated with a decrease in the S/U ratio of membrane phospholipids or with an increase in membrane fluidity (Cossins and Prosser, 1982, Cossins and Sinensky, 1984, Cossins and Raynard, 1987, Hazel and Williams, 1990, Behan-Martin et al., 1993) compared to warm-adapted or acclimated animals, but studies which investigated the correlation between the saturation of membrane lipids and the membrane biophysical state produced both positive and negative results.

Cossins (1977) found that synaptosomal membranes of cold-acclimated goldfish were more fluid than those of warm-acclimated fish, difference correlated with decreased proportions of saturated fatty acids of the major phospholipid classes and an increased unsaturation index in choline phosphoglycerides. The cholesterol content of synaptosomal membranes of goldfish was unaffected by thermal acclimation (Cossins, 1977).

Lee and Cossins (1990) found no difference in fluidity in the intestinal brush-border membranes from 10°C and 30°C-acclimated carp, and no major changes in fatty acid composition, while in the basolateral fraction there was a significant increase in fluidity and in the proportions of unsaturated fatty acids with cold acclimation, which could be also correlated with evidence of functional adaptations of the basolateral membrane during thermal acclimation (Gibson, Ellory and Cossins,

1985). Crockett and Hazel (1995) explored the role of cholesterol in the response to temperature acclimation of these two types of membranes, the basolateral and brush-border membranes from intestinal epithelia of 5°C and 20°C-acclimated rainbow trout. They showed that in trout the brush border membranes from cold-acclimated fish were more ordered (DPH probe) than those from warm-acclimated fish, and this inverse compensation was associated with higher contents of cholesterol in the cold-acclimated membranes and with only minor changes in the fatty acid composition. In contrast, the basolateral membranes display perfect compensation of order (DPH), decreased levels of saturated and increased levels of polyunsaturated fatty acids in the cold-acclimated membranes, but no changes in the cholesterol content or Ch/PL molar ratio with acclimation temperature (Crockett and Hazel, 1995).

Buda *et al.* (1994) found a high degree of compensation (80%) in the structural order of membranes of a mixed brain cell population from cold and warm acclimated carp, acquired through a rapid and reversible process, while the isolated phospholipids exhibited only around 10% compensation in fluidity. The fatty acid composition of the brain total phospholipids did not vary with adaptation to temperature, neither did the molecular species composition of PC, only that of PE.

Farkas, Storebakken and Bhosle (1988) compared Arctic and tropical copepods and found that the phospholipid vesicles from tropical species were more ordered than those from Arctic species, but the difference in fluidity could not be easily correlated with their respective lipid compositions, because the unsaturation of lipids from tropical copepods was higher than of those from Arctic species, although the latter had higher levels of C22:6. Farkas *et al.* (1994) compared cold and warm adapted species of fish and shrimp and found that the S/U ratio and the levels of C22:6 did not show any notable difference, but the phospholipids isolated from the cold-adapted species were more fluid than those from the warm-adapted species (ESR), although the difference was more obvious in the outermost segment of the bilayer. Dey *et al.* (1993b) also showed that the level of polyunsaturated fatty acids was almost identical in species of marine and freshwater fishes adapted to contrasting

temperatures. The conclusion of the work from Farkas and colleagues was that the changes in the phospholipid molecular species, especially in PE, rather than in the overall fatty acid composition, played the decisive role in thermal adaptation, and that changes in the lipid molecules cannot be extrapolated to explain the thermal adaptation in intact membranes (Farkas *et al.*, 1994, Buda *et al.*, 1994).

Work carried out by Thompson (1989), Dey and Farkas (1992) and Dey *et al.* (1993a) on fish erythrocytes showed that these cells were able to change their membrane fluidity with temperature without changing their fatty acid composition or S/U ratio, which suggested that the alteration of the S/U ratio or of the level of polyunsaturated fatty acids does not play a decisive role in membrane lipid adaptation to temperature. The latter paper (Dey *et al.*, 1993a) also investigated the molecular species composition and the sterol/phospholipid ratio and these did not change either. They also found that the response *in vivo* was more efficient than that *in vitro*.

