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Influence of season and temperature on the metabolic costs of survival in the intertidal isopod, Ligia oceanica

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Influence of Season and Temperature on the Metabolic Costs of Survival in the Intertidal Isopod, *Ligia oceanica*

A thesis submitted to the University of Wales, Bangor

by

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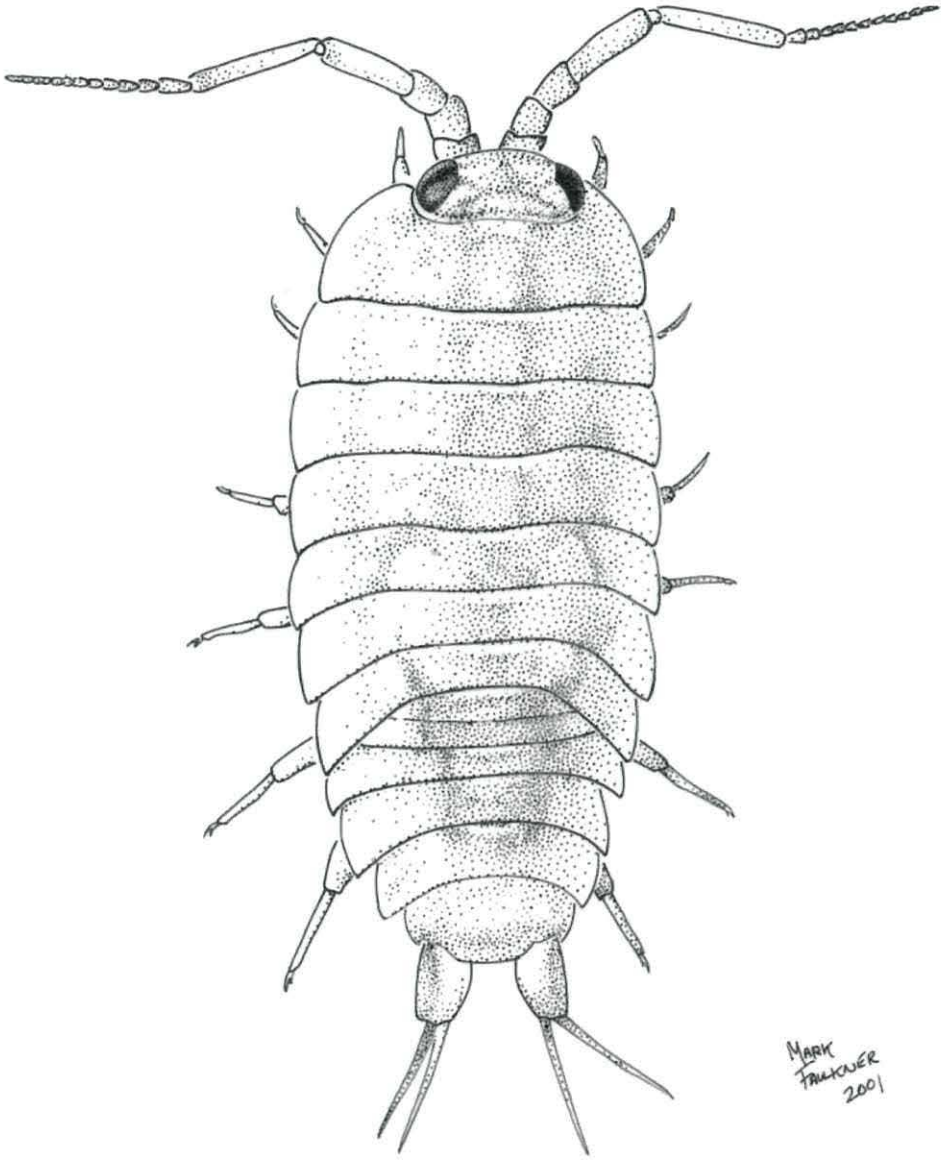

Ligia oceanica (Linnaeus, 1767)

Synopsis

The intertidal isopod, *Ligia oceanica*, inhabits humid micro-habitats at the high water mark of rocky shores, where it experiences elevated temperatures in the summer due to solar radiation. Temperature tolerances vary seasonally with critical maximum temperatures falling from 37.4°C in the summer, to 34.9°C in the winter.

In general, whole-animal rates of oxygen uptake and protein synthesis were higher in winter compared with summer animals. In both seasons, rates of oxygen uptake were more sensitive to temperature change than rates of protein synthesis, due to a compensatory increase in RNA levels in the winter. Patterns of heat-shock protein induction varied between seasons, with acclimatised *Ligia* in the summer showing higher constitutive and *de novo* heat-shock protein synthesis levels than winter *Ligia*. In the summer, animals synthesised heat-shock protein 60 at two induction temperatures of 27°C and 31°C. Animals collected in the winter, however, synthesised hsp60 at the lower induction temperature of 25°C, and continued to express hsp60 at 27 and 29°C. Acclimated *Ligia* showed delayed heat-shock protein induction and an overall attenuated heat-shock response relative to acclimatised animals, regardless of season.

Estimates of the metabolic costs of general protein synthesis revealed that in the winter, costs were relatively expensive and accounted for 70.0% of oxygen uptake rates at 40.9 μmol O_2.mg protein^{-1}. In the summer, metabolic costs were 10-times lower at 3.9 μmol O_2.mg protein^{-1}, accounting for 17.3% of oxygen uptake. Temperature acclimation had no effect on metabolic costs, but winter values, expressed as the proportion of oxygen uptake, showed remarkable similarity to those found in an Antarctic isopod. Metabolic costs of heat shock protein synthesis were 2.4-times higher in the winter than in the summer, showing that energy requirements for survival are higher at a time when energy stores may be restricted.

Contents

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Abbreviations

[^{3}H] Phe	[^{3}H] phenylalanine
β-PEA	β-phenylethylamine
A_s	absolute rate of protein synthesis
A/T	acclimation temperature
ATP	adenosine-triphosphate
BSA	bovine serum albumin
CHX	cycloheximide
CTMax	critical thermal maxima
DNA	deoxyribonucleic acid
GH	growth hormone
Hsc(s)	heat-shock cognate(s)
Hsp(s)	heat-shock protein(s)
kDa	kilo Dalton
k_{RNA}	rate of ribosomal activity
k_s	fractional rate of protein synthesis
$\dot{M}\,O_2$	rate of oxygen uptake
mRNA	messenger ribonucleic acid
PAGE	polyacrylamide gel electrophoresis
PCA	perchloric acid
Phe	phenylalanine
PMSF	phenylmethylsulfonylfluoride
Q_{10}	temperature coefficient

RH	relative humidity
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
R/T	rate/temperature
S_a	specific radioactivity of the free-pool phenylalanine
S_b	specific radioactivity of the protein-bound phenylalanine
SDA	specific dynamic action
SDS	sodium dodecylsulfate
SE	standard error
SMR	standard metabolic rate
tRNA	transfer ribonucleic acid

Chapter One

General Introduction

1.0 *Introduction*

Temperature relationships of organisms are one of the most studied areas in animal physiology (Somero, 1995). The physically pervasive nature of temperature means that it affects animals at all levels of biological organisation, unlike other environmental factors such as light, which exerts an influence at just one organisational level (Lagerspetz, 1987). This is especially true of ectothermic organisms which are of particular interest, as they are essentially thermoconformers and have a somewhat limited ability to alter their body temperature from that of the environment. The gradual move from studying whole-animal physiology to concentrating on biochemical and molecular physiology has helped to elucidate the fundamental mechanisms behind thermal sensitivity, thermal optima and thermal tolerance of whole-animals (Lagerspetz, 1987; Somero, 1995). Studies on whole-animals are still relevant, however, as it is the ability of the organism as an integral system to cope with or tolerate environmental temperature variation, and not as isolated cell cultures, which ultimately dictate its survival and the limits of its ecological distribution.

1.1 *Biological Effects of Environmental Temperature Variation*

Changes in body temperature have profound effects on many biological rate processes. If body temperature moves beyond the normal scope of temperature tolerance for an organism, then major metabolic disruption or death can result (Huey and Bennett, 1990). Within the zone of temperature

tolerance, biological rate processes usually accelerate in a regular manner with an increase in temperature towards the upper limits of thermal tolerance (see page 7), where the rate of increase begins to plateau. Further temperature increases result in a decline in the rate process, and eventually death (Bernaerts *et al.*, 1987). Death can either be as a result of the thermal denaturation of proteins at high temperatures, with a subsequent loss of enzyme activity, and in the case of terrestrial ectotherms, possibly dehydration (Dahlhoff and Somero, 1993; Begon *et al.*, 1996). In the case of cold stenothermic animals, death can be as a result of tissue oxygen demand outstripping the mechanisms of oxygen supply, as seen in the spider crab, *Maja squinado* (Frederich and Pörtner, 2000), and the Antarctic bivalve, *Laternula elliptica* (Peck *et al.*, 2002). The temperature optima for ectotherms is usually just a few degrees below the temperature where thermal death would occur (Huey and Bennett, 1990; Begon *et al.*, 1996), and this is the temperature at which the rate process is at its most efficient (Huey and Bennett, 1990; Crossin *et al.*, 1998). Small temperature changes around the thermal optima tend to make little difference to metabolism and certain indicators of health, such as growth, are also optimal, at least in larval cyprinids (Wieser and Forstner, 1986), terrestrial isopods (Wieser, 1972b) and the American lobster, *Homarus americanus* (Crossin *et al.*, 1998).

Organisms can respond to thermal stress in one of two ways: either through thermoregulation, or by making adjustments to their thermal sensitivity (Huey and Bennett, 1990). Thermoregulation is largely restricted to endotherms (mammals and birds) and offers a degree of independence from

environmental temperature change, although a number of ectotherms are able to thermoregulate by altering behaviour, for example: by being nocturnal, by retreating to sheltered microhabitats, by lowering body temperature through evaporative cooling and through colour change (Edney, 1953; Edney, 1961; Kivivuori, 1994; Crossin *et al.*, 1998; Cerdá and Retana, 2000). The main drawback of endothermy is that a large proportion of the overall metabolic costs are used for the optimal regulation of body temperature (Cossins and Bowler, 1987). In ectotherms, adjustments to temperature change depend on the time-scale of the thermal change, which can either be short-term, for example diel temperature fluctuations, or long-term, which operate on seasonal or evolutionary time-scales. In the present study, consideration will be given to both short- and long-term adjustments, with seasonal differences used as a means of assessing long-term change.

The mechanisms responsible for making adjustments to thermal sensitivity are dependent on the extent of the stress, which in turn is often related to the time-scale of the stressor. Environmental temperature fluctuations which occur within the normal thermal limits of an organism are dealt with by capacity adaptations (Prosser, 1986). Capacity adaptations usually occur over longer time periods and are dependent on relatively large physiological alterations. Tolerance to extreme and rapid temperature change on the other hand requires resistance adaptations (Prosser, 1986), and resistance adaptations are seen as a 'last-ditch' attempt at remaining viable during transient periods of extreme temperature stress. Organisms which are faced with large diel temperature fluctuations, as in the intertidal zone, cannot

effectively alter physiological parameters in such a short time-scale. Additionally, it is also likely that relatively large physiological adjustments carry certain metabolic costs. If environmental temperatures go beyond the effective range for existing capacity adaptations, then in order to remain viable, the organism must rely on transient resistance adaptations. The later scenario is of most relevance to small terrestrial ectotherms such as invertebrates.

Thermal acclimation serves to compensate, to some extent, for changes in environmental temperature (Feder, 1978). Studies involving the acclimation of animals to constant temperatures are valuable for verifying the presence of acclimatory capacities in ectotherms and for the investigation of any subsequent physiological and biochemical mechanisms behind thermal acclimation (Feder, 1985). The process of acclimatisation is similar to that of acclimation in that rate processes can be maintained at a similar rate at varying temperatures, but acclimatisation occurs naturally through seasonal change and involves the variation of more than one physical variable, for example, photoperiod. The processes of acclimation and acclimatisation affect lethal or critical temperatures in every species that has been studied (Brattstrom, 1979), although temperate species show much stronger responses than those from relatively constant temperature environments such as tropical and polar regimes (Vernberg, 1959; Feder, 1978; Kivivuori and Lagerspetz, 1990; Weinstein and Somero, 1998; van Dijk *et al.*, 1999). The attenuated acclimatory response in tropical and polar organisms probably reflects their evolutionary adaptation to a lack of climatic variation. Changes in tolerance limits with acclimation temperature are associated with changes in

rate function, although the timing and magnitude of these relationships usually differ, indicating the mechanisms behind acclimation are not due to a single process (Edney, 1964b). Indeed, there appear to be three main molecular mechanisms which underlie thermal acclimation in animals: the synthesis of alternate isoenzymes, quantitative differences in the levels of existing enzymes, and alterations to membrane lipids and fatty acids (Somero and Hochachka, 1976; Feder, 1983; Clarke, 1987; Guderley, 1990). Seasonal changes in thermal sensitivities are a result of changes in capacity adaptations whereby adjustments are made to the intracellular environment, by the alteration of enzyme concentrations and through the preferential switching to different enzyme variants. For example, the respiratory enzyme, cytochrome c oxidase, increases in concentration in the white muscle fibres of teleosts to compensate for the reduction in activity due to the effects of low temperature (Johnston *et al.*, 1975; Sidell, 1977; Clarke, 1987; Foster *et al.*, 1993b; Weinstein and Somero, 1998). Homeoviscous adaptation, a term coined by Sinensky (1974), is another typical example of a capacity adaptation. It is used to describe the adaptations to the physical properties of plasma membranes in order to maintain their functional and structural integrity at different environmental temperatures. A reduction in temperature results in the increased incorporation of unsaturated fatty acids into phospholipids, thereby increasing membrane fluidity (Sinensky, 1974; Cossins, 1983; Buda *et al.*, 1994; Hazel, 1995; Cuculescu *et al.*, 1999; Ivanov *et al.*, 1999). The purpose of this remodelling is to maintain a relatively constant state for the membrane lipids over a range of temperatures (Guderley, 1990; Cuculescu *et al.*, 1999). This property is of particular importance to neural tissues which are

heavily reliant on membrane processes for normal function as temperature change has been shown to alter the velocity of nervous conduction (Buda *et al.*, 1994). These changes take time to become apparent and may be metabolically expensive to elicit, and as a result, are restricted to relatively long-term time periods such as weeks or months.

1.1.1 *Metabolic Rate*

One of the most studied areas of physiology is the effect of ambient temperature on rates of metabolism (Bernaerts *et al.*, 1987). The metabolic rate, specifically the standard metabolic rate (SMR), is defined as the sum of all the metabolic activities needed to keep an organism alive (basal metabolism), in addition to the instantaneous contributions from any growth or gametogenesis taking place during the period of the experiment under physiologically steady-state conditions and in the absence of food (Bayne *et al.*, 1976). A convenient means of quantifying the effects of temperature on the SMR is through the measurement of oxygen uptake rates, as adenosine-triphosphate (ATP), which fuels most cellular activities, is generated from the tricarboxylic, or citric acid cycle, which requires oxygen. Consequently, measurements of oxygen uptake can give good indirect estimations of metabolic rate (Clarke, 1987). Whole-animal rates of oxygen uptake however, can be extremely variable between individuals due to differences in reproductive state, life-cycle stage, sex, body mass, nutritional status, *etc.* (Aldrich, 1975; Alcarez and Sardá, 1981; Carefoot, 1987; Clarke, 1987; Carefoot, 1990b; Houlihan *et al.*, 1990b; Johnston, 1993). In addition, variability is introduced by the use of different measurement techniques and

by allowing the animals to settle for various lengths of time to the experimental conditions, making comparisons between studies difficult. For example, previous studies have indicated that for the measurement of resting metabolic rate, ectotherms, such as crustaceans, should be allowed at least 12 hours to settle after handling and transportation (Smit *et al.*, 1971; Ivleva, 1973; Aldrich, 1975; Ralph and Maxwell, 1977). Studies also indicate that animals require between 5 and 48 hours to settle in the experimental apparatus (Halcrow and Boyd, 1967; Taylor and Whiteley, 1987; Saint-Paul *et al.*, 1988; Al-Wassia *et al.*, 1989; Chappelle and Peck, 1995; Marsden, 1999).

Metabolic rate usually increases in a predictable manner as temperature increases. A doubling or trebling of the SMR with a 10°C temperature increase is usual in a variety of ectothermic taxa, representing the normal rate of change for thermochemical reactions (Hoar, 1975). The increase in the velocity of a reaction with a 10°C rise in temperature is commonly known as the Q_{10} or van't Hoff coefficient, and can be calculated using the following equation:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2 - T_1}}$$

Where R_1 and R_2 are the velocity constants at the two temperatures T_1 and T_2.

Metabolic adjustments to temperature change in ectotherms include immediate and long-term changes. In general, Q_{10} values are higher at lower temperature ranges in temperate ectotherms, while values higher than three

indicate a higher sensitivity to temperature change. The initial period of temperature change often involves an overshoot in oxygen uptake rates followed by a period of stabilisation taking place over a period lasting from hours to days (Ivleva, 1973; Precht *et al.*, 1973). Typically, when the temperature is increased, there is an immediate increase in rates of oxygen uptake followed by a period of decline, as found in Arctic charr, *Salvelinus alpinus* (Lyytikäinen and Jobling, 1998). Although the rate of oxygen uptake declines after the period of stabilisation, it remains at a higher rate than at the initial temperature. The reverse is also true. On transferral to a lower temperature, rates of oxygen uptake show an initial decrease before increasing to an intermediate but stable value which is still less than the rate before transferral (Dunlap, 1980; Feder, 1985; Cossins and Bowler, 1987). Consequently, periods of acclimation allow for the adjustment and stabilisation of many physiological and biochemical processes.

Moreover, the true adaptive value of acclimation is to extend the physiological temperature range of a population either upwards or downwards, depending on the season (Edney, 1964a), and not to merely metabolically compensate for a fall or rise in temperature. For instance, at higher acclimation temperatures (21°C), reduced metabolic rates are thought to moderate energy expenditure in warm seasons (Feder, 1985). The reduced acclimatory response to relatively cool temperatures (5°C) also reflects the need to conserve energy expenditure (Feder, 1985). The corollary of this is that metabolic compensation in cold environments would be disadvantageous as a high metabolic rate in a cold temperature environment would be energetically

wasteful (Clarke, 1987). Q_{10} values of less than 2 indicate the possibility of homeostatic mechanisms in operation, compensating for changes in temperature (Precht, 1958). Q_{10} values close to one may indicate acclimation, enzyme inactivation, or a number of other factors (Feder, 1976). In contrast, high Q_{10} values are usually indicative of inadequate acclimation periods (Ivleva, 1973).

Mitochondrial activities comprise a significant proportion of oxygen uptake (Peck, 2002). Intraspecific studies by Sommer and Pörtner (1999) investigating changes in mitochondrial function in a subpolar and boreal population of the eurythermal polychaete, *Arenicola marina*, revealed that mitochondrial activity, mitochondrial densities, resting metabolic rates and the subsequent metabolic costs associated with mitochondrial activity were all higher in the population from the higher latitude. A similar situation has been shown to occur due to changes in season and during low-temperature acclimation in the eurythermal teleosts, *Oncorhynchus mykiss* and *Myoxocephalus scorpius*. In both species, low-temperature specific modifications to mitochondrial processes included increases in the oxidative capacities of mitochondria compared to fish at warmer temperatures (Guderley, 1998). Consequently, eurythermal ectotherms appear to compensate at the level of the mitochondria for changes in temperature, and therefore influence both oxidative metabolism and aerobic scope. Such a compensatory mechanism is believed to enhance metabolic costs in ectotherms living at low temperatures (Pörtner *et al.*, 1999).