The relationship between physical state and the lipid composition has also been studied on artificial lipid bilayers prepared using phospholipid or phospholipid and cholesterol mixtures of controlled composition (Coolbear, Berde and Keough, 1983, Stubbs *et al.*, 1981, Hazel *et al.*, 1991). Although these mixtures can not reproduce the complexity of the lipid-lipid and protein-lipid interactions of natural cell membranes, the results obtained showed that there is no simple linear relationship between the degree of lipid unsaturation and membrane physical state.

Wodtke and Cossins (1991) pointed out that only the monitoring of the time-course of the changes that occur after transfer to a new temperature may provide significant correlative evidence of a relationship between homeoviscous adaptation and changes in membrane lipid composition. Papers investigating these aspects (Wodtke and Cossins, 1991, Hazel and Landrey, 1988a, 1988b, Wodtke, 1986, Hagar and Hazel, 1985, Schünke and Wodtke, 1983) showed that rapid changes take place in the lipid composition, membrane physical state and activities of the enzymes involved in the restructuring processes of the particular types of membranes investigated, sometimes with slightly different time-courses during the first days after

transfer. Hazel and Landrey (1988a, 1988b) suggested that some of the changes that are observed in animals in course of acclimation are transient and exceed in magnitude the differences between fully acclimated individuals, idea supported by similar observations in crustaceans (Pruitt, 1988, Chapelle et al., 1979). Rapid activations of desaturases, correlated with increased membrane unsaturation have been also reported for microorganisms (Fujii and Fulco, 1977, Nozawa and Kasai, 1978). De Torrengo and Brenner (1976) did not find any correlation between the increase in desaturase activity in liver microsomes of fish *Pimelodus maculatus* after transfer to a colder temperature and the fatty acid composition of microsomal membranes, in fact they observed an increase in some saturated fatty acids (C16:0) and a decrease in polyunsaturated fatty acids (C20:5, C22:5 and C22:6).

Macartney, Maresca and Cossins (1994) have reviewed the evidence that supports the hypothesis that membrane adaptations are achieved via a rapid activation of the enzymes involved in the restructuring of the membrane lipid architecture, activation triggered by the direct effect of an altered temperature on membrane biophysical state, ideas which have been discussed in earlier papers (Cossins, 1983, Cossins and Raynard, 1987, Maresca and Cossins, 1993). Strong evidence for this hypothesis comes from the papers of Vigh et al. (1993), Los et al. (1993), Joo et al. (1991) (reviewed by Maresca and Cossins, 1993) who demonstrated that a reduction in membrane fluidity either via cooling or via *in situ* catalytic hydrogenation of unsaturated membrane lipids stimulated the transcription of the gene for the desaturase Δ^{12} in a strain of the blue green alga *Synechocystis*, and also that cooling and hydrogenation had additive effects. Given the complexity of the lipid-protein systems that constitute the cell membranes, a variety of restructurings can be imagined, whose significance is not yet understood and which may not be so easily detectable with the techniques available at present.

Another intriguing aspect of our results, apart from the lack of correlation between the changes in membrane fluidity and the changes in fatty acid composition, was why acclimation to 15°C seemed to involve different overall changes in

membrane lipid composition and biophysical state than acclimation to 22°C. We can only speculate that for acclimating to 15°C only some changes in the fatty acid composition are required, while acclimation to 22°C involves different mechanisms, possibly modulation of the interactions between phospholipids and cholesterol, or changes in the relative proportions or molecular species of PC and PE, due to the different level of thermal stress that this temperature imposes on the species studied.

Chapelle *et al.* (1977, 1979) have demonstrated that in marine crabs the rates of phospholipid biosynthesis strongly depend on environmental temperature, with PC biosynthesis being less sensitive to warmer temperatures than that of PE, while the rate of PE biosynthesis is less sensitive to cold temperatures. Similar aspects have been observed in crayfish (Pruitt, 1988) and fish (Hazel, 1990).

Hazel and Prosser (1979) showed that the ratio between the rates of incorporation of acetate in fatty acids (unsaturated and saturated) was actually higher in hepatocytes from warm-acclimated trout than in hepatocytes from cold-acclimated trout, each measured at their respective acclimation temperature. A study of the activity and temperature dependence of the activity and Km of enzymes involved in the restructuring of the membrane lipid composition from cold and warm-acclimated crabs might explain why the S/U of 22°C-acclimated crabs is not different from that of cold-acclimated crabs and is lower than that of 15°C-acclimated crabs.

Our results seem to indicate that the role of cholesterol in the regulation of plasma membrane fluidity in crustaceans during temperature acclimation or acclimatization is more important than it was usually considered (Pruitt, 1990) because of the inconsistency of the results reported. Cholesterol has also other roles in the seasonal cycles of lobsters and crabs, as it has been demonstrated that it is an important precursor in the formation of the moulting hormones (references listed in O'Hara *et al.*, 1985).

7.2. Seasonal Differences

A few interesting seasonal differences were observed, suggesting that the adaptations at plasma membrane level play an important role in seasonal acclimatization, but these are driven by a combination of factors, not by environmental temperature alone (Cossins and Bowler, 1987). The background of seasonal acclimatization appeared to influence the response of ectotherms to laboratory conditions.

The magnitude of the changes induced by temperature acclimation in membrane lipid composition depended on the season in which the experiment was carried out. The increase in the S/U ratio induced by warm-acclimation to 15°C was significant only in winter and spring, while in autumn we did not find any major differences in the fatty acid composition of cold and warm-acclimated crabs. In the stenothermal species, *Cancer pagurus*, the increase observed in winter in the S/U ratio was of a greater magnitude than that observed in spring.

The efficacy of the homeoviscous response at DPH level also differed from one season to another (in *Carcinus maenas* greater response in spring, in *Cancer pagurus* slightly greater response in autumn).

The fatty acid composition of membrane lipids varied on a seasonal basis. Crabs acclimated in spring had more saturated fatty acids and less polyunsaturated fatty acids in their plasma membrane PC and PE than crabs acclimated in autumn, these being differences observed after the laboratory acclimation period. The fatty acid composition of crabs acclimated in winter was either intermediary between autumn and spring, or closer to that of spring crabs. The seasonal differences in the Ch/PL molar ratios were not so marked but followed the trend of being lower in the colder seasons, and to increase more with warm-acclimation in a cold season.

These differences in lipid composition between crabs acclimated at the same temperature, but in different seasons, were not paralleled by significant differences in plasma membrane fluidity. However, an interesting observation was that the rate of

change in membrane fluidity with temperature was significantly lower in the membrane preparations from summer crabs. Therefore, at measurement temperatures above 15-20°C the plasma membranes from summer crabs were more ordered than those from spring and autumn crabs, suggesting the existence of a seasonal adaptation that would enable crabs to cope with rapid increases in temperature.

The analysis of seasonal variation was limited by a few facts: no fluidity measurements were carried out in the winter experiment (beginning of 1993), because the membrane preparations were used entirely for lipid extraction, in an attempt to quantify the phospholipid classes; no reliable fatty acid composition data are available for the summer experiment (1992); and only two acclimation temperatures were used (5°C and 15°C) in the summer '92 and winter '92-'93 experiments.

Other authors have reported correlations between seasonal differences in the lipid composition of various species of crustaceans and their thermal tolerance (Cook and Gabbott, 1972) or a lack of correlation between the S/U index of membrane phospholipids and membrane fluidity (Farkas, Storebakken and Bhosle, 1988).

Seasonal changes have been observed in the morphology and some functional parameters of motor terminals of both vertebrates and invertebrates (Lnenicka, 1993, Lnenicka and Zhao, 1991) which have been related to the different levels of activity in each season.

7.3. Correlations between Changes in the Temperature Dependence of Neuromuscular Function and in Muscle Plasma Membrane Lipid Composition and Fluidity

Most processes involved in neuromuscular transmission are membrane dependent. Based on the results presented in Chapters 3 and 4, only two acclimation temperatures (8°C and 22°C) were selected for the experiments described in Chapter 5, as crabs acclimated to 8°C and 22°C, respectively, displayed a significant difference between the fluidities of their plasma membranes.