1.1.2 *Growth and Protein Synthesis Rates*

Much of our understanding of the relationship between temperature, growth and protein synthesis has been gained from studies on eurythermal teleosts (Fauconneau and Arnal, 1985; Houlihan *et al.*, 1988; Houlihan *et al.*, 1992; Lyndon *et al.*, 1992; Pannevis and Houlihan, 1992; Foster *et al.*, 1993a; Houlihan *et al.*, 1995; Carter *et al.*, 1998). The general understanding of the relationship between protein metabolism and temperature has now been extended to invertebrates including studies on the cephalopod, *Octopus vulgaris* (Houlihan *et al.*, 1990a), the bivalve mollusc, *Mytilus edulis* (Hawkins *et al.*, 1986; Hawkins *et al.*, 1989) and several crustacean species (Carefoot, 1973b; Houlihan *et al.*, 1990b; Whiteley *et al.*, 1996; El Haj and Whiteley, 1997).

Protein turnover includes both protein synthesis and protein degradation (Waterlow *et al.*, 1978) and is dependent on both nutritional status and the stability of the protein at the temperature it is adapted to operate at (Clarke, 1987). For example, net growth (protein accretion) requires rates of protein synthesis to exceed rates of protein degradation, whereas during protein loss, for example during starvation, degradation exceeds synthesis (Sugden and Fuller, 1991; Houlihan *et al.*, 1993a). Under maintenance conditions, rates of protein synthesis will be equivalent to rates of degradation, with no net gain or loss of body protein. Protein degradation rates are difficult to measure, but in fish, protein synthesis rates can be measured and used as a determinant of growth as a close relationship exists between these two variables (Houlihan *et al.*, 1988; Houlihan, 1991; Foster *et al.*, 1993a; Houlihan *et al.*, 1995;

McCarthy *et al.*, 1999). Cold adapted proteins tend to be more sensitive to temperature change as their molecular structure is more unstable than proteins from temperate and tropical organisms when measured at common temperatures, although this is probably not the case when compared at their normal operating temperatures (Johnston *et al.*, 1975; Clarke, 1987; Weinstein and Somero, 1998). Consequently, rates of protein turnover in an organism exposed to an acute temperature increase will increase above and beyond rates of protein turnover in the same organism which has been acclimated or acclimatised to the same temperature. The importance of protein synthesis in capacity adaptation processes such as temperature acclimation has been demonstrated on studies in fish. For example, starvation results in a net loss of protein in the carp, *Cyprinus carpio*, as described above, and a subsequent inability to acclimate to new temperatures (Gerlach *et al.*, 1990), while treatment with a protein synthesis inhibitor interferes with the ability to adjust heat resistance in the teleost, *Rhodeus amarus* (Künnemann, 1973).

Many estimates of rates of protein synthesis in fish and other animals such as molluscs and crustaceans have been made by measuring the *in vivo* incorporation of a radiolabelled essential amino acid in the free- and protein-bound fractions using the flooding dose technique of Garlick *et al.* (1980). This method avoids any problems associated with variable free-pool radioactivity and amino acid recycling. Typically, rates of protein synthesis are presented fractionally, *i.e.* as a proportion of the total protein content per day. Protein synthetic rates of individual tissues can vary enormously, with the highest

rates of protein synthesis in various teleosts (Haschemeyer and Smith, 1979; Pocrnjic *et al.*, 1983; Fauconneau, 1985; Fauconneau and Arnal, 1985; Houlihan *et al.*, 1988), the shore crab, *Carcinus maenas* (Houlihan *et al.*, 1990b) and the mollusc, *Octopus vulgaris* (Houlihan *et al.*, 1990a) being found in the gills, whereas lower rates of synthesis are found in muscle, especially the white muscle fibres of fish. However, the reasons for these variations in fractional rates of protein synthesis between different tissue types are unclear, although the slow, glycolytic muscle fibres of fish are poorly perfused and could account for poor circulation of the radiolabel (Houlihan *et al.*, 1988). The highest rates of whole-body protein synthesis are usually found in larval or juvenile animals where fractional rates of synthesis can reach 5% per day or even higher (Houlihan *et al.*, 1995), but in adult animals this figure is usually much lower, for example, between 0.37 and 1.15% per day in *Carcinus maenas* (El Haj and Houlihan, 1987). In eukaryotes, rates of protein synthesis are determined by two factors: the concentration of ribosomal RNA, which accounts for approximately 80% of the total cellular RNA (Green *et al.*, 1994) (termed the capacity for protein synthesis, or RNA:protein ratio) and the activity of the ribosomes (k_{RNA}) (Houlihan, 1991; Houlihan *et al.*, 1995). The capacity for protein synthesis increases in the whole-body with decreasing water temperature (Foster *et al.*, 1992; Mathers *et al.*, 1993; McCarthy and Houlihan, 1997; McCarthy *et al.*, 1999) and is thought to be a compensatory mechanism countering the effects of reduced k_{RNA} values at lower temperatures.

A large proportion of the SMR is thought to be taken up by the costs involved in protein synthesis and evidence for this comes from the strong correlation between oxygen uptake and protein synthesis rates in various teleosts, from both *in vitro* and *in vivo* studies (Houlihan *et al.*, 1990b; Houlihan, 1991; Houlihan *et al.*, 1993a; Houlihan *et al.*, 1995; Smith and Houlihan, 1995; Smith *et al.*, 2000). Collectively, these experiments show that both variables increase proportionally with body mass, have similar Q_{10} values and show similar decreases in rate following starvation. Additionally, the post-prandial increase in oxygen consumption (termed the specific dynamic action response, or SDA response), is mirrored by a parallel increase in rates of protein synthesis in both fish and crustaceans (Tandler and Beamish, 1979; Jobling, 1983; Carefoot, 1990a; Brown and Cameron, 1991; Carter and Brafield, 1992; Whiteley *et al.*, 2001). SDA is not thought to be due to the mechanical costs of processing of the food or to deamination, but is more likely to represent the metabolic cost of growth (Jobling, 1983; Carefoot, 1990a; Brown and Cameron, 1991; Whiteley *et al.*, 2001). Moreover, work using the protein synthesis inhibitor, cycloheximide (CHX), has shown strong correlations between rates of oxygen uptake and rates of protein synthesis in vertebrates and invertebrates, including chickens (Aoyagi *et al.*, 1988), isopod crustaceans (Whiteley *et al.*, 1996) and also isolated trout hepatocytes (Pannevis and Houlihan, 1992). CHX inhibits protein synthesis by preventing peptide chain elongation (Emmerich *et al.*, 1976), so that the reduction in oxygen uptake after administration of CHX can be taken as the proportion of oxygen uptake needed for peptide chain elongation (Pannevis and Houlihan, 1992). CHX does not inhibit mitochondrial protein synthesis, but the

contribution of mitochondrial protein synthesis to overall protein synthesis is relatively small (between 3 and 5%) (L.S. Peck, personal communication). Of the few studies carried out to date on invertebrates, metabolic costs of protein synthesis have been estimated to account for up to 66% of the resting metabolic rate, but his value is dependent on thermal experience. For example, the Antarctic isopod *Glyptonotus antarcticus* living at 0°C has metabolic costs of protein synthesis of 885 mmol ATP.g protein^{-1} synthesised that are 4-times higher than the temperate isopod, *Idotea rescata* at 14°C with values of 237 mmol ATP.g protein^{-1} synthesised (Whiteley *et al.*, 1996). The marked difference in metabolic costs was attributed to RNA synthesis, which is thought to form the fixed energetic costs of overall protein synthesis, whereas protein synthesis forms the variable costs (Smith *et al.*, 1999). Specifically, the fixed component is thought to be due to the activation of tRNA and the production of rRNA (Pannevis and Houlihan, 1992). As rates of protein synthesis increase, the fixed component accounts for a smaller proportion of the total energy expenditure. Consequently, at lower temperatures, the proportion of metabolism that is attributable to protein synthesis rates is greater than at higher temperatures (Smith and Houlihan, 1995).

1.1.3 *Heat-Shock Proteins*

If behavioural thermoregulation and capacity adaptations alone are not sufficient in dealing with thermal stress, then more appropriate physiological adjustments are initiated. For instance, an acclimatory response to rapidly varying temperature, such as those which occur on a diel basis, is probably

useless and even counterproductive, as acclimation occurs over a period of days or weeks (Hochachka and Somero, 1984; Feder, 1985). The major changes in physiology that accompany acclimatory and acclimatisatory changes, occur on a seasonal basis (Huey and Bennett, 1990). The adjustments may be metabolically expensive and they usually occur over periods of weeks and months being accompanied by other environmental cues, such as varying photoperiod and food availability. The production of heat-shock proteins (hsps) through the heat-shock response serves to rescue perturbed proteins from thermal denaturation, acting as a resistance adaptation to aid survival at elevated temperatures. The broad function of hsps is to prevent inappropriate aggregations of proteins which have been damaged through stress. They are a highly conserved group of proteins and have been found in most organisms studied (Lindquist, 1986; McLennan and Miller, 1990; Parsell and Lindquist, 1994; Feder and Hofmann, 1999). Some hsps function as molecular chaperones and prevent aggregations by promoting the re-folding and compartmentalisation of denatured proteins, whilst others help in the degradation process of proteins which are beyond repair (Parsell and Lindquist, 1994; Roberts *et al.*, 1997). Although termed heat-shock proteins, hsps can be induced by almost any non-thermal stress including cellular energy depletion, extreme ion concentrations, other osmolytes, gases, ethanol, viral infection, amino-acid analogues and damage to DNA (Craig and Gross, 1991; Feder and Hofmann, 1999).

The threshold induction temperature of hsps is correlated with the normal range of temperatures experienced by a species. Typically, the threshold

induction temperature of hsps is higher during the summer months in temperate species, and in congeneric species living at lower latitudes. For example, species living in closer proximity to equatorial regions have higher hsp induction temperatures than those living in temperate regions. This observation is true for a range of animal species, including the marine mussels, *Mytilus trossulus* and *M. californianus* (Hofmann and Somero, 1996a; Roberts *et al.*, 1997), the marine snail, *Tegula spp.* (Tomanek and Somero, 2002), the Saharan desert ant, *Cataglyphis spp.* (Gehring and Wehner, 1995), and the eurythermal teleosts, *Gillichthys mirabilis* (Dietz and Somero, 1992; Dietz, 1994) and *Oryzias latipes* (Oda *et al.*, 1991). The plasticity of the heat-shock response, as displayed in temperate organisms which undergo seasonal variations in temperature, demonstrate that hsp synthesis is subject to acclimatory manipulation and is not genetically fixed (Somero, 1995).

The fact that hsps are rapidly induced in times of stress is indicative of their importance as an emergency response (Parsell and Lindquist, 1994). This notion is further reinforced in studies where increases in levels of hsps are mirrored by rapid and parallel increases in thermotolerance (Li and Werb, 1982), and also in studies where thermal tolerances of closely related species correlate well with their hsp synthesis response (Bosch *et al.*, 1988; Bosch *et al.*, 1991; Parsell *et al.*, 1993). Indeed, hsps may be the only major classes of proteins synthesised at, or near the limits of thermal tolerance of an organism (Morimoto *et al.*, 1990). However, the exact role of specific hsps in studies of whole-animal thermal tolerance are compounded by the complex myriad

interactions of the stress response. This has been demonstrated on work with fish hepatocytes where cells cultured at different temperatures revealed no differences in their hsp threshold induction temperatures (Koban *et al.*, 1987). Extrapolation of the results of laboratory studies on isolated cell cultures to whole organisms is clearly too simplistic (Somero, 1995), and researchers have realised that future work needs to concentrate on the heat-shock response of organisms in their natural situations. Consequently, recent studies have started to look at the temperature of hsp induction in wild populations and have compared the induction temperature, magnitude and time-course of the hsp response to seasonal change (Feder and Hofmann, 1999). These studies provide a valuable insight into some of the factors which dictate the ecological distribution of a species (see section 1.1.4).

The overall hsp stress response rarely consists of the expression of a single hsp from a single tissue, given that there are multiple hsps in each hsp family, and multiple hsp families. In addition, there are multiple non-hsp mechanisms of stress alleviation, such as osmotic stress protectants (trehalose in insects), homeoviscous adaptation, and the expression of isozymes or allozymes of enzymes (Feder and Hofmann, 1999). Most hsp studies operate within the confines imposed on them by the lack of development of suitable methods for addressing these complex issues. As a result, Feder and Hofmann (1999) believe that these studies can only offer comparatively vague, correlative answers to the specific questions they ask. However, these authors also state that continual advances in molecular techniques are gradually improving this

situation, but for the moment, correlative studies remain the only option for most workers.

The major classes of hsps can be broadly divided into four families: the hsp90 family, the large molecular weight hsps, from 83-90 kDa; the hsp70 family, from 66-78 kDa; the hsp60 family; and ubiquitin and the small hsps, which vary from 8 to 30 kDa (Morimoto *et al.*, 1990; Lindquist, 1993). The hsp90 family are essential for growth at normal temperatures, and they are further induced by heat. The exact nature of their role in thermo-protection however, is unclear (Lindquist, 1993). The hsp70 proteins belong to a family of highly conserved proteins and they are one of the most prominent heat-induced proteins found in organisms studied to date (Parsell and Lindquist, 1994). There is a strong correlation between their synthesis patterns and the ability of an organism to tolerate increased temperatures as shown in a number of species, such as bivalve molluscs (Roberts *et al.*, 1997), fish (Fader *et al.*, 1994), intertidal gastropods (Tomanek and Somero, 1999; Tomanek and Somero, 2000; Tomanek and Somero, 2002) and insects (Dahlgaard *et al.*, 1998). Heat-shock proteins are involved in protein folding, unfolding, assembly and disassembly, and it is thought they stabilise fully or partially unfolded target proteins by binding to their hydrophobic surfaces, preventing improper associations (Parsell and Lindquist, 1994). The hsp60 family are some of the most abundant proteins at normal temperatures, serving as molecular chaperones by promoting the folding of newly synthesised proteins. In addition, hsp60 prevents the aggregation of denatured proteins, and hsp60 becomes more important in protein re-folding as temperatures increase

(Parsell and Lindquist, 1994). Ubiquitin and the low molecular weight hsps are less conserved than the higher molecular weight hsps (Arrigo and Landry, 1994). Ubiquitin has been implicated in the regulation of proteolysis, as ubiquitin will target denatured proteins which are unable to be re-folded by the functions of other hsps for degradation (Lindquist, 1986).

1.1.4 *Thermal Tolerances: Ecological Consequences*

The ecological consequences of hsp synthesis and associated changes in thermotolerance have been investigated in a number of studies in which comparisons have been made between species from relatively thermostable environments and those from unstable environments. Some of these studies highlight the fact that merely being able to express hsps when subjected to thermal stress is just a small part of the role that hsps play in countering the effects of thermal stress. Investigation of the different characteristics of the heat-shock response (for example, the induction temperature, the maximal induction temperature, the time-course of hsp synthesis, the temperature at which hsps stop being synthesised, the response to acclimation and acclimatisation and the expression of specific hsps) in natural conditions, may help explain how organisms are adapted to their specific thermal niches.

An ectotherm in a thermostable habitat can, of course, experience high temperatures, as shown by Gehring and Wehner (1995) in their study of hsp synthesis and thermotolerance in two species of desert ant, *Cataglyphis spp.* The ability of these ant species to survive the extremely high daytime temperatures (above 50°C for at least 10 minutes) of their desert environment

comes, in part, from the synthesis of hsps. The assumption that the threshold induction temperature for the desert species would be far higher than for an ant species from a temperate environment was, somewhat surprisingly, incorrect. Gehring and Wehner (1995) found that the induction temperature for maximal hsp synthesis in the desert species was at 37°C, just 2°C higher than in the temperate species, *Formica spp.* This enabled the desert species to synthesise hsps in preparation for the acute temperature stress they would experience on leaving the nest. Although hsp synthesis is a rapid process, it cannot be mobilised rapidly enough for this extreme situation where the almost instantaneous temperature differential can be greater than 30°C. So, it appears hsps can be synthesised in anticipation of the thermal stress.

Studies using intertidal invertebrates are especially interesting, as closely related species living in relatively close proximity in different littoral zones have been found to show great differences in their levels of thermotolerance. Typically, species that inhabit the high intertidal zone, where temperatures are more variable, are more tolerant of acute heat-stress than their lower intertidal counterparts. This has been demonstrated in limpets (genus *Collisella*) (Sanders *et al.*, 1991), in marine snails (genus *Tegula*) (Tomanek and Somero, 1999; Tomanek and Somero, 2000; Tomanek and Somero, 2002) and in the desert teleost *Poeciliopsis spp.* (White *et al.*, 1994). This is managed either through a combination of differential expression of a broader range of hsps, or with differences in the time-course and magnitude of the heat-shock response or through a mix of both (Hofmann, 1999).

At the other extreme, Bosch *et al.* (1988), have shown a complete lack of any hsp response and great sensitivity to temperature stress in a freshwater hydroid from a thermally stable habitat. A closely related species from a more thermally variable habitat was able to synthesise hsps when heat-stressed and showed a subsequent degree of thermotolerance. A similar lack of hsp expression and thermotolerance has also been found by Hofmann *et al.* (2000), in the Nototheniid, *Trematomus bernacchii*, an Antarctic teleost from a thermally stable and uniformly cold environment. Given the importance of the heat-shock response in the ability to withstand acute thermal stress and the high degree of evolutionary conservation of the genes responsible for these proteins (Lindquist, 1986; Parsell and Lindquist, 1994; Somero, 1995; Feder and Hofmann, 1999), it is interesting that these examples do not synthesise hsps in response to thermal-stress.

There is almost no question as to the importance of hsp synthesis in the enhancement of thermal tolerance, but the general consequence of hsp expression at normal temperatures appears to reduce fitness (Feder *et al.*, 1992; Krebs and Loeschke, 1994a). Energetic costs of hsp production probably place an additional energetic burden on organisms, as their synthesis does not directly contribute to growth or reproduction, while hsps are synthesised preferentially over normal proteins reducing the competition for protein synthetic factors such as ribosomes (Parsell and Lindquist, 1994; Roberts *et al.*, 1997). Another time when expression of hsps may reduce fitness is during embryogenesis, when organisms show extraordinarily high rates of protein synthesis (Marsh *et al.*, 2001). Any interference with normal

rates of protein synthesis at this stage in development may have profound consequences for the continued viability of the individual. Consequently, hsps are not expressed during these especially sensitive periods of development (Giudice, 1985; Heikkila *et al.*, 1985). A number of studies have looked at the possibility of a negative consequence of hsp expression on fitness, measured in terms of reduced viability and fecundity of organisms expressing hsps at normal environmental temperatures (Feder *et al.*, 1992; Sanchez *et al.*, 1992; Krebs and Loeschke, 1994a). Consequently, it appears that hsp expression does have a fitness cost. Coleman *et al.* (1995), have reasoned that in a thermally stable environment, individuals which are unable to express hsps would have an energetic advantage over those which could express hsps, and over time, natural selection would favour those individuals that have lost the ability to express hsps. These authors also argue that if natural selection has optimised the costs of hsp synthesis with the benefits of their expression, then *in situ* studies of the stress response may be invaluable in helping to link the functional aspects of hsp synthesis (the specific molecular mechanisms of thermoprotection) with the ecological aspects (the biogeographic distribution of organisms).

1.2 *Seasonal Effects on Physiological Variables*

The major abiotic factors which alter with season in temperate climates are environmental temperature and photoperiod (Guderley, 2002). The biotic factors, such as food availability, predation and competition, tend to follow the abiotic factors (Berg and Bremset, 1998). Many of the studies on seasonal effects and organismal physiology have been carried out on temperate

species of fish (Dawson and Grimm, 1980; Marchant and Peter, 1986; Paul *et al.*, 1993; Fader *et al.*, 1994; Berg and Bremset, 1998; Lecklin and Nikinmaa, 1999). Generally, feeding, growth and reproduction occurs in the summer when days are longer, food is more readily available and temperatures are more suitable for the survival of juveniles (Marchant and Peter, 1986; Conover, 1992). The winter months are characterised by reductions in metabolic rate which is thought to be an adaptation to the reduced availability of food (Karås, 1990). In fish, other changes which are indicative of the winter state include increased liver mass relative to body mass (Foster *et al.*, 1993a), increased concentrations of white muscle RNA (Foster *et al.*, 1993a), reduced levels of fat, protein and neutral lipids used for energy reserves (Paul *et al.*, 1993; Berg and Bremset, 1998), lower levels of constitutive hsp70 expression (Fader *et al.*, 1994), and reductions in serum levels of certain steroid hormones and growth hormone (Marchant and Peter, 1986; Kime *et al.*, 1991).