The conclusion of the results presented in Chapter 5 was that temperature acclimation altered the temperature dependence of the parameters of neuromuscular function measured in a compensatory manner.

The compensation observed in the temperature-dependence of axonal conduction velocity was approximately 38% in *Carcinus maenas*, whereas *Cancer pagurus* displayed an inverse acclimation response (%AR = -23%), due to slightly higher average conduction velocity in axons of warm acclimated crabs than in axons of cold-acclimated crabs at warm temperatures. An intriguing fact was that conduction was blocked at lower temperatures in axons of warm-acclimated crabs than in those of cold-acclimated crabs. These results may reflect only a lower resistance to *in vitro* conditions, rather than a greater thermal sensitivity of axons from warm-acclimated crabs.

The extent of the compensation of muscle membrane resting potential after three weeks of warm-acclimation was different in the two species: nearly perfect compensation in *Cancer pagurus* and nearly no compensation in *Carcinus maenas*. The initial effect of the transfer to warmer temperatures (time-course experiment) was also different suggesting that in *Cancer pagurus* the mechanisms involved in the maintenance of the membrane resting potential are poorly functioning, until they adjust to the warmer temperature, while in *Carcinus maenas* only the thermodynamic effect is observed. Gladwell, Bowler and Duncan (1975) showed that in crayfish the muscle fibre membranes were gradually depolarized during exposure to lethal high temperatures. This resulted from an increase in the passive permeability of the sarcolemma, combined with the progressive thermal inactivation of the Na^+-K^+-activated ATPase which maintains the resting potential (Gladwell, Bowler and Duncan, 1975). The thermostability of the cation pumps and of the mechanisms that control the passive permeability of the muscle membrane of the eurythermal *Carcinus maenas* is presumably greater than in *Cancer pagurus* and they can function at warmer temperatures without major adjustments.

The temperature ranges of maximum excitatory junction potentials amplitudes were adaptively shifted towards warmer temperatures, from approximately 8-12°C for cold-acclimated crabs, to 18-22°C for warm-acclimated *Cancer pagurus* and to 14-16°C for warm-acclimated *Carcinus maenas*. The temperature ranges of maximum amplitude were broader for the facilitated EJP (A2), due to parallel increases in facilitation at the extreme cold and warm temperatures. Re-acclimation to cold of warm-acclimated *Carcinus* restored the typical profiles for cold-acclimated crabs.

Our findings were not in good agreement with the conclusions of Stephens (1990) that crustaceans have optimal neuromuscular function at their acclimation temperature, and are able to achieve a certain independence of temperature by increasing facilitation at temperatures above and below the acclimation temperature. They seemed to be more similar to those reported by Harri and Florey (1979) for excitatory junction potentials induced by the stimulation of the slow axon to the crayfish closer muscle, which had maximum amplitudes at temperatures lower than the warm-acclimation temperature and maximum facilitation over a broader range around the acclimation temperature.

Warm-acclimated *Carcinus* appeared to have optimal neuromuscular function at temperatures around 15-16°C, equivalent to approximately 50% compensation, whereas warm-acclimated *Cancer* displayed an overall poor neuromuscular function after three weeks at 22°C. Although the EJP amplitudes were slightly greater at warm temperatures than at cold ones, we could not describe this as optimal function.

To what extent these compensatory changes in the effects of temperature on neuromuscular function can be related to the changes at plasma membrane level presented in Chapters 3 and 4 and discussed above, is again a controversial matter. The fact that different types of membranes of fish, lizards and mammals which hibernate, the giant median axon of crayfish and the peripheral nerves of garfish, cockroach and earthworms exhibit homeoviscous compensatory changes in bilayer fluidity or membrane fatty acid composition following temperature acclimation (Macdonald, 1990) suggested that these changes may also play an important role in

the adaptation to temperature of neural function. Observations of parallel changes in either lipid composition or membrane fluidity and in parameters of neuromuscular function have been reported, but an unequivocal causal link has not been established. One example is the work of Cossins, Friedlander and Prosser (1977) who reported changes in behaviour heat resistance in goldfish following cold or warm acclimation, correlated in direction and overall time-course with changes in the fluidity and lipid composition of brain synaptosomes. Macdonald (1990, 1988) concluded in his reviews that bilayer fluidity, hence homeoviscous adaptation of membranes, have only a little influence on the cable properties of the cells, and also on the electrophysiology and biochemistry of the Na+ and other electrically-gated ion channels, and there is no direct evidence that post-synaptic processes and the functioning of the Ach-R channel in particular are influenced by the homeoviscous adaptation of membrane lipids. The bilayer fluidity may affect indirectly the channel kinetics and turnover of membrane constituents (Macdonald, 1990).

These conclusions are not too surprising if one takes into account the complexity of the relationships that may exist between temperature-induced changes in membrane lipids, membrane physical state and the function of membrane protein complexes involved in membrane excitability, such as ion channels and ion pumps (Cossins and Raynard, 1987), and the direct influence of temperature upon the function of these ion channels and pumps (Hochachka, 1988).

For example, Harper *et al.* (1990) observed a great extent of compensation in the conduction velocity of the vagus nerve of carp, following temperature acclimation, combined with a small compensation in the fluidity of brain membrane fractions, but with no differences in the fatty acid compositions of brain phospholipids extracted from cold and warm-acclimated animals. Macdonald (1994) suggested as an explanation for the compensation in conduction velocity the findings of Hodgkin (1975), who showed that the temperature coefficient of the dielectric is negative for saturated lipids, and positive for unsaturated lipids, hence the degree of saturation will

affect membrane capacitance (Cm). However, other factors must be involved since Cm increases much less per °C than predicted in this way (Macdonald, 1994).

Kivivuori, Laine and Lagerspetz (1984) found that temperature acclimation induced a shift of approximately 4°C in the break point of the temperature dependence curves of the resting membrane potential of crayfish medial giant axon. This shift was paralleled by an approximately 4°C shift in the fluorescence polarization curves of the same membranes. This supported the view of Romey, Chicheportiche and Lazdunski (1980) that the breaks in the temperature dependences of electrical functions of nerve cells at mid-temperatures are due to membrane lipid phase transitions, which would also alter the membrane fluidity. This was demonstrated by Connoly et al. (1985a, 1985b) who showed that in the protozoan Tetrahymena pyriformis the membrane resting potential displayed changes in the slope of the Arrhenius plots, which occurred at temperatures very close to the growth temperature and to those of the phase transitions in the membrane lipid bilayer. However, in crustacean axonal or muscle membranes, the relationship between the temperatures at which the break points in the resting membrane potential occurred and the acclimation temperatures was not clear and consistent (Stephens and Atwood, 1982, Stephens, 1985a, 1985b, Stephens, 1988). Our results did not even show any break points, neither in the temperature dependence of the resting membrane potential, nor that of membrane fluidity.

Attempts to modify the membrane potential using other fluidizing agents, such as arachidonic acid, in cells which displayed homeoviscous adaptation as a response to those agents, were ineffectual (Saum, McGee and Love, 1981, Takenaka et al., 1986). On the other hand, cholesterol enriched axons showed an action potential with a slower time-course and a lower amplitude, effects similar to those of cooling or increased pressure (Redmann et al., 1979).

7.4. Correlations between Changes in the Temperature Dependence of Neuromuscular Function, the Whole Animal Thermal Tolerance and the Muscle Plasma Membrane Lipid Composition and Fluidity

The results presented in Chapter 5 also showed that, although both species had the ability to develop compensatory responses, the neuromuscular function of *Cancer pagurus* was more thermally sensitive than that of *Carcinus maenas*. This correlated well with the conclusion of Chapter 6, that the stenothermal species, *Cancer pagurus*, was more thermally sensitive (significantly lower CTMax, at all acclimation temperatures) than the eurythermal *Carcinus maenas*. The critical thermal maximum was chosen as an ecologically significant index of whole organism thermal tolerance, which would allow a comparison between the two species studied, as well as with other species.