Many of these seasonal changes in physiology are assumed to be due to the effects of temperature, but this is not necessarily the case. For example, the increase in serum growth hormone (GH) levels in goldfish during the summer months correlate well with increased water temperature, but increased GH serum levels also correlate well with increased photoperiod (Marchant and Peter, 1986). Indeed, photoperiod and diet can have similar effects on rates of metabolism as increases in temperature (Guderley, 1990). Investigations into the metabolic rate of organisms living in constant temperature environments have revealed that strong seasonal variations in food availability may be the

cause of changes in rates of oxygen uptake. For example, increased rates of oxygen uptake in the benthic amphipod, *Monoporeia affinis*, correlate well with food availability in the relatively constant temperature environment of the Baltic Sea (Lehtonen, 1996). A similar situation has also been demonstrated in Antarctic krill, *Euphausia superba* (Torres *et al.*, 1994) and in the mollusc, *Clione limacina* (Conover and Lalli, 1974). Similarly, brook trout, *Salvelinus fontinalis*, and brown trout, *Salmo trutta*, maintained at a constant temperature of 10°C, exposed to natural daylight and fed *ad libitum* showed seasonal variation in rates of oxygen uptake that could not be attributed to changes in temperature or food availability (Beamish, 1964). Instead, maximum rates of oxygen uptake coincided with the natural spawning period for the species.

These examples illustrate the difficulties in separating the effects of temperature from other seasonal effects on whole-animal physiology, and it is improbable that the acclimation of an organism from one season to the temperature typical of another season will override the seasonal effect. However, temperature acclimation in itself is important as a tool with which to separate out the specific effects of temperature during seasonal changes in organismal physiological status.

1.3 *Temperature Variation in the Intertidal Zone*

The intertidal zone can be considered as the furthest terrestrial extension of the marine environment, with most of the plant and animal life being of marine origin (Nybakken, 1997). The intertidal zone of rocky shores is a structurally simple and relatively uniform environment, but one that is potentially

extremely stressful and prone to great environmental variability (Carefoot, 1973b; Bally, 1987; Willows, 1987b). Both marine and terrestrial conditions prevail (Koop and Field, 1980), as by definition, the intertidal zone is an area of the shore that is periodically immersed through tidal action (Hofmann, 1999), and then subjected to periods of aerial exposure, making it one of the harshest environments to inhabit.

One of the most important factors affecting the survival of intertidal organisms is exposure to elevated temperatures (Edney, 1961; Lewis, 1963; Miller and Vernberg, 1968; Koop and Field, 1980; Nybakken, 1997). In oceans or any other large bodies of water, temperature remains within a relatively restricted range (Bliss, 1968) and rarely exceed the lethal limits for most organisms (Nybakken, 1997). In contrast, the aerial environment is characterised by potentially large and rapid changes over a wide range of temperatures (Edney, 1968; Willows, 1987b; Nybakken, 1997; Roberts *et al.*, 1997). For example, in temperate areas such as the UK, air temperature can vary seasonally by up to 50°C (Whiteley *et al.*, 1997). As a consequence, exposure to hsp-inducing, or even lethal heat-stress is highly likely in the intertidal zone (Nybakken, 1997; Feder and Hofmann, 1999). The animals which inhabit the intertidal zone are of particular interest to physiologists, as they are uniquely adapted to the differing physical environments of the marine and terrestrial world and offer a convenient means of examining the biological consequences of living in such contrasting environments (Hofmann, 1999). One such example of an intertidal dweller is *Ligia oceanica*, a characteristic

macroinvertebrate of the supralittoral zone of rocky shores in temperate regions.

1.4 *Ligia oceanica*

The semi-terrestrial isopod *Ligia oceanica* belongs to the suborder, Oniscoidea. Of the nine isopod suborders, only the Oniscoideans have managed to successfully evolve to a terrestrial existence (Takeda, 1984). The genus *Ligia* is regarded as a transitional stage in the evolution from the marine to the terrestrial habitat in the Isopoda, which invaded the land through littoral forms (Edney, 1954; Carefoot, 1973a; Takeda, 1984; Warburg, 1987), and are seen as the most primitive form of terrestrial isopod (Takeda, 1984). *Ligia spp.* are common in coastal environments around the world (Furota and Ito, 1999) and inhabit the humid high littoral or supralittoral zones (Tsai *et al.*, 1997), living in rock crevices or under stones above the high water mark. *Ligia spp.* are essentially nocturnal, and come out to feed on macroalgae and encrusting diatoms in large numbers at night during low tides (Nicholls, 1931; Carefoot, 1984).

The Isopoda possess a number of morphological and physiological adaptations which aided them in their move to the terrestrial environment. For example, dorso-ventral compression allows for stability on land, biphasic moulting reduces water loss, the possession of a brood pouch allowing for a degree of independence from bodies of water and nitrogenous waste being released as gaseous ammonia (Edney, 1954; Bliss and Mantel, 1968; Edney, 1968). Moreover, they have evolved other adaptations to reduce water loss,

for example, the development of harder, thicker and more complex cuticles in more xeric isopod forms, and the evolution of the pleopods from the primitive gill-like structures of the Ligiids to the pseudotracheated forms of the Porcellionids and Armadilliliids (Edney, 1961; Warburg, 1968; Takeda, 1984; Warburg, 1987). However, behavioural adaptations are used to avoid desiccating conditions, and may have been instrumental in enabling the Oniscoideans to invade the terrestrial environment (Mayes and Holdich, 1975).

The distribution of terrestrial isopods is principally dictated by the physiological constraints imposed by humidity. This is of particular importance to semi-terrestrial species such as *Ligia*, which rapidly lose water through their relatively permeable exoskeleton. Subsequently, water loss ultimately defines their limits of existence on the upper shore. One important behavioural adaptation which minimises water loss is gregariousness. This behaviour is strongest in the Ligiids (Takeda, 1984) and becomes less strong as isopods become better morphologically adapted to minimising water loss. But perhaps the most important behavioural adaptation to maintaining a terrestrial existence is through being nocturnal and negatively phototactic (Edney, 1954; Warburg, 1968; Warburg, 1987), and consequently avoiding the higher temperatures and desiccatory effects of daytime exposure. The normal nocturnal behaviour and negative phototaxes of *Ligia oceanica* can apparently be interrupted by periods of deliberate insolation in an attempt to reduce body temperature through evaporative water loss. Studies by Edney (1953) and Warburg (1968) have shown that the relative humidities of the shingle

microhabitats that *L. oceanica* occupy can easily reach saturation point during the summer periods (see Figure 1.1), when mortality is at its highest in *Ligia* (Willows, 1987b). The lethal temperature of *L. oceanica* in saturated air is about 32.5°C (Edney, 1953). When temperatures in the microhabitat approach the lethal temperature, Edney (1953) claimed that *L. oceanica* become positively phototactic and emerge into conditions where humidity and air temperature may be lower, but where they are exposed to direct insolation, and presumably, greater risk of predation. The subsequent evaporative water loss and cooling from air convection can reduce body temperatures by as much as 8°C (Edney, 1953; Edney, 1961). The larger body size of *L. oceanica* relative to its terrestrial ancestors enables it to sustain a rate of evaporative water loss per unit area for longer than an equivalent smaller isopod, translating to greater possible reductions in body temperature (Edney, 1968). A larger body size also acts as a more effective buffer against environmental temperature variation than would be the case for a smaller animal, due to the smaller surface area to volume ratio (Nybakken, 1997). The risks of desiccation from this profligate use of water are much reduced in the humid intertidal zone and enable *L. oceanica* to use evaporative water loss as a very effective mechanism of reducing body temperature, but only for short periods of time. As with other crustaceans which are subject to seasonal temperature variation, growth and reproduction in *Ligia spp.* are restricted to the warmer months when temperatures are higher, days are longer and food is more readily available (Willows, 1984; Willows, 1987b; Whiteley *et al.*, 1997) In addition, they experience large and rapid environmental temperature fluctuations in the summer months and rely on evaporative cooling and the

synthesis of heat-shock proteins to survive elevated temperatures, which result from solar heating effects of the sun (Edney, 1953; Whiteley *et al.*, 1997).

Intertidal isopods such as *Ligia oceanica* make a good model animal for the study of the effects of temperature on the physiology of intertidal organisms for a variety of reasons. Previous work by Whiteley *et al.* (1996), compared the metabolic costs of protein synthesis in two species of marine isopod which inhabit very different thermal environments. The giant Antarctic isopod, *Glyptonotus antarcticus*, a stenothermal species and inhabitant of cold Antarctic waters has an upper lethal temperature of 6°C. Metabolic costs of protein synthesis in *G. antarcticus* at 0°C were found to be 4-times higher than for the temperate, eurythermal isopod, *Idotea rescata* at 4°C. The increased costs of protein synthesis at the lower temperatures experienced by *G. antarcticus* suggests that the ability to synthesise proteins at these temperatures may be limited by the expensive energetic costs involved. A natural extension of this study is to compare the metabolic costs of protein synthesis in a semi-terrestrial isopod species which experiences even greater variation in environmental temperature on a seasonal and diel basis to that experienced by the polar isopod species living at a stable temperature. Other studies on intertidal organisms have used sessile, or relatively non-motile organisms such as the bivalve mollusc, *Mytilus edulis* (Hawkins *et al.*, 1987; Hawkins *et al.*, 1989; Sanders *et al.*, 1992), the limpet, *Collisella spp.* (Sanders *et al.*, 1991) and the marine snail, *Tegula spp.* (Tomanek and Somero, 1999; Tomanek and Somero, 2000). Although *L. oceanica* are highly

mobile, they are still restricted to the relatively narrow supralittoral band of the shore, primarily because of their limited morphological and physiological adaptations to the terrestrial environment and their subsequent susceptibility to desiccation. *Ligia* avoids thermal extremes during the summer months by moving between microclimates within the shingle. However, desiccation is a potential threat and escape to a less humid microhabitat is not always a solution, and therefore the animals can be exposed to elevated temperatures in excess of 30°C (see Figure 1.1). Finally, like many other intertidal organisms, different isopod species occupy the various zones that make up the intertidal zone, enabling studies between closely related organisms from different thermal niches possible.

The aim of this thesis is to study the effects of short- and long-term temperature variation on the relationship between rates of metabolism, rates of protein synthesis and patterns of heat-shock protein synthesis in an intertidal crustacean living in a highly variable thermal environment, in order to determine the mechanisms responsible for influencing survival during thermal stress. Protein synthesis has been shown to be crucial to the ability of an organism to acclimate to new temperatures, but the synthesis of new proteins can be metabolically costly, and therefore not appropriate during short-term temperature changes characteristic on the shore. As a result, metabolic costs of protein synthesis in winter animals may be greater than for warm acclimated animals and animals collected in the summer, suggesting a strong seasonal effect on the ability to survive elevated temperatures. Consequently, heat-shock responses in isopods caught in the wild and acclimated in the

laboratory in winter and summer will be related to the relative costs of protein synthesis and more specifically, to the energetic costs of heat-shock protein synthesis, to determine the metabolic costs of survival on the shore.

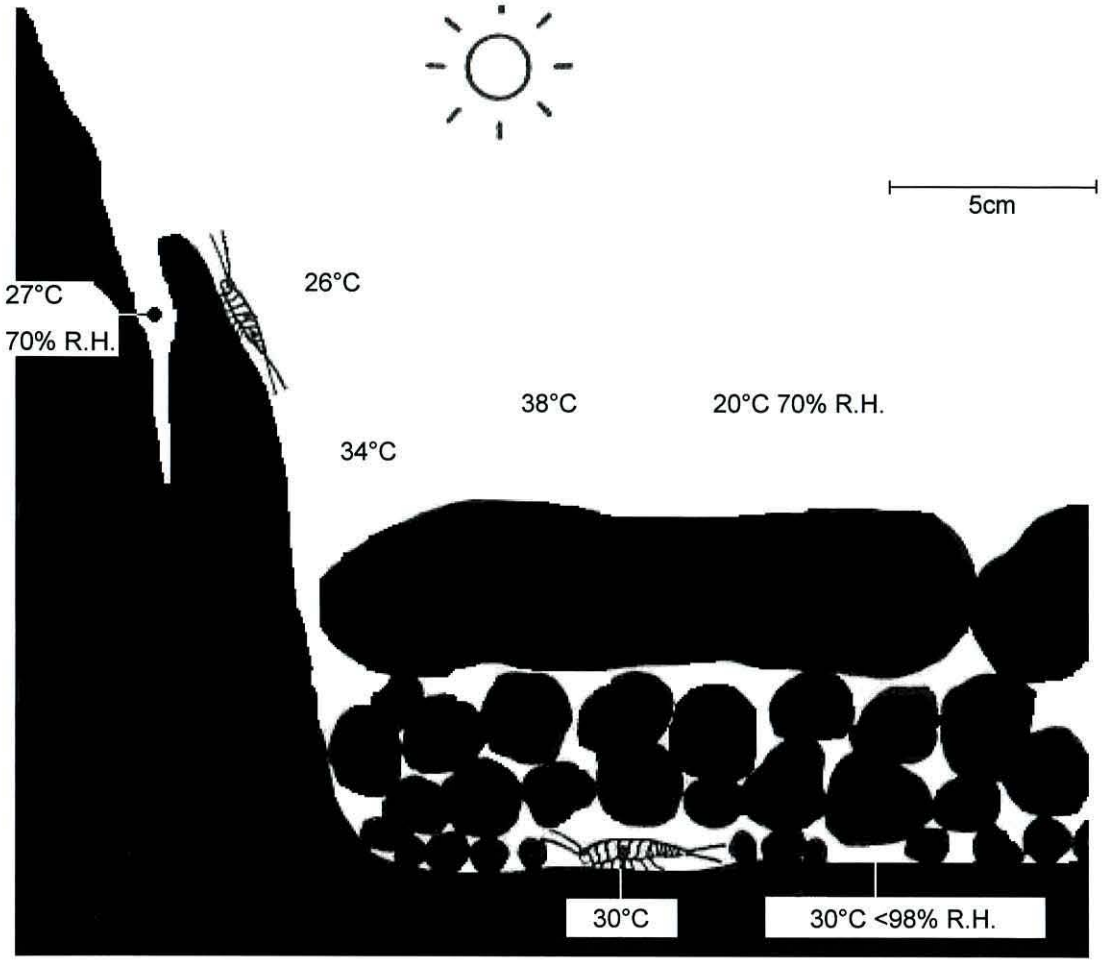


Figure 1.1. A diagrammatic representation of the environmental conditions experienced by *Ligia oceanica* inhabiting different microclimates on a shingle shore at the base of a sand stone cliff in Pembrokeshire during the summer. R.H. represents relative humidity. Adapted from Edney (1953).

Chapter Two

Seasonal Differences in Rates of Metabolism and Protein Synthesis in *Ligia oceanica*: Effects of Temperature Acclimation

2.1 *Introduction*

Acclimation or acclimatisation to relatively long-term temperature change is considered a capacity adaptation (Huey and Bennett, 1990) and is largely restricted to organisms which inhabit environments where gradual temperature change, happening over periods of weeks or months, occurs with some degree of regularity. Typically, ectotherms from temperate regions display the ability to acclimate to the relatively predictable cycle of physical changes brought about by season. Acclimation is of little value in constant temperature environments (Feder, 1978), and previous work investigating the acclimatory ability of cold stenothermal organisms such as crabs, isopods and fish has shown an inability of these species to acclimate to an increased temperature environment (Kivivuori and Lagerspetz, 1990; Weinstein and Somero, 1998; Pörtner *et al.*, 1999; van Dijk *et al.*, 1999). The same has been shown for stenothermal animals from warm regions, as Miller and Vernberg (1968) demonstrated an inability to adapt to temperatures less than 20°C in a species of tropical fiddler crab. Sublittoral organisms such as the European lobster, *Homarus gammarus*, which experience predictable seasonal thermal variation, but where diel temperature change is buffered by the aqueous environment can also display limited acclimatory ability (Tully *et al.*, 2000). Subsequently, lobsters appear to occupy an intermediate position regarding acclimatory ability between the extremes of the temperate eurytherms and the warm and cold adapted stenotherms. The adaptive value of acclimation to

ectotherms is to offer a degree of physiological independence from the acute effects of alterations in the environmental temperature (Foster *et al.*, 1992).

During the winter months, the adaptive significance of the acclimatory response in ectotherms which remain active is to physiologically compensate for low temperatures. For example, this would involve an increase in the metabolic rate above the expected level to allow the organism a degree of temperature independence. The reverse is true in the summer where a reduction in the metabolic rate is thought to be a mechanism for moderating energy expenditure at high temperatures (Feder, 1985). In broad terms, temperature acclimation compensates for the temperature effects on biochemical rates of reaction allowing for a more constant level of activity within the normal range of temperatures experienced by an organism (Haschemeyer, 1969). Chronic exposure to constant temperatures will eventually result in acclimation to that temperature, if the capacity for an acclimatory response is present. The period of acclimation usually takes from days to weeks, for example, a minimum of 40 days is thought to be sufficient time for thermal acclimation in fish (Foster *et al.*, 1992). Whereas acclimation is usually performed in the laboratory under controlled conditions with the alteration of just a single factor, the natural seasonal process of acclimatisation involves additional diel temperature change, climatic variability (wind, rain), changing photoperiod and light intensity, exposure to ultra violet radiation and changes in food availability amongst other variables.

This raises the interesting question of whether the alteration of a single abiotic variable, *i.e.* temperature, is sufficient to change the physiological status of animals from different seasons and whether acclimation to a constant temperature can alter the seasonal influences on metabolism and rates of protein synthesis. Studies on Antarctic invertebrates such as the Antarctic echinoid, *Sterechinus neumayeri* (Brockington and Clarke, 2001; Brockington and Peck, 2001), and the Antarctic bivalve, *Laturnula elliptica* (Brockington, 2001), which experience relatively constant annual temperatures have revealed that their metabolic activity was largely influenced by activity associated with feeding, growth and spawning. Moreover, seasonal differences in fiddler crabs (genus *Uca*) were still evident even after thermal acclimation (Vernberg, 1959), suggesting temperature alone was insufficient for acclimatisation. Some of the largest physiological changes in organisms which experience seasonal cycles are due to reproduction (Clarke, 1987), and it is known that growth and reproduction in temperate Crustacea is limited to the summer months (Whiteley *et al.*, 1997), as during the winter, crustaceans enter a state of torpor, or maintain a scope for reduced activity.

The seasonal differences apparent in ectothermic organisms are due to a number of interacting factors and the best studied of these is metabolic rate (Edney, 1964b; Feder, 1985; Saint-Paul *et al.*, 1988; Chappelle and Peck, 1995). Fewer studies have looked at the relationship between season and rates of protein synthesis. As there is reported to be a strong correlation between rates of oxygen uptake and rates of protein synthesis, it is of great interest to know if both of these variables can be experimentally manipulated

through temperature acclimation. In the following chapter, the acclimatory capacities of *Ligia oceanica* from the summer and winter will be investigated by acclimating isopods to various temperatures and comparing rates of metabolism and protein synthesis, in order to characterise any seasonal differences in the relationship between these two variables.

2.2 *Materials and Methods*

2.2.1 *Collection and Maintenance of Animals*

Ligia oceanica were collected from the rocky supralittoral shores of Rhosneigr (O/S grid reference: 231787, 372452) and Cemlyn Bay (O/S grid reference: 233075, 393820) on the West and North coasts of Anglesey, respectively (Figures 2.1 and 2.2). Medium-sized to large (>200 mg) intermoult males were selected over small, moulting individuals and females, as the production of gametes in mature female organisms can cause protein demand for this one pathway to exceed all others (Clarke, 1987). Moulting animals were detected by calcium deposits seen as white flecks on the ventral surface and males were easily identified by the presence of copulatory spicules, extensions of the endopodites located within the pleopods. Isopods collected for the preliminary time-course experiments were collected in October 1998. Winter animals were collected in January 1999 and summer animals were collected in June 1999.