The CTMax of both species was increased by warm-acclimation. The magnitude of the response (ΔCTMax/ΔAcclimation Temperature) was greater in *Cancer pagurus* (0.46) than in *Carcinus maenas* (0.23) suggesting a relationship between the magnitude of the response and the amount of stress the new temperature represented for each species. No seasonal difference was found in CTMax between summer and autumn.

The increase in CTMax following warm-acclimation can be related to the compensatory changes in neuromuscular function (Chapter 5) and to the compensation in membrane fluidity (Chapter 3) and in some aspects of lipid composition (Chapter 4). On the whole, the efficacies of these acclimatory responses ranged from approximately 20% to 60%.

The thermal sensitivity should decrease from whole organism level to isolated tissues and to cellular level (Lagerspetz, 1987) but in our case the values of the CTMax were higher than the temperatures of axonal conduction blockage and of marked decrease in the amplitudes of the EJPs. This can be explained based on the observation of Bowler, Gladwell and Duncan (1973) that the isolated muscle of 25°C

adapted crayfish was much more thermally sensitive than the whole animal, probably due to the absence, in the isolated preparation, of the homeostatic mechanisms which one supposes exist in the whole organism and delay the temperature effect. Furthermore, an isolated muscle would most probably not be supplied with oxygen nor have waste products removed as efficiently as in the whole animal (Bowler, Gladwell and Duncan, 1973).

Other authors showed that increased lipid unsaturation was associated with greater cold tolerance (Cook and Gabbott, 1972) or with decreased resistance to high lethal temperatures (Cossins, 1976). Also the lower heat tolerance of an Arctic amphipod (*Orchomene plebs*), compared to a Baltic isopod (*Saduria entomon*) was associated with greater neuronal and branchial membrane fluidity of the Arctic species, which lives at colder temperatures than the Baltic one (Lahdes, Kivivuori and Lehti-Koivunen, 1993, Kivivuori and Lagerspetz, 1990). The role played by the membrane physical state in thermal tolerance has been suggested by studies which showed that heat death in the crayfish *Astacus* was associated with a dramatic increase in the permeability of excitable membranes to cations (Bowler, Gladwell and Duncan, 1973, Gladwell, Bowler and Duncan, 1975) and by observations that in fish and planktonic crustaceans the ability to remain active during winter appeared to be related to the ability to modify the composition of cellular membrane lipids in an adaptive manner (Farkas, Nemecz and Csengeri, 1984, Pruitt, 1988, Farkas, 1979). Cossins, Friedlander and Prosser (1977) showed that in goldfish the reduction in heat resistance during cold acclimation was correlated in direction and overall time course with changes in the fluidity of synaptosomes (increased) and in the saturation of the membrane phospholipids (decreased). The heat resistance was assessed by monitoring the temperatures of hyperexcitability, loss of equilibrium and coma induction, which are behavioural features related to synaptic block.

However, a direct causal link can not be established. For example, Cossins (1976) found that altering the photoperiod from 'long summer day' to 'short winter

day' induced an increase in the unsaturation of the phospholipids of crayfish muscle microsomes, but no change in heat tolerance.

7.5. Comparison between the Stenothermal and Eurythermal Species

The same problem, of a lack of a direct causal link, appears if we try to correlate the difference in thermal sensitivity between the stenothermal and the eurythermal species with inter-specific differences in other parameters measured at cellular or functional level, in an attempt to find the weak link in the stenotherms. The greater thermal sensitivity of *Cancer pagurus* at whole organism level (Chapter 6) can be related to the observed poorer neuromuscular function in warm-acclimated *Cancer pagurus* (lower amplitudes of EJP and lower facilitation at warm temperatures, initial depolarization of muscle membranes after transfer to a warmer acclimation temperature) compared to warm-acclimated *Carcinus maenas* and the lower temperatures of axonal conduction blockage in *Cancer pagurus* (Chapter 5), to the lower extent of compensation in membrane fluidity following warm-acclimation and the greater fluidity of muscle plasma membranes (Chapter 3), and to the generally lower Ch/PL molar ratios (Chapter 4) compared to those of *Carcinus*, independent of the acclimation temperature.

The differences observed in the extent of compensation of the resting potential of muscle fibres (Chapter 5, the time-course experiment) and also in the amount of facilitation in warm-acclimated crabs suggested that there are differences between the adaptive strategies at membrane level of the stenothermal and the eurythermal species, or at least in the sequence and time-course of events. Although the actual differences found in membrane lipid composition and fluidity are not always consistent and their extent seems to depend on season and the absolute value of the acclimation temperature, they constitute evidence that some aspects of the membrane structure are different between stenothermal and eurythermal species, which may influence their

ability to adjust the function of membrane protein complexes when subjected to temperature fluctuations.

A comparison of a cold-water stenothermal (*Salvelinus alpinus*) and a eurythermal (*Rutilus rutilus*) species of fish (Schwarzbaum, Wieser and Niederstätter, 1991, Schwarzbaum, Wieser and Cossins, 1992) led to the conclusion that these two co-existing species of fish use different strategies of membrane adaptation to environmental temperature, with respect to kidney function. The eurythermal species responded to low-temperature acclimation predominantly by altering the properties of the (Na^+-K^+)-ATPase (ouabain-binding sites, specific activity, thermal stability), whereas the stenothermal species responded by altering bulk membrane fluidity and residual K^+ efflux.

Proteins from eurythermal species display stable values of Km over the range of temperature they experience in natural conditions, while proteins from stenothermal species show rapid increases in Km at temperatures outside a narrow range of temperature around their habitat temperature (Somero and Hochachka, 1976, Somero, 1995). The higher thermal sensitivity of membrane-bound enzymes, compared to cytoplasmic enzymes (Bowler and Manning, 1994) and the demonstrations of acclimatory shifts in the sensitivity of membrane-associated enzymes to thermal denaturation, and of significant correlations between membrane fluidity and the activity of several of these enzymes, particularly the Na^+-K^+- and Ca^{2+}-ATPases (Hazel and Williams, 1990) suggest that the biophysical state of the lipid bilayer and its regulation in case of alterations in the environmental parameters plays an important role in maintaining membrane function. However, the interactions between the lipid bilayer and the membrane-bound or associated proteins may affect the protein conformation, the flexibility required for the conformational changes involved in their functioning as enzymes, channels or receptors, and their rotational and lateral mobility in several ways, not only through the microviscosity of the lipid matrix. These include electrostatic interactions and surface potential at the lipid headgroup region, bilayer thickness, cross-sectional or interfacial area per lipid

molecule, lateral compressibility and elastic curvature (experimental evidence reviewed in Cossins, 1994).

Where the nature and direction of the response was similar, the significance of the few differences observed in the extent of the response to warm-acclimation between the two species was not straightforward. Some of them, such as the greater increase in the S/U ratio of membrane PC and PE in 15°C-acclimated *Cancer pagurus* in winter, the greater %HR of *Carcinus maenas* in spring, or the greater increase in CTMax of *Cancer pagurus* in summer or autumn, can be explained, again in a speculative manner, on the basis of the internal seasonal clock suggested by Roche and Pérès (1984), who said that the significance a certain externally imposed temperature will have for an animal depends on how this temperature is perceived relative to the range of temperatures seen as normal by that animal in the season when the experiment is carried out.

Another hypothesis is that the constant temperature to which crabs are subjected in laboratory conditions is normal to the stenothermal species and individuals will develop a response within their genetic limits, while for the eurythermal species it will be seen as an unnatural situation and it might reduce the individuals capacity to develop a response. The results of the time-course experiment (Chapter 5) could be interpreted as a decline in the neuromuscular performance of both species after the third week at 22°C and raised the question whether prolonged exposure to a constant high temperature may induce some thermal damage in the species studied or reduce their ability to cope with fluctuations in temperature.