Animals were transported immediately back to the University of Wales, Bangor, where they were placed into opaque containers with a diameter of 30cm and a depth of 11 cm. No more than 30 animals were placed in a single container in an effort to reduce mortality due to overcrowding. The containers were furnished with rocks and 300 ml of seawater and placed on a shallow incline to provide equal areas of seawater and dry rock. Lids were placed on each container to help maintain a saturated, humid atmosphere, important to the survival of terrestrial isopods. The containers were placed in cooled incubators (Gallenkamp models INF-631-Q and INF-781-T) maintained at the experimental temperature and set at a light/dark cycle of 12/12. Acclimated animals were fed twice weekly on a diet of potato shavings and *Fucus spp.* fronds. Containers were cleaned weekly and dead animals and uneaten food were removed. Acclimatised animals used straight from the shore were held at the appropriate *in situ* temperature of collection, without food, for a maximum of 2 days prior to experimentation.

2.2.2 *Experimental Procedures*

2.2.2.1 *Whole-Animal Rates of Oxygen Uptake*

Rates of oxygen uptake were measured using constant pressure respirometry as described by Spencer-Davies (1966). This method maintains the gases of the respiration and compensation chambers at a constant temperature and pressure, removing the complexities of pressure measurement inherent in Warburg manometry, so changes in volume through oxygen uptake can be recorded directly.

2.2.2.2 *Measuring Oxygen Uptake Rates in Ligia oceanica*

Each respirometer consisted of three main components: the respiration and compensation chambers, the manometer block, and the standard micrometer head (see Figure 2.3).

Both the respiration and compensation chambers had a working volume of 45 cm^3, and contained two small tubes. One tube was filled with 0.5 ml of 6N potassium hydroxide for the absorption of expired CO_2, whilst the other tube contained 0.5 ml of seawater to maintain a high relative humidity. A small piece of fluted ashless filter paper (Whatman 40) was added to the tube containing KOH to increase the surface area available for CO_2 absorption. Ashless filter paper was used as this does not produce CO_2. Another piece of Whatman 40 filter paper soaked in seawater was added to the base of each chamber to maintain the high relative humidities within the chamber. The respiration chamber had been wrapped in dark tape to minimise the any possibility of stress to the animals caused by sudden movements during measurements of oxygen uptake where adjustments to the micrometer were necessary.

The manometer block consisted of a solid block of Perspex into which a manometer bore, two connecting bores and a micrometer bore had been drilled. A horizontal line was etched across both arms of the manometer bores, approximately 3 cm from their base, and on both sides of the block to negate parallax. This line denoted the upper limits for the manometer fluid and was crucial when taking measurements of oxygen uptake. It acted as a

reference point for the return of the manometer fluid as volume changes were compensated by adjustments to the micrometer. Both respiration and compensation chambers were open to the outside via their respective connecting bores. To create a closed system, the crossbar was simply tightened, creating an airtight seal over both connecting bores. The only connection between the two chambers was via the manometer bore, which was filled with manometer fluid. The micrometer bore connected directly to the respiration chamber and was sealed at one end by the chamber and at the other by the micrometer spindle. The micrometer was mounted into the manometer block simply by a tight push-fit. The spindle had a rubber syringe tip glued onto its end and this provided an airtight seal.

For the measurement of oxygen uptake rates, the respiration chamber containing an individual isopod and the compensation chamber were pushed firmly onto their respective rubber bungs. To ensure an airtight fit, a small quantity of silicon grease was applied to the inside rims of both chambers before they were connected to the manometer block. With the crossbar in the open position, the respirometer was set up in a water bath maintained at the experimental temperature, ensuring the respiration and compensation chambers were both submerged. The respiration chamber containing the animal had been covered in tape to help minimise stress and disturbance to the animal through it detecting outside movements such as occurred when adjustments to the micrometer were made. A minimum of 30 minutes was allowed for thermal equilibration of respiration and compensation chambers, after which time the crossbar was closed to seal the apparatus. The rate of

thermal equilibration between the respirometry chambers would therefore be more rapid where the temperature differential was greatest, *i.e.* at the higher experimental temperatures, where thermal equilibration was achieved within 30 minutes. Micrometer measurements were taken at either 3 or 5 minute intervals for a total of 30 minutes.

Changes in volume within the respirometer chamber were determined by sealing the chamber via the crossbar to produce a closed system. With an isopod in the respiration chamber, and at the point where the crossbar was closed, the volume in the respiration chamber equalled that of the compensation chamber. As the animal respired, CO_2 was absorbed by the KOH, and the volume in the respiration chamber dropped. The decrease in volume was seen as the manometer fluid moved up the bore on the side of the respiration chamber. By carefully advancing the micrometer the required amount, the level of the manometer was reset by aligning both arms of the manometer fluid with the horizontal lines etched onto the manometer block. At this point, the volume in the respiration chamber once again equalled that of the compensation chamber. Micrometer measurements in micrometer advances per minute ($mm.min^{-1}$), were converted into volume measurements by multiplying by a factor 65.04. As the diameter of the micrometer bore was 9.1 mm, a 1 mm advancement of the micrometer resulted in a volume change of 65.04 mm^3.

2.2.2.3 *Whole-Animal Fractional Rates of Protein Synthesis*

Rates of protein synthesis were measured in whole-animals using the procedure described by Garlick *et al.* (1980), as detailed by others (Houlihan *et al.*, 1986; Houlihan *et al.*, 1988; Houlihan *et al.*, 1990a; Houlihan *et al.*, 1990b; Foster *et al.*, 1992; Whiteley *et al.*, 1996). This method involves the administration of a radiolabelled amino-acid, [^{3}H] phenylalanine (Phe), in a single large flooding dose. This has the effect of flooding all precursor pools of amino-acids, so they have similar specific radioactivities during the period of incorporation into protein. Phe has been previously shown to be an essential amino acid in Crustacea (van Marrewijk and Zandee, 1975).

2.2.2.4 *Experimental Protocol*

Animals were injected with crab saline, containing 150 mmol.l^{-1} of cold L-Phe and 50 µCi.ml^{-1} L-[2,3,4,5,6-^{3}H] Phe (Amersham, sp. act. 112 Ci.mmol^{-1}) using a Hamilton syringe at a dose of 1 µl.50 mg^{-1} body weight. Injections were made into the haemocoel through the dorsal surface at the pereon-pleon junction, just to the right or left of the alimentary canal. Prior to injection, a small volume of haemolymph (1-2 µl) was taken up into the syringe to ensure the radiolabel was delivered into the haemocoel of the animal. The syringe was left in place for 10 seconds before removal to ensure adequate circulation of the injected solution. Animals were left at the relevant experimental temperature for a period of 1 hour post-injection, before being snap-frozen in liquid N_2 and stored at –80°C for later analysis of levels of Phe and the specific activity of the free and protein-bound fractions.

2.2.2.5 *Analysis of Protein Synthesis Rates*

Isopods were ground into a fine powder using a pestle and mortar pre-cooled in liquid nitrogen (N_2). 2% perchloric acid (PCA) was added to the homogenate to precipitate the free-pool amino-acids, and the supernatant was stored at –20°C for later conversion of Phe to β-phenylethylamine (β-PEA). The remaining pellet was washed twice in 2% PCA to remove any free-pool Phe residues. Protein-bound Phe was obtained by dissolving the PCA homogenate in 0.3N sodium hydroxide at 37°C for 1 hour. A 20 µl sub-sample was removed at this point for protein determination, initially using the method of Bradford (1976), but later using a modification of the Lowry technique because of its greater accuracy (Sigma procedure no. P 5656) with bovine serum albumin (BSA) as a standard (Lowry *et al.*, 1951). Absorption levels were read at a wavelength of 750 nm in a Labsystems Multiskan MS plate reader.

Protein and nucleic acids were precipitated from the alkali digest by adding 12% PCA. The acid-soluble fraction was removed for determination of RNA levels by ultraviolet absorption at 232 and 260 nm (Ashford and Pain, 1986) using either a Cecil CE 4400/UV VIS double beam scanning spectrophotometer or a Shimadzu UV-260 UV-visible recording spectrophotometer. The pellet was re-suspended in 10% PCA and incubated at 70°C for 25 minutes to precipitate DNA for determination by the diphenylamine technique using salmon testes DNA as a standard, and measuring absorption at 595 and 700 nm (Cecil Instruments CE 303 grating

spectrophotometer). The remaining pellet was hydrolysed in 6 N HCl at 110°C for 24 hours, and the acid removed by evaporation over several days.

Amino acids were re-suspended in citrate acid buffer (pH 6.3) prior to conversion into β-PEA. The Phe in the free and protein-bound fractions was enzymatically converted to β-PEA and assayed using a fluorescence technique described by Suzuki and Yagi (1976), where standard solutions of β-PEA were used to quantify the recovery of converted Phe from both fractions. The fluorescence was read at an excitation of 386 nm and an emission of 495 nm (Perkin-Elmer LS-5 or LS-3 luminescence spectrometers), or at an excitation of 355 nm and an emission of 460 nm (Wallac VICTOR^2 1420 Multilabel Counter). The specific activities of protein-bound and free-pool β-PEA were determined by liquid scintillation using a Wallac WinSpectral 1414 scintillation counter and Optiphase 'Hisafe' scintillant.

2.2.3 *Experimental Regime*

2.2.3.1 *Time-Course Experiments for Protein Synthesis Rates*

A preliminary set of experiments was carried out to establish the relationship between temperature and incorporation rates of [^{3}H] Phe from the free-pools into the protein-bound fraction, and for the determination of a suitable time-scale for maximum rates of protein synthesis at high levels of specific activity in the free-pools. As incorporation rates are dependent on temperature, it was necessary to establish the effect of acclimation temperature on the specific activities of both free-pools and protein-bound fractions to give a more accurate determination of incubation times. In order to accurately measure

rates of protein synthesis, the specific activities of the free-pools must be elevated and remain stable, or show a slow linear decline with time. The labelling of body protein should also be linear with time (Houlihan *et al.*, 1995). Houlihan *et al.* (1988), outlined three important assumptions when using the flooding-dose technique for measuring rates of protein synthesis. Firstly, the high concentration of Phe should not affect rates of protein synthesis, and this has been shown in work by Loughna and Goldspink (1985). Secondly, the radiolabelled amino-acid should equilibrate rapidly with the intracellular free-pools and this has been demonstrated many times in work with fish (Houlihan *et al.*, 1986; Houlihan *et al.*, 1988; Houlihan *et al.*, 1995). Finally, the specific activity of the free-pool should remain constant, or at increased levels but showing a slow linear decrease, and this has again been demonstrated in teleosts (McMillan and Houlihan, 1988; Foster *et al.*, 1992; Pannevis and Houlihan, 1992; Mathers *et al.*, 1993), in the mollusc *Octopus vulgaris* (Houlihan *et al.*, 1990a) and also in crustaceans (El Haj and Houlihan, 1987; Hewitt, 1992; Whiteley *et al.*, 1996).

2.2.3.2 *Time-Course Experiments*

Ligia oceanica acclimated at either 10, 15 or 20°C for 4 weeks were divided into three groups to include 3 different incorporation times 30, 60 and 90 minutes. Animals were deprived of food for 2 days before experimentation and were injected with the [^{3}H] Phe/cold Phe cocktail according to the methods described earlier, at a dose of 1 µl.50 mg^{-1} body weight and left for the appropriate period of time before being snap-frozen in liquid N_2 for the analysis of protein synthesis rates.

2.2.3.3 *Effect of Acclimation Temperature on Rates of Oxygen Uptake and Protein Synthesis*

Ligia oceanica were collected in January and June 1999 from Rhosneigr on the West coast of Anglesey and returned to the University of Wales Bangor for 4 weeks acclimation to various temperatures. Animals collected in January were divided into four groups of thirty animals, and acclimated at 5, 10, 15 and 20°C. Twenty animals were also held at 25°C, but mortality was 100% after one week. Animals collected in June were divided into five groups of thirty and acclimated at 5, 10, 15, 20 and 25°C. After 4 weeks acclimation, whole-animal rates of oxygen uptake and whole-animal fractional rates of protein synthesis were measured simultaneously in the same animals, according to the techniques described earlier.

As there were only three respirometers available, a maximum of twelve individual respirometry experiments could be made in any one day, six in the morning and six in the afternoon. This meant that a complete set of respirometry measurements could be taken for two acclimation groups in a single day. For each acclimation temperature, half of the respirometry measurements were taken in the morning, and the other half in the afternoon.

Two water baths were set up at the appropriate temperatures. Six animals from each acclimation temperature were separated out from the main group and deprived of food for two days but still held at their respective acclimation temperatures. Increases in metabolic rate caused by the SDA response were eliminated by the daily removal of faecal pellets from the containers holding

the six isopods. 24 hours before respirometry measurements were due to be taken, animals were blotted dry and weighed, prior to being placed into individual respiration chambers with one animal per chamber, still at their respective acclimation temperatures. Lids were kept on the respirometry chambers to help maintain high humidities. The following morning, three respiration chambers were carefully connected to the manometer blocks, making sure the isopods were not disturbed. The respiration and compensation chambers of the assembled respirometers were immersed in a water bath set at the acclimation temperature, and allowed to thermally equilibrate to the experimental temperature one hour before respirometry measurements were taken. The apparatus was then sealed and readings were taken every 5 minutes for a period totalling 30 minutes. Once a complete set of readings had been taken, the respiration chambers containing the isopods were removed, and the isopods were injected with radiolabel for the measurement of whole-animal fractional rates of protein synthesis. Animals were returned to their respiration chambers which had been removed from the manometer block, and with the lids in place, maintained at their relevant acclimation temperatures for a further 60 minutes before being removed and snap frozen in liquid N_2 prior to processing. The respiration chambers from another acclimation temperature, each containing a single isopod, were then connected to the respirometers, and treated in exactly the same way as the first group before respirometry measurements were taken.

2.2.4 *Calculations*

Whole-animal oxygen uptake and absolute rates of protein synthesis were scaled to represent an animal of one gram fresh weight using a weight exponent of 0.7 (Whiteley *et al.*, 1996).

Rates of protein synthesis were calculated using the equation from Garlick *et al.* (1980):

$$k_S = \frac{S_b}{S_a} \times \frac{24}{t} \times 100$$

where k_s is the fractional rate of protein synthesis (%.day^{-1}), S_a and S_b are the specific radioactivities of the free-pool and protein-bound Phe (dpm.nmol^{-1}) respectively, and t is the incubation time (h).

The RNA:protein ratio was calculated using the equation from Preedy *et al.* (1988):

$$RNA : protein = \frac{RNA \times 1000}{protein}$$

where RNA:protein, or capacity for protein synthesis, is expressed as µg RNA.mg protein^{-1}.

The translational efficiency of the RNA was calculated using the equation from Preedy *et al.* (1988):

$$k_{RNA} = \frac{k_S \times 10}{RNA : protein}$$

the rate of RNA activity (k_{RNA}), is expressed as (µg protein.µg $RNA^{-1}.day^{-1}$).

Absolute rates of protein synthesis were calculated using the equation from Houlihan *et al.* (1990b):

$$A_S = \frac{k_S}{100} \times protein$$

where A_s is the absolute rate of protein synthesis (mg $protein.day^{-1}$).

All statistical analyses within seasons were performed using single factor analysis of variance (ANOVA) plus Tukey's test with significance set at the 5% level ($P = < 0.05$) and between seasons using two way analysis of variance (Two Way ANOVA) plus Tukey's test, also with the significance set at the 5% level ($P = < 0.05$).

2.3 *Results*

2.3.1 *Time-Course Experiments for Protein Synthesis Experiments*

The mean specific activities of the free-pool fractions are shown in Figure 2.4. Mean values in *Ligia oceanica* acclimated at 10°C were 8,045 ± 746 dpm.nmol^{-1} after the first 30 minutes. Values stabilised to 6,112 ± 1,741 and 6,530 ± 2,673 dpm.nmol^{-1} after 60 and 90 minutes, respectively. At an acclimation temperature of 15°C, the mean free-pool specific activity decreased dramatically from 14,947 ± 4,503 dpm.nmol^{-1} after 30 minutes, to 6,925 ± 2,203 dpm.nmol^{-1} after 60 minutes. Mean free-pool specific activities of *Ligia* acclimated at 20°C changed from 6,972 ± 2,099 dpm.nmol^{-1} after 30 minutes to 3,808 ± 785 and 4,096 ± 653 dpm.nmol^{-1} at 60 and 90 minutes, respectively. At all three acclimation temperatures, there were no significant differences between the mean values at each time interval.

The specific activities of the protein-bound fractions at 10, 15 and 20°C are shown in Figure 2.5. Rates of incorporation of the radiolabel into tissue proteins were linear with time for all acclimation temperatures. Incorporation rates were generally elevated at 15°C compared with the rates at 10 and 20°C, but the differences between mean values were not significant.

Figure 2.6 shows the resulting fractional rates of protein synthesis (k_s) for animals held at all acclimation temperatures over the course of the radiolabel incorporation period. Lowest k_s values were consistently recorded for 10°C

acclimated *Ligia* with a mean of 1%.day^{-1} over the 90 minute period. Highest mean k_s values for all acclimation temperatures were recorded after 60 minutes of radiolabel incorporation, with mean k_s ranging from 1.3 ± 0.5 at 10°C, to 2.9 ± 0.8%.day^{-1} at 15°C acclimation.

The time-courses for all three variables in 10°C acclimated animals is summarised in Figure 2.7. This figure shows the relationships between the free-pool specific activity, the protein-bound specific activity and the k_s values during 90 minutes of incubation. The specific activity of the free-pools remained stable and elevated over 90 minutes of the incorporation period, whilst protein-bound specific activity increased linearly. There were no significant differences at either 60 or 90 minutes from the mean values at 30 minutes for any of the variables.

2.3.2 *Effect of Acclimation Temperature on Oxygen Uptake Rates*

Figure 2.8 shows the relationship between whole-animal rates of oxygen uptake and temperature for *Ligia* collected in the winter. This Figure shows that there was an increase in mean rates of oxygen uptake ($\dot{M}O_2$) with increasing temperature, ranging from 20.3 ± 0.6 µmol.animal^{-1}.day^{-1} at 5°C to 114.2 ± 10.9 µmol.animal^{-1}.day^{-1} at 20°C. The overall Q_{10} was 3.2. At all temperatures, mean $\dot{M}O_2$ was significantly different from the mean at 5°C (single factor ANOVA plus Tukey. 5 and 10°C: *d.f.* = 1, F = 65.94, P = < 0.001; 5 and 15°C: *d.f.* = 1, F = 78.45, P = < 0.001; 5 and 20°C: *d.f.* = 1, F = 73.74, P = < 0.001).

Ligia collected in the summer also showed an increase in rates of oxygen uptake with increasing temperature, rising from 14.9 ± 0.7μmol.animal^{-1}.day^{-1} at 5°C, to 87.7 ± 8.3 μmol.animal^{-1}.day^{-1} at 25°C, and giving a Q_{10} of 2.4 (Figure 2.8). Mean $\dot{M}O_2$ was significantly higher at all temperatures compared to the mean at 15°C. (single factor ANOVA plus Tukey. 5 and 15°C: *d.f.* = 1, *F* = 120.55, *P* = < 0.001; 10 and 15°C: *d.f.* = 1, *F* = 6.82, *P* = < 0.026; 20 and 15°C: *d.f.* = 1, *F* = 5.57, *P* = < 0.04; 25 and 15°C: *d.f.* = 1, *F* = 22.03, *P* = < 0.001).

Mean $\dot{M}O_2$ was generally higher in *Ligia* collected in the winter than in the summer at all acclimation temperatures (Figure 2.8). The differences in the mean values among the different levels of season was greater than would be expected by chance after allowing for the effects of differences in temperature (Two Way ANOVA plus Tukey. *d.f.* = 1, *F* = 52.57, *P* = < 0.001). The differences between the mean values among the different levels of season were also greater than would be expected by chance allowing for the effects of seasonal differences (Two Way ANOVA plus Tukey. *d.f.* = 3, *F* = 60.52, *P* = < 0.001). The interaction between season and temperature was also found to be statistically significant (Two Way ANOVA plus Tukey. *d.f.* = 3, *F* = 7.60, *P* = < 0.001).

2.3.3 *Effects of Acclimation temperature on Fractional Rates of Protein Synthesis and Associated Variables*

In winter *Ligia*, there was no change in mean k_s between the acclimation temperatures of 5 and 20°C (Figure 2.9), with a mean value of 0.4 ± 0.2

%.day^{-1} at 10°C, and a value of 1.2 ± 0.3 %.day^{-1} at 20°C. Q_{10} between 5 and 20°C was 1.8. Mean values for RNA activity (k_{RNA}) also showed little change between 5 and 20°C, with a value of 0.2 ± 0.1 µg protein.µg RNA^{-1}.day^{-1} at 10°C, and a value of 1.2 ± 0.3 µg protein.µg RNA^{-1}.day^{-1} at 20°C. Q_{10} for k_{RNA} between 5 and 20°C was 2.0. The RNA:protein ratio remained at around 24.3 µg RNA.mg protein^{-1}, and was not affected by temperature.

In summer animals, there was little change in k_s between 5 and 20°C (Figure 2.9). However, mean k_s increased 4.5-times between 20 and 25°C, from 0.2 ± 0.1%.day^{-1} to 1.0 ± 0.5 %.day^{-1}, giving a Q_{10} of 20.5 between 20 and 25°C. There was a significant difference between the means at 15 and 20°C (single factor ANOVA plus Tukey. *d.f.* = 1, $F = 5.18$, $P = 0.04$). Mean k_{RNA} values showed little change between 5 and 25°C, staying at around 0.7 µg protein.mg RNA^{-1}.day^{-1} and resulting in a Q_{10} of 1.0. However, there was a significant difference between the means at 15 and 20°C (single factor ANOVA plus Tukey. *d.f.* = 1, $F = 6.79$, $P = 0.02$). Between 5 and 15°C, RNA:protein ratios remained at around 5.7 µg RNA.mg protein^{-1}. At 20°C, the RNA:protein ratio increased to 7.6 ± 0.4 µg RNA.mg protein^{-1}, and at 25°C, it increased further to 11.0 ± 1.2 µg RNA.mg protein^{-1}. Significant differences existed between the means at 10 and 15°C (single factor ANOVA plus Tukey. *d.f.* = 1, $F = 9.29$, $P = 0.01$), at 20 and 15°C (single factor ANOVA plus Tukey. *d.f.* = 1, $F = 21.44$, $P = < 0.001$), and at 25 and 15°C (single factor ANOVA plus Tukey. *d.f.* = 1, $F = 25.12$, $P = < 0.001$).

Comparisons between mean whole-animal k_s values of winter and summer *Ligia* (Figure 2.9) revealed significant differences due to the effects of season (Two Way ANOVA plus Tukey. *d.f.* = 1, $F = 6.29$, $P = 0.02$). For example, mean k_s of winter *Ligia* at 15°C was 1.7-times higher at 0.7 ± 0.1 *versus* 0.4 ± 0.1 %.day^{-1}, and at 20°C k_S was 5.5-times higher at 1.2 ± 0.3 *versus* 0.21 ± 0.04 %.day^{-1}. The interaction between mean k_s values of winter and summer *Ligia* was also found to be statistically significant (Two Way ANOVA plus Tukey. *d.f.* = 3, $F = 2.93$, $P = 0.04$). Mean k_{RNA} values of summer and winter *Ligia* remained similar at all temperatures, however, there were significant differences between the mean RNA:protein values of winter *versus* summer *Ligia*, allowing for any differences due to the effects of temperature (Two Way ANOVA plus Tukey. *d.f.* = 1, $F = 41.01$, $P = < 0.001$).

2.4 *Discussion*

2.4.1 *Validity of the Flooding Dose Technique*

Garlick *et al.* (1980), specified two criteria which must be fulfilled for the flooding dose technique to work. Firstly, there must be a rapid equilibration of the injected radiolabel with the intracellular free-pools, and secondly, the specific activity of the free-pools must remain constant, or show a slow, linear decline over the incorporation period. Both of these criteria were met in this study on *Ligia oceanica*, validating the use of the flooding dose technique over the injection of a single tracer dose or constant infusion method, with which there are associated difficulties. For example, the specific activities of

the amino acids can vary between compartments, making it difficult to determine the specific activity at the site of protein synthesis. As the flooding dose technique floods all precursor pools with the labelled amino acid, the difference in specific activity between the plasma and tissues is minimised (Garlick *et al.*, 1980). Also, as the specific activity of the free-pool amino acids decreases linearly with time, protein synthesis rates can be determined by measurement at just two time points (Garlick *et al.*, 1980). In contrast, the injection of a single tracer dose requires the use of a complex time course (Waterlow *et al.*, 1978), necessitating the sacrifice of many animals. One problem with the flooding-dose technique is that an individual animal can only be used once, so the monitoring of the same animal at different treatment conditions or for varying periods of time is impossible. This problem is avoided by the use of the stable isotope [^{15}N], where animals are fed with a known quantity of [^{15}N]-enriched protein (Houlihan *et al.*, 1995). Comparisons of the radioactivity of what was ingested, and what was excreted (by collecting all nitrogenous waste) reveal the proportion of radioactivity retained by the organism, allowing for an estimate of rates of protein synthesis. Apart from being an expensive and difficult technique to apply, the situation is complicated by the fact that terrestrial isopods excrete gaseous ammonia, making collection of the gaseous component of the nitrogenous waste more difficult. Another problem with the flooding-dose technique, with particular reference to whole-animal studies involves the inclusion of internal standards between sample processing. This is difficult to do where all the tissue from a whole-animal is processed, leaving no spare tissue for a standard for future protein determinations.

At all acclimation temperatures, protein-bound specific activity increased linearly with time. The mean protein-bound specific activities over the time-course were 2.5, 4.2, and 2.9 dpm.nmol^{-1} Phe for 10, 15, and 20°C, respectively. This compares well to other whole-body *in vivo* studies of protein synthesis. Whiteley *et al.* (1996), recorded protein-bound specific activities of around 2 and between 1 and 2 dpm.nmol^{-1} Phe for the marine isopods *Glyptonotus* and *Idotea* respectively. Conceição *et al.* (1997) recorded specific activities of between 9 and 28 dpm.nmol^{-1} Phe in larval turbot and Mathers *et al.* (1993) recorded activities of between 0.5 and 1.4 dpm.nmol^{-1} Phe in trout fry. The reasons for the relatively high specific activities recorded by Conceição *et al.* (1997), in larval turbot have been discussed by Houlihan (1991). The proportion of the gut relative to rest of the body is much greater in larval fish, and rates of protein synthesis in the gut are much higher. As fish grow, the relative proportions of the gut decrease, and so the proportion of high protein synthesis rates in the gut also decrease.

For the remaining determinations of fractional protein synthesis rates in *Ligia*, an incorporation time of 60 minutes was used. At this time interval, measurements of k_s were at maximum levels with high, yet stable levels of specific activity in the free-pools. Similar time intervals have been used in previous estimates of protein synthesis rates in crustaceans using the flooding dose technique. For example, Hewitt (1992), used incubation times of 45 and 60 minutes in the brown tiger prawn *Penaeus esculentus* at 30°C, and Whiteley *et al.* (1996), used 60 minutes in the eurythermal temperate isopod, *Idotea rescata*, at 4 and 14°C. The incubation period was extended to 2 hours

in the Antarctic isopod *Glyptonotus* held at 0°C, used in the same study, to compensate for the temperature related decrease in incorporation rates. A similar problem was not encountered in *Ligia* at 10°C, because k_s values remained fairly constant between 30 and 90 minutes (Figure 2.7). In each case, the time required for the incorporation of the radiolabel in crustaceans was greatly extended from the incorporation times of between 2 and 10 minutes allowed for the uptake of radiolabel in rats (Garlick *et al.*, 1980). Rates of mammalian protein synthesis have been shown to be between 4 and 21% higher than rates in crustaceans (Hewitt, 1992), as the body temperature of eutherian mammals is usually considerably higher than crustacean poikilotherms, at 37°C.

2.4.2 *Whole-Animal Rates of Oxygen Uptake*

In the present study, *Ligia oceanica* collected in the winter and acclimated to changes in temperature showed different responses in $\dot{M}O_2$ to summer animals. Comparisons of the $\dot{M}O_2$ between summer and winter *Ligia* at various acclimation temperatures showed that mean winter values were generally higher than those of summer collected animals at common acclimation temperatures (Figure 2.8). In addition, winter animals held at 25°C were unable to acclimate to this temperature, and all animals died within a week, showing a reduced thermal tolerance compared to isopods caught in the summer.

The process of acclimation is known to influence lethal or critical temperatures in many species studied to date (Brattstrom, 1979). For example, Newell *et al.*

(1976), increased the upper thermal tolerance of *Ligia oceanica* from 27.5°C to 33.4°C by acclimating animals to various temperatures for different periods of time. Moreover, the period of time required for acclimation also varied with temperature, as more time was required to achieve the same increase in thermal tolerance at lower acclimation temperatures (*i.e.* 3 weeks at 12°C) than at higher acclimation temperatures (6 days or less at 26°C). In the present study, isopods were acclimated to a specific temperature for at least 4 weeks, which has previously been shown to be sufficient time for complete acclimation to the temperature change in teleosts (Lyytikäinen and Jobling, 1998), and isopod crustaceans (Luxmoore, 1984). Bernaerts *et al.* (1987), found similar temperature-induced modifications to upper lethal limits in his study on four strains of the brine shrimp, *Artemia*. The upper lethal limits of these strains varied according to the maximum temperatures found in the water bodies they were collected from. The highest lethal limits were found in the strains which inhabited the lakes with the highest water temperatures. For example, the strains which had an upper lethal limit of 35°C came from a water body where maximum recorded water temperatures were about 35°C. Strains which had an upper lethal limit of 45°C came from water bodies where maximum water temperatures were higher. For some of the strains of *Artemia*, which were known to have originated from the same stock, the ability to adapt to an increase in temperature within two years must have meant that adaptation was already genetically encoded (Bernaerts *et al.*, 1987).

The effects of acclimation temperature on upper tolerance limits are correlated with changes in rate functions, but as the time and extent of these

relationships usually differs, it is unlikely that the effects are due to a single process of acclimation (Edney, 1964a). Organisms which have the ability to rapidly adapt to higher lethal temperatures do so by expressing different isoenzymes. In a situation where a single enzyme is unable to support eurythermia, multiple variants of the enzyme may occur with the ability to catalyse the same reaction at different environmental temperatures (Somero and Hochachka, 1976). Feder (1983), found that the effects of acclimation temperature in amphibians were mediated through the dependence on differing proportions of isoenzymes that are present at all acclimation temperatures. He outlined three molecular mechanisms underlying thermal acclimation in animals. Firstly, the synthesis of alternate isoenzymes as mentioned above. Secondly, quantitative differences in the levels of existing enzymes. Finally, through alterations in membrane phospholipids and fatty acids. For instance, cold acclimation in the rough-skinned newt, *Taricha granulosa*, had the effect of decreasing the saturation of lipids through increasing levels of stearic acid, thus increasing the fluidity of cell membranes at low temperatures (Feder, 1983). The resultant compensation effects served to allow normal physiological function over a range of temperatures.

In this study, $\dot{M}O_2$ levels in winter *Ligia* were higher than those in the summer, showing some compensation for the lower winter temperatures out in the field which persists in the lab. Newell *et al.* (1976), found regions of reduced temperature sensitivity with their studies on the oxygen uptake of *Ligia oceanica*, from between 15 and 25°C at low acclimation temperatures, to between 20 and 35°C at high acclimation temperatures (see Discussion in

Chapter 3). Wieser (1972a), in his study on the terrestrial isopods, *Oniscus asellus* and *Cylisticus convexus*, found that metabolic rate was least affected by the temperature within the range to which the isopods were adapted. The adaptive significance of having no acclimatory ability at lower temperatures may be to extend the physiological temperature range of a population during colder seasons (Edney, 1964b), and the ability to acclimate at warmer temperatures may be to moderate energy expenditure during the warmer months (Feder, 1985).

Comparing whole-animal rates of oxygen uptake in *Ligia* with other work in the literature shows values to be remarkably similar. Table 2.1 shows mean $\dot{M}O_2$ values of various terrestrial, semi-terrestrial, and marine isopod species, scaled to a standard body mass of one gram fresh weight. The somewhat higher values from Edney's (1964b) work are likely to be due to differences in the experimental set-up. A period of just 30 minutes was allowed for isopods to settle after the stress of handling, and the nutritional state of the animals was not mentioned. As Carefoot (1990a), points out, overnight acclimation of *Ligia pallasii* to the experimental conditions of the respirometry equipment is crucial for $\dot{M}O_2$ values to return to resting levels. Elevated $\dot{M}O_2$ values are not recorded exclusively in animals which are obviously active and disturbed and it can take several hours for $\dot{M}O_2$ values to return to resting values.

2.4.3 *Whole-Animal Rates of Fractional Protein Synthesis*

In contrast to whole-animal rates of oxygen uptake, whole-animal rates of protein synthesis changed little with acclimation temperature in both seasons

(Figure 2.9). However, towards the upper acclimation temperatures of both seasons, increased Q_{10} values show an increase in the rates of protein synthesis. For example, between 15 and 20°C in winter *Ligia*, the Q_{10} was 3.0, and between 20 and 25°C in the summer, the Q_{10} was 20.5. This suggests a possibility of the activation of some energy-demanding process towards the upper acclimation temperatures of both summer and winter *Ligia*, for example, the induction of the heat-shock protein response (see Chapter 4).

Acclimation appears to have shifted the rate/temperature curve (R/T) of winter collected *Ligia* to the left of the R/T graph for summer collected *Ligia*. This has the effect of reducing the temperature at which the winter animals started to show an increase in temperature sensitivity, compared to the summer animals, *i.e.*, 15°C for winter isopods, and 20°C for summer isopods. At the acclimation temperatures of 15 and 20°C, k_s values were significantly higher in the winter *versus* summer animals. The seasonal differences in protein synthesis rates can be attributed to the protein synthetic capacities, which were higher in the winter compared with the summer animals. Therefore, *Ligia* in the winter compensated for the effects of temperature on RNA activities, by increasing ribosomal RNA levels, as found in a number of other ectothermal animals during cold acclimation (Houlihan *et al.*, 1988; Houlihan *et al.*, 1993a; Houlihan *et al.*, 1995; McCarthy *et al.*, 1999; Smith *et al.*, 2000). Interestingly, this study shows that even after 4 weeks of acclimation to low temperatures, RNA:protein levels remained unchanged in summer animals. One explanation for this observation, being that other factors may also be involved in promoting this response, or simply that RNA:protein adjustments are more long-term.

By taking simultaneous measurements of $\dot{M}O_2$ and k_s in this way, it is possible to correlate rates of synthesis to rates of metabolism in response to acclimation temperature. In the winter and the summer there was a poor correlation between the two variables, with mean $\dot{M}O_2$ showing an increase with temperature, and k_s showing little increase with temperature until the upper end of the temperature range. The discrepancy between the two responses can mean one of two things: either metabolic costs of protein synthesis differ between summer and winter animals, and possibly between acclimation temperatures, or that energy is re-distributed from protein synthesis to reproduction and/or growth. These speculations will be addressed in Chapter 5. The main conclusion of the present chapter, however, is that winter animals appear to compensate for the low temperatures by increasing the capacity to synthesise proteins (*i.e.* increased RNA:protein ratios), and therefore winter animals are characterised by higher rates of k_s, especially at 15 and 20°C, even after acclimation.

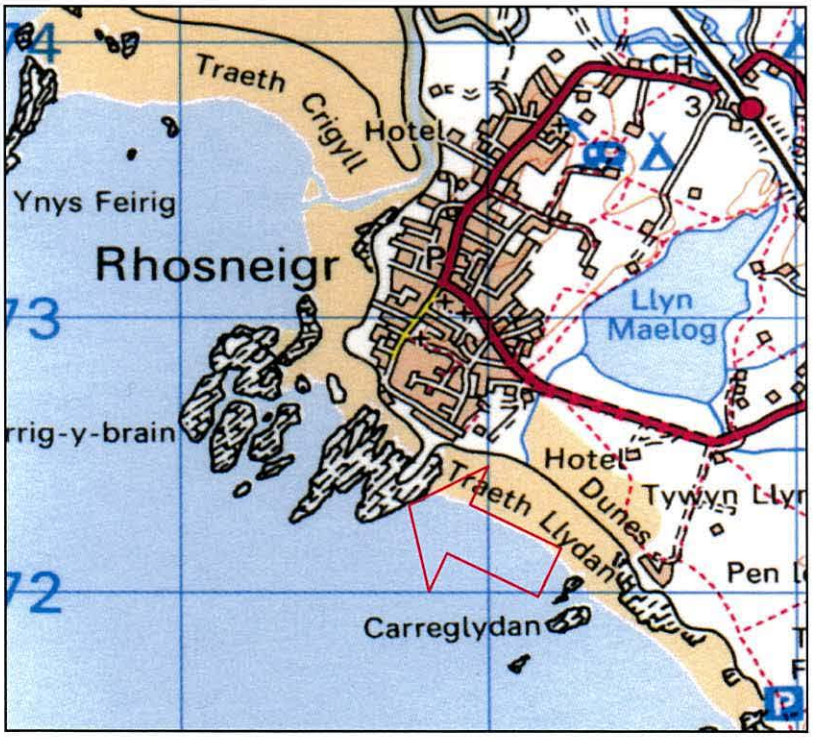
Table 2.1. Mean $\dot{M}O_2$ values of various terrestrial, semi-terrestrial and marine isopod species scaled to represent an animal of one gram fresh weight and acclimated at various temperatures using a weight exponent of 0.70 (Whiteley *et al.*, 1996). A/T refers to the acclimation temperature (°C). $\dot{M}O_2$ values are expressed as means and as $\mu mol.animal^{-1}.h^{-1}$. Q_{10} values correspond to the incremental temperature range in the A/T column (for example, 5-10°C, 10-15°C, 15-20°C).

Season	Species	n	A/T	$\dot{M}O_2$	Q_{10}	Reference
Winter 1999	*L. oceanica*	6 6 5 6	5 10 15 20	0.9 2.0 3.5 4.8	- 5.6 3.0 1.9	This Study
Summer 1999	*L. oceanica*	6	5 10 15 20 25	0.6 1.3 1.9 2.5 3.7	- 4.3 2.3 1.7 2.1	
Summer 1988-90	*L. pallasii* *P. scaber* *A. vulgare* *O. asellus*	20	15 15 15 15	2.2 1.5 2.0 2.5	-	(Carefoot *et al.*, 1990)
Winter 1973	*L. oceanica** *L. oceanica*†	17 18	5 5	0.6 1.9	-	(Newell *et al.*, 1976)
Unknown	*L. pallasii*	-	15	0.9	-	(Carefoot, 1990)
Autumn	*I. rescata*	11 19	4 14	3.0 5.9	- 2.0	(Whiteley *et al.*, 1996)
-	*G. antarcticus*	19	0	1.7	-	
Unknown	*A. vulgare*	16 24 19	10 20 30	35.6 50.3 61.1	- 1.4 1.2	(Edney, 1964)
	P. laevis	19	10 20 30	29.2 53.0 74.7	- 1.8 1.4	

* Starved for 20 days.
† Access to food for 20 days.

Figure 2.1. **(a)** Ordnance Survey Landranger Map (114) showing the location of Rhosneigr on the West coast of Anglesey (O/S grid reference: 231787, 372452). **(b)** The red arrow indicates the area of collection. One grid square is equivalent to 1 km^2. The black arrow indicates True North.