Otto (1973) assessed the effect of acclimation to constant temperature in comparison with that of acclimation to various fluctuating regimes (in which he used lethal, supralethal and sublethal temperatures as peak temperatures, and also varied the time at the peak temperature) on the thermal tolerance of the mosquito fish *Gambusia affinis* and he found that only the cyclical regimes with sublethal exposures to lethal or supralethal peak temperatures produced tolerances comparable to those observed for animals in the field (Otto, 1974). In the field, he observed western

mosquito-fish in water at 42°C, which was approximately 4°C above their laboratory upper incipient lethal temperature of 38.3°C. This was consistent with other findings that acclimation to cyclical temperature regimes increased the rate and scope of thermal acclimation (Heath, 1963, Hutchison and Ferrance, 1970, Feldmeth, Stone and Brown, 1974). Maness and Hutchison (1980) demonstrated the involvement of heat hardening (defined as a transient increase in thermal tolerance induced by a brief exposure to a high sublethal temperature) in the temperature adaptation of species from habitats with extreme fluctuations in diurnal temperatures. These may utilize heat hardening to survive lethal environmental temperatures and expand their thermal range of activity. The transient increases in thermal tolerance are usually associated with synthesis of heat shock proteins (Lagerspetz, Anneli-Korhonen and Tiiska, 1995).

Acclimation to either constant, diurnally cycling or randomly fluctuating temperature had different effects on goldfish erythrocytes (Houston and Schrapp, 1994, Houston and Gingras-Bedard, 1994). The fluctuating regime, which resembled the natural circumstances, was associated with increased numbers of juvenile and developing cells, with higher potential for reorganization and decreased numbers of mature cells.

Dahlhoff and Somero (1993) demonstrated in abalone the existence of a relationship between acclimatory ability and the biogeographical distribution of congeneric species.

7.6. Concluding Remarks

We consider that the work presented in this thesis brings original and useful information in an area which has been little investigated. There are only few attempts of multidisciplinary approaches on the same experimental system, trying to relate responses at different levels of organization.

Only few studies refer to stenothermal species, mostly Antarctic ones (Somero, 1991, Johnston and Altringham, 1988, Lahdes, 1995). Even fewer papers have compared the effects of thermal acclimation in stenothermal and eurythermal species of vertebrates (Schwarzbaum, Wieser and Niederstätter, 1991, Schwarzbaum, Wieser and Cossins, 1992) or invertebrates (Dahlhoff and Somero, 1993, Blundon, 1989).

At the organization level scale, the organism level is the upper level of interest for physiologists and the first level of interest for ecologists. The intersection of these interests, which integrates the information from lower organization levels for ecological correlations, became the object of study of new branches in biosciences called physiological ecology, or molecular ecology. These have gained more and more grounds and importance over the last two decades and the number of papers which investigate aspects of thermal biology at more than one level of organization in the same groups of animals has increased (Bennett, 1987).

More information on the abilities of species to acclimate to altered conditions of temperature and on the mechanisms involved in the acclimation process could improve the models used to predict the effects of global warming and of various forms of thermal pollution of fresh and marine waters on animals with different thermal strategies and on whole ecosystems. The assessment of the seasonal variations in the ability of ectothermic animals to respond to changes in temperature is also of importance, especially for predicting the impact of thermal pollution, which may exceed the limits of tolerance of some species in seasons with more extreme temperatures.

Further work could include completing the seasonal study of thermal tolerance and of lipid composition and fluidity of plasma membranes. Simultaneous measurements of all the cellular, functional and whole organism parameters in crabs in course of acclimation, maybe combined also with measurements of the activities and temperature dependences of Km of the enzymes involved in the restructuring of lipid

bilayer architecture, could provide evidence for causal relationships between changes in these parameters, as pointed out in a recent review by Cossins (1994), but would require a larger number of animals and more than one researcher.

Measurements on crabs taken directly from their natural environment in different seasons will be of use for the understanding of the cellular modifications involved in seasonal acclimatization and of the influence this has upon the response to laboratory acclimation at constant temperature.

A more detailed investigation of the temperature dependence of neuromuscular function in crabs acclimated to cold and warm temperatures has already been carried out in our research group on the same combination of species (Pearson, unpublished observations), in parallel with heterothermal acclimation experiments, when half of the body is maintained at cold temperature and the other half at warm temperatures, with the central nervous system maintained at either cold or warm temperature. These experiments will provide information about the role of CNS in the developing of acclimatory responses in peripheral tissues and assess the extent of cellular adaptation versus systemic adaptation.

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