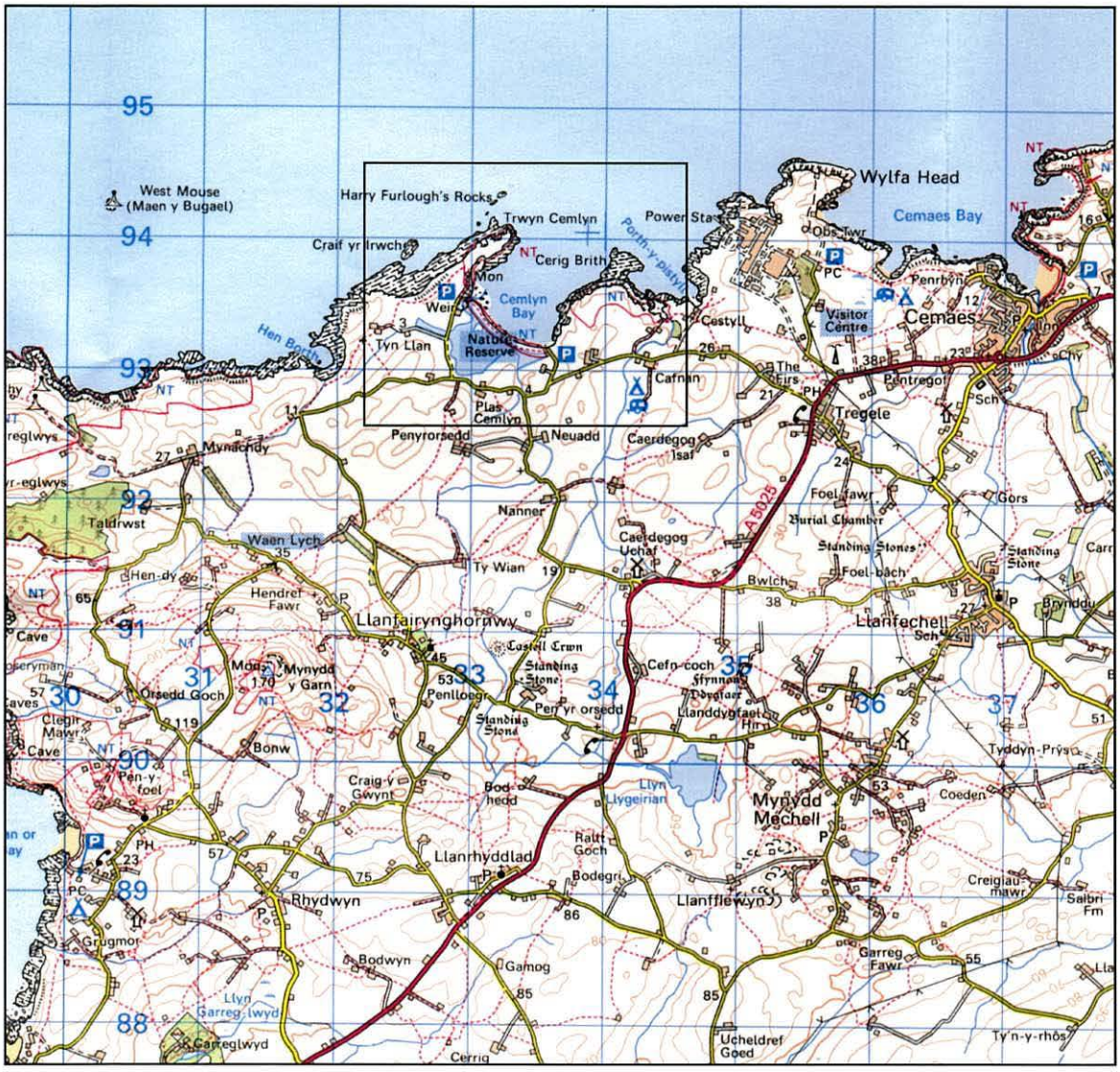

a
Llanfihangel yn Nhowyn
Fadog
Treflesg
Llanfairyneubwll
Llyn Traffwll (Reservoir)
Llyn Penrhyn
Traffwll
Llechylched
Gwaenfynydd
Settlement
Dowyn
Ynys-las
Carnau
Tai-croesion
Bodennog
Capel Gwyn
Ty Mawr
Rhosbeddau
Bryn-y-bar
Plas Cymyran
Valley Airfield
LC Tywyn Trewan Common (Cyttir Tywyn Trewan)
Cefnysgwydd Bach
Bryn Hyfryd
Eilian Ho
Glan Tywyn
Afon Crigyll
Silver Bay
Traeth Cymyran
Cymyran Bay
Dyfria
Inscribed Stone
Dunes
Pentre-traeth
Ty Newydd
Lain-wen Fm
Traeth Crigyll
Hotel
CH
Plas Llanfaelog
RHOSNEIGR
Burial Chamber
Neuadd
Pencarnisiog
Ynys Feirig
Rhosneigr
Llanfaelog
Cerrig-y-Gwyr
Llyn Maelog
Bryn Du
Treruffydd
Cerrig-y-brain
Traeth Llydan Dunes
Tywyn Llyn
Penseri
Ty'n Croes
LC
Rhosbadd
Pen Lon
Bryncian
Carreglydan
Tywyn Fferam
Ffenam-fawr
Bryngwyn
Penhenllys
Bodgedwydd
Porth Nobla
Rhosmor
Gongl-gam
Cairn
Barclodiad y Gawres Chambered Cairn (restored)
Trecastell
Henllys-fawr
Porth Trecastell
Gate House
Bodelwa
Merddyn-y-bit
Ynysoedd Duon
Plas Llangwyfan
Clafdy
29
30
31
32
34
35
36
70
71
72
73
74
75
76
77


b
Traeth Crigyll
Hotel
CH
Ynys Feirig
Rhosneigr
Llyn Maelog
rrig-y-brain
Hotel
Traeth Llydan Dunes
Tywyn Llyn
Pen
Carreglydan
72
73
74

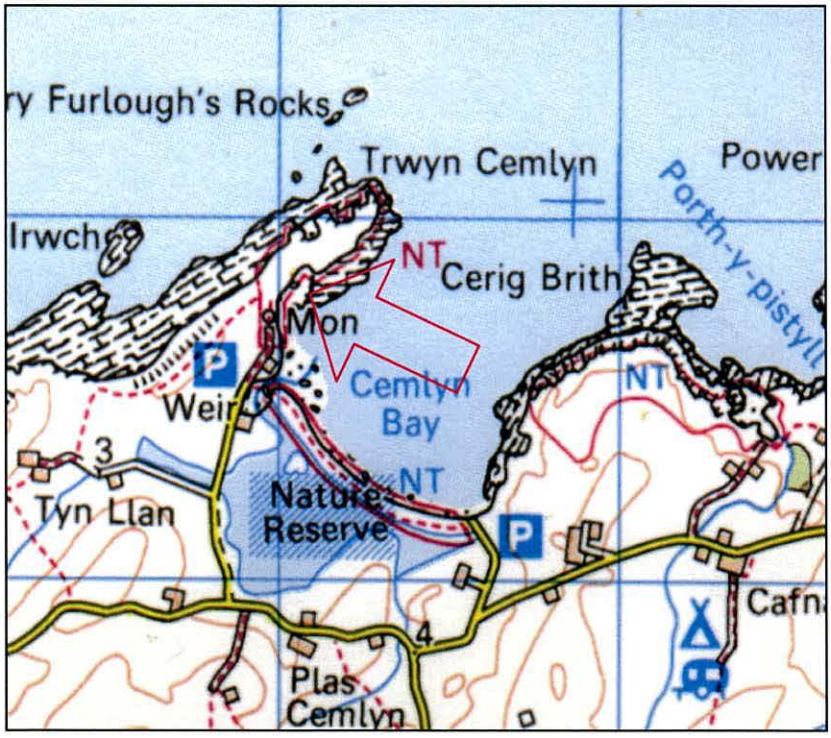
True North

Figure 2.2. **(a)** Ordnance Survey Landranger Map (114) showing the location of Cemlyn Bay on the North coast of Anglesey (O/S grid reference: 233075, 393820). **(b)** The red arrow indicates the area of collection. One grid square is equivalent to 1 km^2. The black arrow indicates True North.

a



b



True North

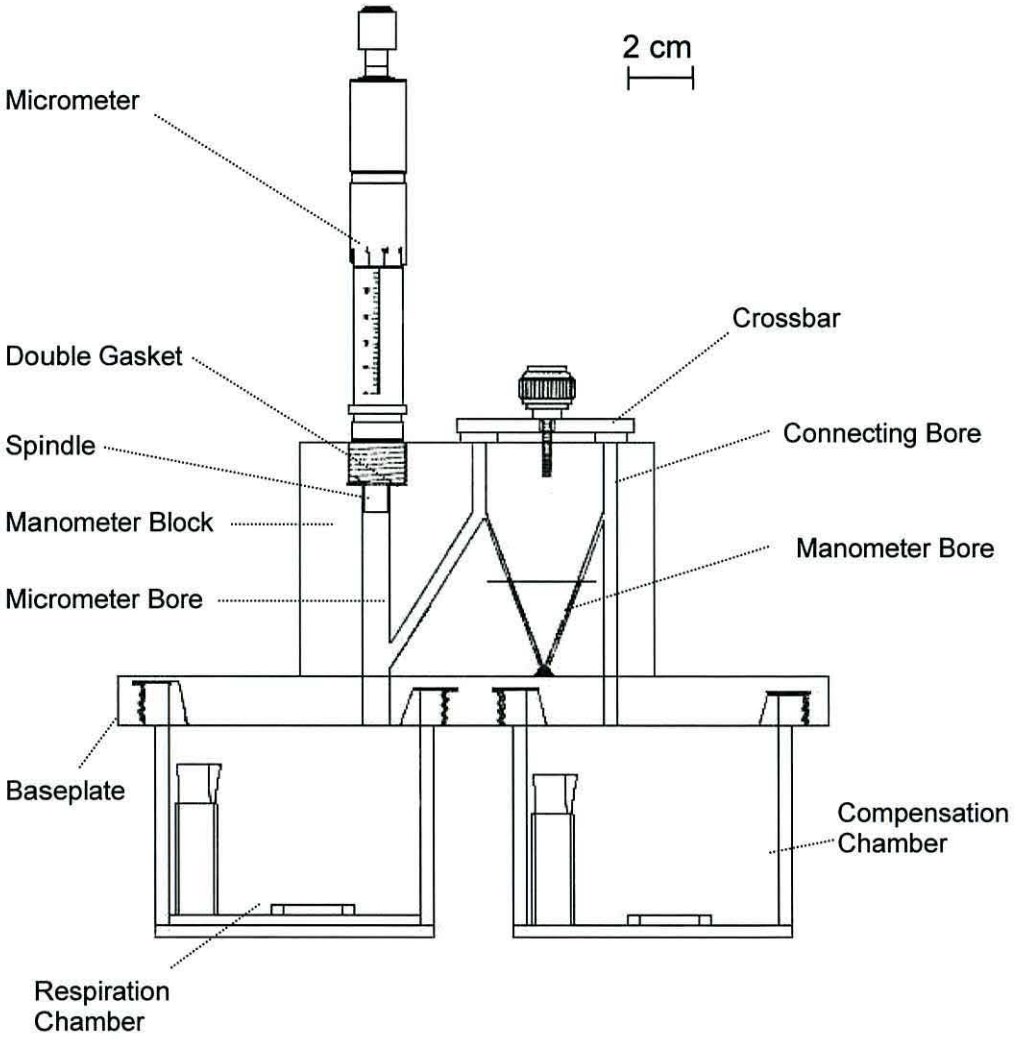


Figure 2.3. The constant pressure respirometer. Adapted from Spencer Davies (1966).

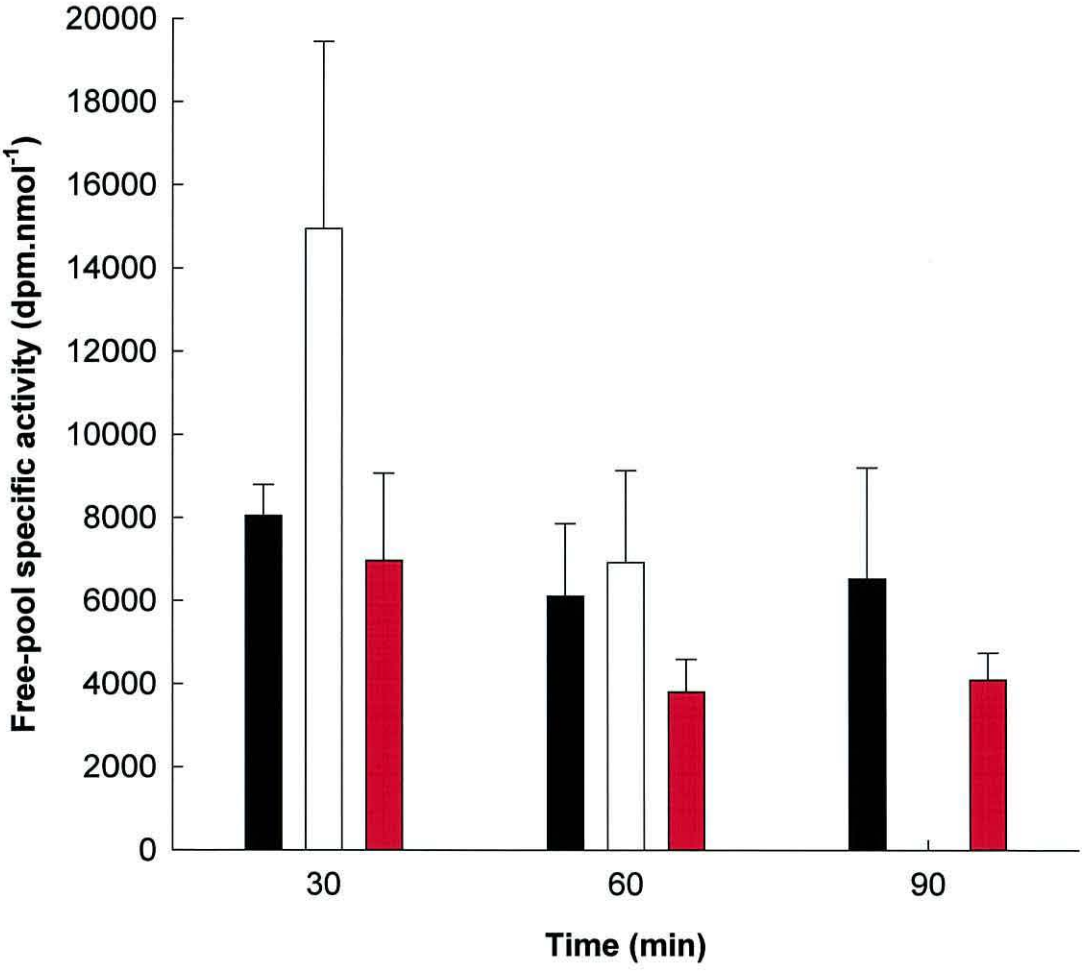


Figure 2.4. Time course of free-pool specific radioactivities in *Ligia oceanica* acclimated at 10°C (black), 15°C (white) and 20°C (red) over an incorporation period of 90 minutes (60 minutes at 15°C). At 10°C, n = 5, 5 and 4 at 30, 60 and 90 minutes, respectively. At 15°C, n = 4 and 5 at 30 and 60 minutes, respectively. At 20°C, n = 4, 5 and 4 at 30, 60 and 90 minutes, respectively. Values are means ± SE.

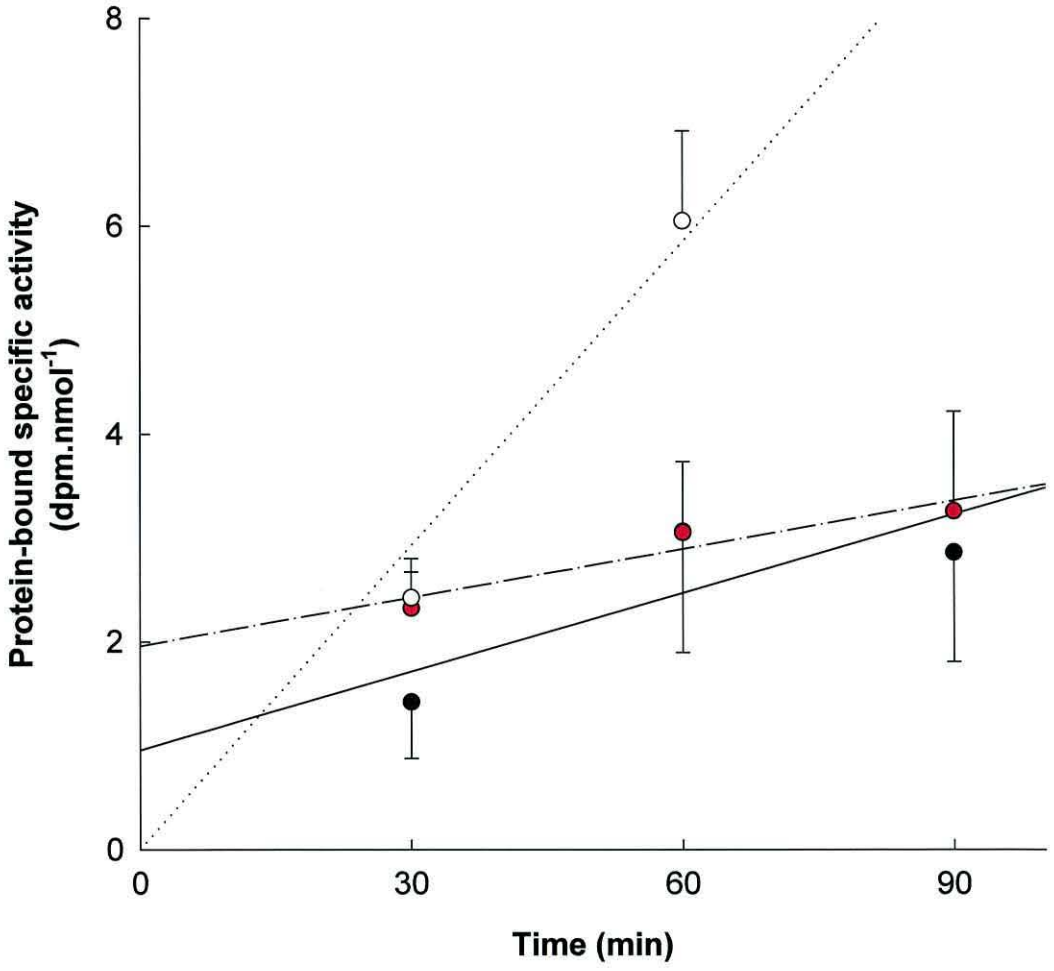


Figure 2.5. Time course of the protein-bound specific radioactivities in *Ligia oceanica* acclimated at 10°C (black circles, solid line), 15°C (white circles, dotted line) and 20°C (red circles, dashed line) over an incorporation period of 90 minutes (60 minutes at 15°C). The relationship between time and protein-bound specific activity can be described by the following linear regression equations for 10, 15 and 20°C acclimated animals, respectively: $y = 0.96 + 0.03\ x$ ($d.f.$ = 1, F = 1.26, P = 0.28, r^2 = 0.10), $y = 1.97 + 1.30\ x$, and $y = 0.02 + 1.95\ x$ ($d.f.$ = 1, F = 0.87, P = 0.37, r^2 = 0.73). The number of observations is given on Figure 2.4. Values are means ± SE.

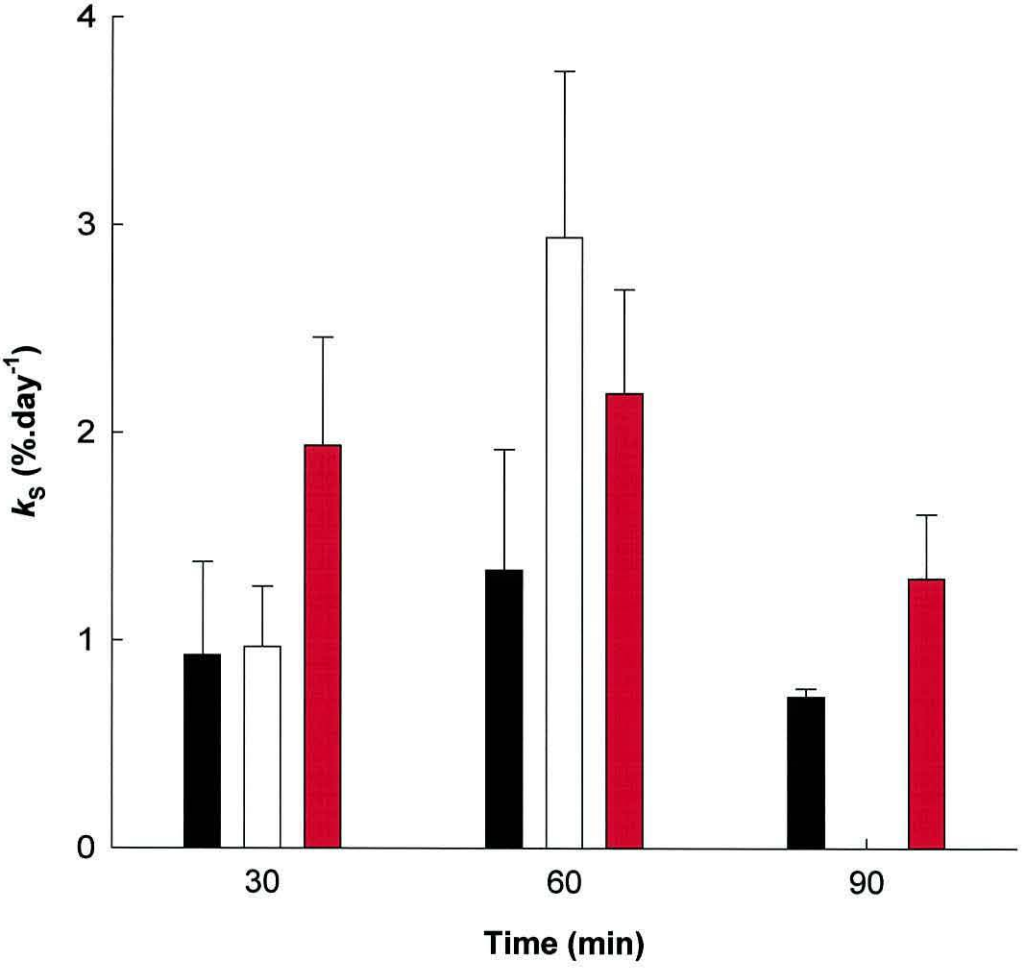
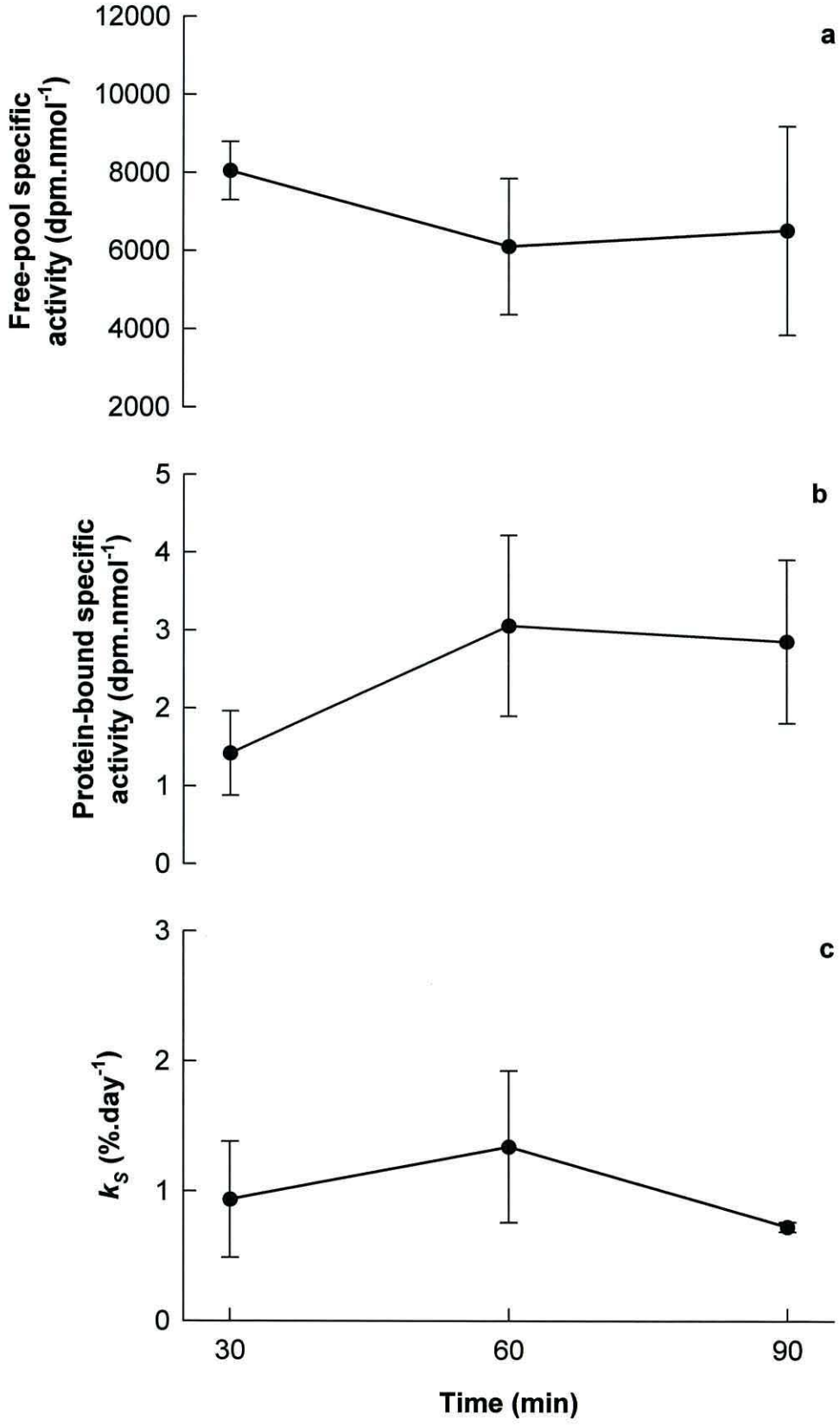


Figure 2.6. Relationship between whole-animal fractional rates of protein synthesis (k_s) in *Ligia oceanica* acclimated at 10°C (black), 15°C (white) and 20°C (red). The number of observations is the same as given on Figure 2.4. Values are means ± SE.

Figure 2.7. **(a)** Specific radioactivities of free-pool phenylalanine and **(b)** protein-bound phenylalanine 30, 60 and 90 minutes post-injection in *Ligia oceanica* acclimated at 10°C, and **(c)** corresponding fractional rates of whole-body protein synthesis (k_S). Values are means ± SE.


a
b
c
Free-pool specific activity (dpm.nmol-1)
Protein-bound specific activity (dpm.nmol-1)
ks (%.day-1)
12000
10000
8000
6000
4000
2000
5
4
3
2
1
0
3
2
1
0
30
60
90
Time (min)

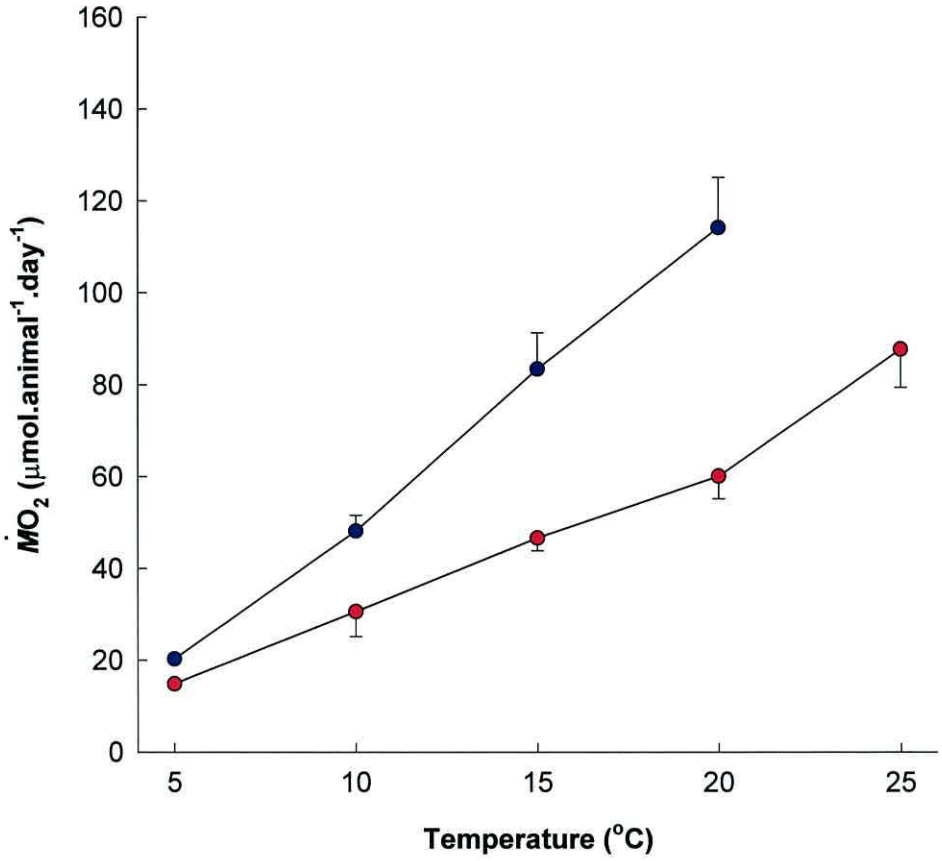
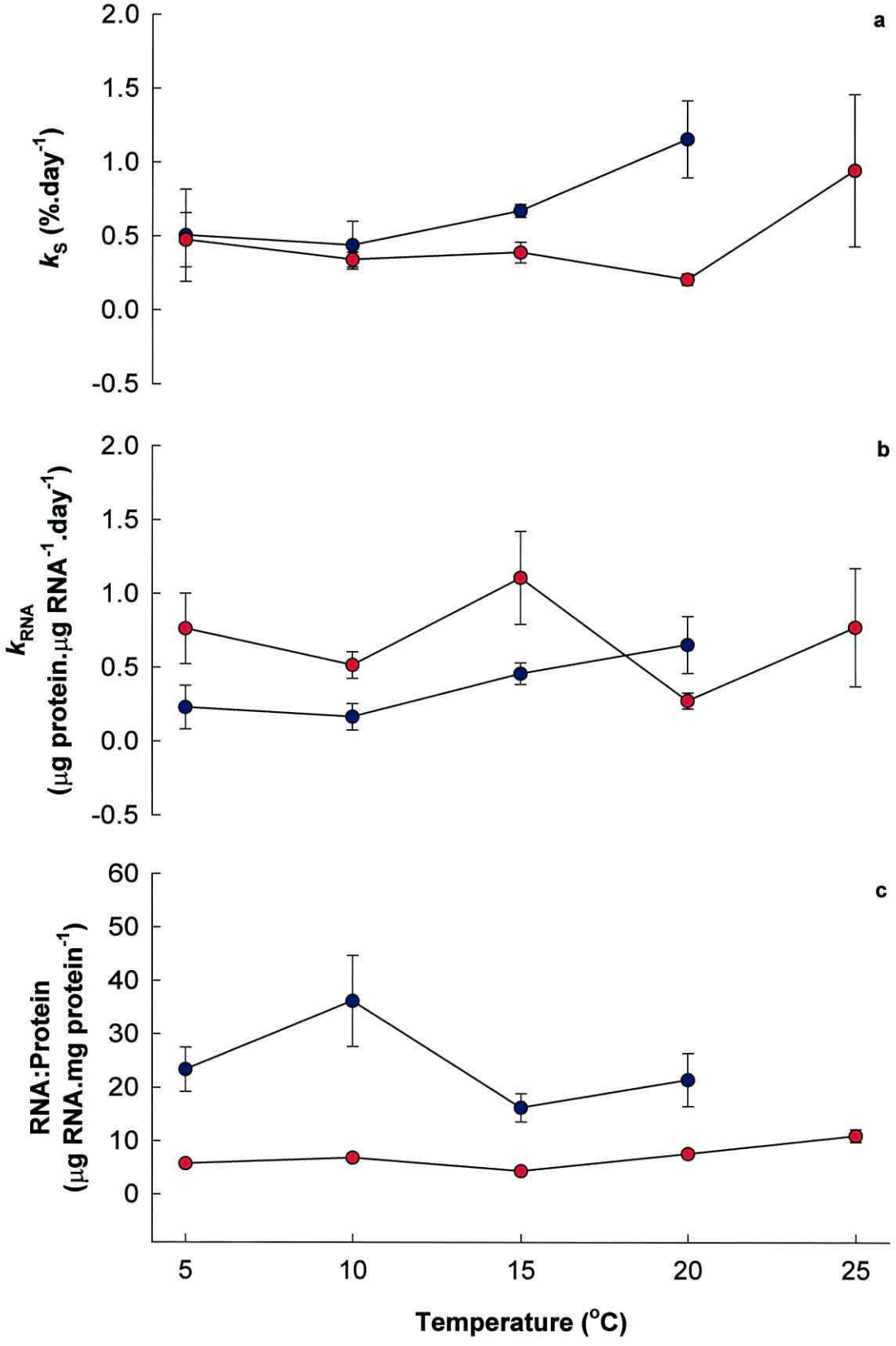


Figure 2.8. The relationship between whole-animal rates of oxygen uptake ($\dot{M}O_2$) and acclimation temperature in *Ligia oceanica* collected in the winter (blue circles) and in the summer (red circles). For both seasons at all temperatures, n = 6 except for winter animals at 15°C where n = 5. Values are expressed as means ± SE.

Figure 2.9. **(a)** Whole-animal fractional rates of protein synthesis (k_s) and associated variables, including **(b)** RNA activity (k_{RNA}) and **(c)** RNA:protein ratios for *Ligia oceanica* collected in the winter (blue circles, n = 5) and in the summer (red circles, n = 7), and acclimated to various temperatures. Values are means ± SE.


a
b
c
k_S (%.day^{-1})
k_{RNA} (µg protein.µg RNA^{-1}.day^{-1})
RNA:Protein (µg RNA.mg protein^{-1})
2.0
1.5
1.0
0.5
0.0
-0.5
60
50
40
30
20
10
0
5
10
15
20
25
Temperature (°C)

Chapter Three

Seasonal Differences in Rates of Metabolism and Protein Synthesis in *Ligia oceanica*: Effects of Acute Temperature Change and Influence on Thermal Tolerances

3.1 *Introduction*

The effects of acute changes in environmental temperature on rates of metabolism are well documented. The classic intraspecific metabolic responses of cold- and warm-acclimated ectothermic animals to acute temperature change are summarised in Figure 3.1 (Randall *et al.*, 1997). Typically, for any given temperature, the rate process of the cold-acclimated species is greater than that of the warm acclimated ectotherm. The acute response to temperature change is the first stage in temperature adaptation. The displacement of the two slopes in Figure 3.1 indicates quantitative differences in enzyme concentrations through acclimatory or acclimatisatory processes in response to chronic constant temperature exposure (Randall *et al.*, 1997). Exposure to acutely variable temperatures is probably more representative of the thermal habitat of animals living on the shore. This is especially true of temperate intertidal ectotherms which experience large and rapid diel temperature fluctuations during the summer months (Nybakken, 1997; Roberts *et al.*, 1997) (see Figure 3.2).

In contrast to the metabolic studies described above, relatively few studies have looked at the simultaneous effects of temperature on both metabolic rate and rates of protein synthesis (Fauconneau and Arnal, 1985; Mathers *et al.*, 1993; Whiteley *et al.*, 1996). Protein synthesis is known to be closely correlated with metabolism (Houlihan *et al.*, 1990b), and studies of these two temperature-dependent rate processes (by determination of whole-animal

rates of protein synthesis and oxygen uptake in response to acute temperature exposure) may reveal more about their relationship with each other. Individually, measurements of rates of oxygen uptake are important in revealing general measures of metabolic activity and in giving indications of all the metabolic processes active during the period of measurement (Clarke, 1987). However, when used in conjunction with other physiological measurements, such as rates of protein synthesis, metabolic rates can give important insights into energetics and energy partitioning (Lyytikäinen and Jobling, 1998) and may help in explaining the various patterns of the distribution of organisms.

The ability to adapt to seasonal temperature changes via capacity adaptations are important in temperate ectothermic species, which experience the relatively predictable annual thermal cycles associated with summer and winter. In particular, protein synthesis plays a crucial role in the process of thermal acclimation, as different quantities and forms of enzymes are required to operate at different temperatures leading to increased synthesis (Hochachka and Somero, 1984; Clarke, 1987; Somero and Hand, 1990; Somero, 1995). For example, increases in the concentrations of key, rate-limiting enzymes are a common feature of cold adaptation, thereby compensating for the reduced reaction rates inherent at colder temperatures. In addition to the expression of different quantities and forms of enzymes with season, growth and reproduction also tend to be restricted to the warmer months, as food is more readily available (Whiteley *et al.*, 1997). As a result, much of the seasonal variation in the physiological variables that occur in

animals between summer and winter is the result of redirection of resources towards gametogenesis, and this is especially true of female organisms (Clarke, 1987).

Previous work on crabs and isopods has shown a strong correlation between the ability to acclimate to warmer temperatures and an increase in critical thermal limits (Edney, 1964a; Stillman and Somero, 2000). To complicate matters, exposure to an alternating cycle of high and low temperatures can further increase critical thermal limits (Edney, 1964a; Edney, 1964b; Feder, 1985). This last scenario is most likely during the summer months where the temperature differential between night and daytime is far greater than during the winter months (see Figure 3.2). This situation is also of greater relevance to animals during the summer, which are far more likely to experience temperatures approaching their thermal limits.

As season can have a profound effect on the thermal responses of ectotherms to acute temperature change, the following chapter characterises the response of whole-animal rates of oxygen uptake and protein synthesis in *Ligia oceanica* caught in the summer and winter. Acute responses were examined in animals straight from the shore in both winter and summer, and in animals caught in the winter and acclimated at 15°C for 4 weeks, to examine the immediate effects of temperature change and the associated effects of any acclimatory response. Acute changes in temperature are more in keeping with the situation on the shore when the animals are exposed to

rapid changes in environmental temperature, and will therefore provide an insight into the response of protein metabolism to *in situ* conditions.

3.2 *Materials & Methods*

Three groups of *Ligia oceanica* were used to investigate the effects of short-term, acute changes in temperature on whole-animal rates of protein synthesis and oxygen uptake. In group one, animals were collected in June 2000 and held overnight without food at the ambient *in situ* air temperature (20°C). The second group were collected in January 2001 and were also held overnight without food at the ambient air temperature at the time of collection (5°C). The third group of animals were also collected in January 2001, but acclimated for 4 weeks at 15°C prior to experimentation, as explained in Chapter 2.

For the measurement of rates of oxygen uptake, animals were transferred to the respiration chambers of the respirometers 24 hours before measurements were due to be taken, and maintained in an open system at the *in situ* temperature. This allowed time for the animals to settle after the stress of handling (Smit *et al.*, 1971; Ivleva, 1973; Aldrich, 1975; Ralph and Maxwell, 1977; Whiteley *et al.*, 1996). The respiration chambers were then connected to the manometer block, and held for 90 minutes at the experimental temperature with the respirometers still unsealed. This allowed time for thermal equilibration of the respiration and compensation chambers. The

crossbar was then closed, and respirometry measurements were taken in the following 30 minutes, after which time the [^{3}H] Phe cocktail was injected into the animals. In each case the animals were left for a further 60 minutes before being snap frozen in liquid N_2.

3.2.1 *CTMax Experiments*

Ligia oceanica were collected from Rhosneigr on the West coast of Anglesey and returned to the University of Wales Bangor. Animals were held overnight at the *in situ* ambient air temperature recorded at the time of collection (summer 2000 = 15°C, n = 14, and winter 2001 = 5°C, n = 16). Animals were placed collectively in a clear Perspex container approximately 150x200x100mm (WxLxH) which was then submerged in a constant temperature water bath. Starting from the ambient temperature of collection, the temperature was raised by 1°C every 10 minutes, a rate of temperature increase that is faster than would occur in the field (see Figure 3.2). A digital thermometer with a remote sensor was placed in the container to independently determine ambient air temperature. Animals were carefully inspected at each temperature increment, and tested to see if they had lost the righting reflex by overturning them with a pointer (Lagerspetz and Bowler, 1993). Any animals that had lost the righting reflex were removed and placed in a recovery container at the ambient collection temperature.

3.2.2 *Testicular Index*

Forty *Ligia oceanica* were collected from the rocky shores of Rhosneigr. Twenty were collected in January 2001 and another twenty in June 2001.

They were immediately brought back to the University of Wales, Bangor where they were blotted dry prior to their testes being removed and weighed. The ratio of the testicular weight to the body weight was expressed as a percentage of the body weight.

3.3 *Results*

3.3.1 *Whole-Animal Rates of Oxygen Uptake*

3.3.1.1 *Acclimatised Ligia oceanica*

There was a steady increase in whole-animal rates of oxygen uptake in summer acclimatised animals with temperature (Figure 3.3), with an overall Q_{10} value of 2.3. Significant differences from mean $\dot{M}O_2$ levels at 20°C (106.5 ± 6.4 µmol.animal^{-1}.day^{-1}) were observed at all other temperatures (single factor ANOVA plus Tukey. 5 and 20°C: *d.f.* = 1, *F* = 109.67, *P* = < 0.001; 10 and 20°C: *d.f.* = 1, *F* = 30.46, *P* = < 0.001; 15 and 20°C: *d.f.* = 1, *F* = 11.15, *P* = 0.007; 25 and 20°C: *d.f.* = 1, *F* = 17.36, *P* = 0.002). The highest rates of oxygen uptake were recorded at 25°C (170.7 ± 14.0 µmol.animal^{-1}.day^{-1}).

Figure 3.3 also shows an increase in $\dot{M}O_2$ with temperature in winter acclimatised animals, with values increasing showing very similar increases to those of summer *Ligia* between 5 and 20°C. Significant increases from mean $\dot{M}O_2$ at 5°C were recorded for all other temperatures except between 5 and 10°C (single factor ANOVA plus Tukey. 5 and 15°C: *d.f.* = 1, *F* = 16.54, *P* =

0.002; 5 and 20°C: *d.f.* = 1, F = 15.71, P = 0.003; 5 and 25°C: *d.f.* = 1, F = 176.81, P = < 0.01). The Q_{10} between 5 and 20°C was 2.2, but a dramatic increase in oxygen uptake at 25°C where $\dot{M}O_2$ was at its highest (264.2 ± 18.5 µmol.animal^{-1}.day^{-1}) gave a Q_{10} of 5.7 between 20 and 25°C.

Summer and winter caught *Ligia* had similar mean $\dot{M}O_2$ values between 5 and 20°C, where Q_{10} values for both summer and winter animals were 2.2 (Figure 3.3). A dramatic increase in rates of oxygen uptake in winter *Ligia* between 20 and 25°C was mirrored by a large Q_{10} value (5.7 *versus* 2.6 for winter and summer *Ligia*, respectively). There were significant seasonal differences between mean values of summer and winter *Ligia* when the effects of temperature were accounted for (two way ANOVA plus Tukey. *d.f.* = 1, F = 12.03, P = 0.001). Similarly, there were significant temperature differences between mean values of summer and winter *Ligia* when the effects of season were accounted for (two way ANOVA plus Tukey. *d.f.* = 4, F = 73.36, P = < 0.001). The interaction between season and temperature was also significant (two way ANOVA plus Tukey. *d.f.* = 4, F = 5.35, P = 0.001).

3.3.1.2 *Acclimated Ligia oceanica*

The relationship between $\dot{M}O_2$ and exposure to acute temperature change in winter collected *Ligia*, acclimated at 15°C is shown in Figure 3.4. Rates of oxygen uptake increased steadily with temperature between 5 and 20°C, where mean $\dot{M}O_2$ values increased from 28.7 ± 2.54 to 87.6 ± 13.7 µmol.animal^{-1}.day^{-1}, respectively, to give a Q_{10} value of 2.1. There was a

dramatic increase in $\dot{M}O_2$ between 20 and 25°C, where mean $\dot{M}O_2$ almost doubled (173.2 ± 16.3 µmol.animal^{-1}.day^{-1} at 25°C), giving a Q_{10} of 3.9. There were significant differences between the mean values at 5 and 15°C (single factor ANOVA plus Tukey. *d.f.* = 1, F = 44.31, P = < 0.001), and between the mean values at 25 and 15°C (single factor ANOVA plus Tukey. *d.f.* = 1, F = 33.14, P = < 0.001).

When *Ligia* collected in the winter and acclimated at 15°C are compared with acclimatised animals from the same season, there was little difference in mean $\dot{M}O_2$ values between 5 and 20°C (Figure 3.4), giving Q_{10} values of 2.1 and 2.2, respectively. At 25°C, there was a dramatic increase in rates of $\dot{M}O_2$ for both acclimatised and acclimated *Ligia*, giving Q_{10} values of 5.7 and 3.9, respectively. Acclimatised *Ligia* had $\dot{M}O_2$ values 1.5-times higher than acclimated animals, with mean $\dot{M}O_2$ being 264.2 ± 18.5 and 173.3 ± 16.3 µmol.animal^{-1}.day^{-1}, respectively. Analysis of the effect of season on the differences between the mean values of acclimated and acclimatised *Ligia* showed these differences were significant (two way ANOVA plus Tukey. *d.f.* = 1, F = 12.54, P = < 0.001). The effects of temperature on the mean values between acclimated and acclimatised animals were also significant (two way ANOVA plus Tukey. *d.f.* = 4, F = 60.57, P = < 0.001). The interaction between season and temperature was also significant (two way ANOVA plus Tukey. *d.f.* = 4, F = 3.67, P = 0.011).

3.3.2 *Whole-Animal Fractional Rates of Protein Synthesis*

3.3.2.1 *Acclimatised Ligia oceanica*

Figure 3.5 shows the relationships between k_s, and the closely associated variables of RNA activity (k_{RNA}) and RNA:protein ratio with temperature for summer and winter acclimatised animals. None of the three dependent variables in summer acclimatised *Ligia* showed any significant change from the mean at 20°C. Mean k_s ranged between 2.3 ± 0.6 %.day^{-1} at 20°C to 5.7 ± 1.5 %.day^{-1} at 15°C. Q_{10} between 5 and 25°C was 1.0. Mean k_{RNA} remained at around 3.5 µg protein.µg RNA^{-1}.day^{-1} with a Q_{10} of 1.0 between 5 and 25°C, and the RNA:protein ratio remained at around 11.3 µg RNA.mg protein^{-1}. Winter acclimatised *Ligia* also showed no significant changes in any of the dependent variables from their respective means at 5°C with temperature. Mean k_s ranged between 0.3 ± 0.1 %.day^{-1} at 25°C to 3.1 ± 2.2 %.day^{-1} at 5°C, giving a Q_{10} of 0.3. Mean k_{RNA} ranged between 0.1 ± 0.02 µg protein.µg RNA^{-1}.day^{-1} at 25°C, to 2.6 ± 1.8 µg protein.µg RNA^{-1}.day^{-1} at 5°C, giving a Q_{10} of 0.2. The RNA:protein ratio remained at around 13.4 µg RNA.mg protein^{-1} between 5 and 20°C, with a slight but non-significant increase to 20.5 ± 4.2 µg RNA.mg protein^{-1} at 25°C.

In general, k_s values between animals collected in the summer and the winter were similar (Figure 3.5), except at 15 and 25°C. At 15°C, k_s of summer collected *Ligia* was 4.4-times higher than that of winter collected animals, with mean values of 5.7 ± 1.5 *versus* 1.3 ± 0.5 %.day^{-1}, respectively. At 25°C, k_s of summer collected *Ligia* was 10.6-times higher than that of winter collected animals (3.2 ± 0.6 *versus* 0.3 ± 0.1 %.day^{-1}, respectively). There was a

significant difference between the mean k_s values of summer and winter *Ligia* as a function of season (two way ANOVA plus Tukey. *d.f.* = 1, *F* = 7.91, *P* = 0.008). Mean k_{RNA} values were similar at all temperatures except at 25°C, where summer RNA activity was 24-times higher than winter activity (3.4 ± 0.4 *versus* 0.1 ± 0.02 µg protein.µg $RNA^{-1}.day^{-1}$, respectively). A significant difference existed between the mean k_{RNA} values of summer and winter *Ligia* as a function of season (two way ANOVA plus Tukey. *d.f.* = 1, *F* = 11.86, *P* = 0.001). Mean RNA:protein ratios were also similar at all temperatures, except for 25°C, where winter values 2.2-times higher (20.5 ± 4.2 *versus* 9.4 ± 0.9 µg RNA.mg $protein^{-1}$, respectively). However, there was a significant difference between mean RNA:protein ratios of summer and winter *Ligia* as a result of season (two way ANOVA plus Tukey. *d.f.* = 1, *F* = 6.61, *P* = 0.01).

3.3.2.2 *Acclimated Ligia oceanica*

Winter acclimated animals showed an increase in k_S with increasing temperature (Figure 3.6), with mean values at all temperatures significantly different from those at 15°C (single factor ANOVA plus Tukey. 5 and 15°C: *d.f.* = 1, *F* = 23.21, *P* = < 0.001; 10 and 15°C: *d.f.* = 1, *F* = 7.40, *P* = 0.02; 25 and 15°C: *d.f.* = 1, *F* = 12.82, *P* = 0.01). Q_{10} between 5 and 15°C was 2.9. Mean k_{RNA} values similarly increased with temperature, increasing three-fold between 5 and 10°C with a Q_{10} of 3.6. Mean values at 5 and 25°C were significantly different from the mean value at 15°C (single factor ANOVA plus Tukey. 5 and 15°C: *d.f.* = 1, *F* = 14.45, *P* = 0.003; 25 and 15°C: *d.f.* = 1, *F* = 9.52, *P* = 0.02). The RNA:protein ratio remained unchanged (around 16.3 µg

RNA.mg protein^{-1}) at all temperatures except for a significant decrease at 10°C (12.9 ± 0.8 µg RNA.mg protein^{-1}).

A comparison between k_s and the associated variables of k_{RNA} and RNA:protein ratio of winter acclimated and winter acclimatised *Ligia oceanica*, is given in Figure 3.6. The animals acclimated to temperature change were the only group to show an increase in k_s with increasing temperature, with a Q_{10} of 2.9 between 5 and 25°C. At 5°C, mean k_s values of acclimated *Ligia* were 6-times lower than corresponding values for acclimatised animals (0.5 ± 0.1 *versus* 3.1 ± 2.2%.day^{-1}). At 15°C, k_s values for both groups were similar with mean values of 1.3 ± 0.5 %.day^{-1} for acclimatised animals, and 1.3 ± 0.2 %.day^{-1} for acclimated animals. k_{RNA} values showed a similar trend to k_s values. Q_{10}'s for acclimated and acclimatised animals between 5 and 25°C were 3.6 and 0.2 respectively. RNA:protein ratios were greater in acclimated animals between 5 and 10°C, but less at 25°C.

The relationship between absolute rates of protein synthesis (A_s) and $\dot{M}O_2$ in winter collected, 15°C acclimated *Ligia* is shown in figure 3.7. The relationship can be described by the linear regression equation: $y = 63.34 + 44.09\ x$ (r^2 = 0.57, *d.f.* = 14.79, $P = 0.003$). A correlative estimation of the metabolic costs of whole animal rates of protein synthesis reveal that 264.2 mmol ATP.g protein^{-1} synthesised are required in winter *Ligia*.

3.3.3 *CTMax*

Figure 3.8 shows the relationship between temperature and the loss of the righting reflex in animals collected in both seasons. Winter animals began to lose the righting reflex at 33°C compared to 33.8°C for summer animals. 50% of the righting reflex was lost at 34.2 and 36.3°C for winter and summer animals, respectively, a difference of 2.1°C. The temperature at which all animals had lost the righting reflex was 34.9 and 37.4°C for winter and summer animals, respectively, a difference of 2.5°C. The range of temperatures over which the righting reflex was lost was 2.9°C for winter animals, and 4.4°C for summer animals.

3.3.4 *Testicular Index*

The differences between the testicular indices of summer and winter *Ligia* were highly significant ($P = < 0.005$). The testicular index of *Ligia* from the summer (n = 20) was 0.7 ± 0.1% and from the winter (n = 20) it was 0.5 ± 0.04% (the proportion of the testicular weight to body mass).

3.4 *Discussion*

The responses of both summer and winter acclimatised *Ligia* to acute temperature changes were different when tested at 25°C. In the present study, oxygen uptake values in winter acclimatised *Ligia* between 5 and 20°C, were similar, but generally higher to those of summer acclimatised animals. This observation agrees with results of earlier studies on the effects of acute

temperature changes on rate processes where cold acclimated or winter animals have higher $\dot{M}O_2$ levels than summer or warm acclimated animals at common temperatures (Marzusch, 1952; Dawson and Bartholomew, 1956; Precht *et al.*, 1973; Armitage and Lei, 1979; Prosser, 1986). Winter acclimatised *Ligia* were able to respond to acute exposure at 25°C with a dramatic increase in $\dot{M}O_2$, possibly indicating the switching on of some energetically demanding process, such as heat-shock protein production for example, as this was probably approaching the upper limit of the temperature range, as shown by the CTMax values. The $\dot{M}O_2$ of summer *Ligia* also increased between 20 and 25°C, although not to the same extent as with winter *Ligia* (as indicated by comparison of the Q_{10} values: 2.6 *versus* 5.7 for summer and winter *Ligia*, respectively). An exposure temperature of 25°C is more likely to occur with summer *Ligia*, as this scenario is highly improbable in the winter, where temperatures rarely exceed 10°C (Figure 3.2).

Increased metabolic rates in animals chronically exposed to low temperatures are a common feature of ectotherms from temperate latitudes (Huey and Bennett, 1990). As *Ligia* are still active during the winter months, it would be advantageous for them to be able to adapt to temperature change and maintain metabolic performance and energy production, even when temperatures are reduced. *Ligia* collected during the winter had increased rates of oxygen uptake in response to acute temperature changes over the temperature range (5 - 25°C) compared to summer collected animals. It must be remembered that the initial period after a temperature change is very unstable with metabolic rates undergoing many changes (Ivleva, 1973). More

specifically, the initial period of temperature change usually involves an overshoot, followed by a period of stabilisation which can last from minutes to hours (Precht, 1958).

Studies comparing acclimated with acclimatised animals are few and far between, making comparisons with other work very difficult. Even though acclimatised animals are not acclimated to a specific temperature, they have never-the-less, experienced chronic temperature exposure. The measurement of rate functions in response to acute temperature change often yields highly variable results. The reasons are numerous, but include the lack of knowledge concerning the thermal history of the animal in question (Edney, 1964b), which is particularly appropriate for acclimatised animals. Although the immediate and precise thermal history of acclimatised animals is unknown, animals collected in the summer are known to have been exposed to higher mean temperatures and to a greater range of temperature variation than animals collected in the winter, as shown by comparisons of the air temperatures recorded from the shore over a 7 day period in the summer (see Figure 3.2). However, the situation is complicated, and other exogenous factors such as day length, humidity and food availability will also influence the physiological status of the acclimatised isopods.

Many investigations on the physiological differences brought about by the change in season have been carried out on teleosts (Dawson and Grimm, 1980; Marchant and Peter, 1986; Montecchia *et al.*, 1990; Schuter and Post, 1990; Kime *et al.*, 1991; Conover, 1992; Lenhardt, 1992; Foster *et al.*, 1993a;

Encina and Granado-Lorencio, 1997), but the results are not always consistent. Interestingly, many of the seasonal differences in metabolic rate in teleost fish may be related to gonadal development (Beamish, 1964; Johnston, 1993; Paul *et al.*, 1993). Johnston (1993), found significant changes in the oxygen uptake rates of sexually mature summer and winter acclimatised *Notothenia coriiceps*, an Antarctic teleost, with summer animals having higher oxygen uptake rates. Juvenile fish, however, showed no differences in oxygen uptake between seasons (Johnston, 1993). Also, fish acclimated to simulated summer and winter conditions showed similar rates of oxygen uptake to their acclimatised counterparts (Johnston, 1993). In contrast, Beamish (1964) found seasonal variations in the oxygen uptake of male brook and brown trout. Rates of oxygen uptake increased from a relatively low value in March/April, to maximum levels in October/November. Beamish found that the increase in the standard metabolic rate corresponded with the start of the spawning period and the maturation of the gonads. Paul *et al.* (1993), found the whole-body energy content of male and female yellowfin sole was at its lowest at the end of the overwintering phase, and at its highest during the spawning period. However, the ovarian index was at its highest just prior to spawning, but the testicular index was at its highest during the winter, and at its lowest during spawning. Consequently, the high metabolic rates coincided with maturation of the ovaries rather than the testes. In the present study, only male isopods were studied. In the males there were significant differences ($P = < 0.005$) in the ratio of testicular weight to body weight between *Ligia* collected in the summer and in the winter ($0.7 \pm 0.1\%$ *versus* $0.5 \pm 0.04\%$, $n = 20$). *Ligia* are essentially semelparous, with breeding mainly

confined to the early spring (March and April) (Sutton *et al.*, 1984; Willows, 1984; Willows, 1987b). However, second broods can be produced as late as May, and as such, the higher testicular index of summer animals may indicate that they were probably reproductively active. However, it is not known to what extent testicular development affected whole-animal rates of protein synthesis and oxygen uptake in male *Ligia*.

In addition to the changes in metabolic rate imposed by the reproductive cycle, seasonal changes in metabolism can also be influenced by food availability. For example, Lehtonen (1996), showed that seasonal variation in the metabolism of a benthic amphipod, *Monoporeia affinis*, was related to the availability of food and higher metabolic rates were recorded when food was more readily available. Karås (1990), found that juvenile perch had standard metabolic rates (SMR) reduced by as much as 50% in winter, compared to animals in the summer when day lengths were at a maximum. He concluded that reduced food availability may cause this winter adaptation of reduced SMR to cover the fundamental energetic costs of maintenance, and that as a consequence of low metabolic costs and reduced food availability, this increased the ability for survival. Changes in day length are also thought to be important, as Whiteley *et al.* (1997), showed that day length and temperature are important cyclical factors of seasonality in crustaceans, which impose seasonal variations on growth and reproduction. Both variables were confined to the warmer months, when temperatures were higher, day length was increased, and food was more readily available. Luxmoore (1981), found that growth rates were high during the summer season in temperate isopods, with

moulting forming a major component of energy expenditure, and that summer was also the period when reproduction occurred, with juvenile isopods being released at a time of maximum food availability. Variation in food availability may be a common occurrence for *Ligia*, and Willows (1987a) suggested that different populations of *Ligia* may experience an unpredictable supply of water-borne detritus, thereby becoming more critically reliant on the seasonally variable energy fixed locally by autotrophs. In addition, biochemical differences have been observed. Hofmann and Somero (1995), showed seasonal differences in levels of ubiquitin conjugates and hsp70 collected from gill tissue in the mussel, *Mytilus trossulus*. Levels of both were higher in mussels collected during the summer than in those collected during the winter. It would appear to be advantageous to have a certain amount of temperature independence during the winter months. Although there may be occasions when temperatures rise to unexpected levels for winter, a concomitant increase in metabolic rate could prove detrimental to an animal's ability to survive when food availability remains poor.

Winter collected *Ligia* acclimated to 15°C, were the only group to increase rates of protein synthesis with increasing temperature. The summer and winter acclimatised groups both appeared insensitive to acute temperature change. Summer collected animals showed no increases in either RNA activity or the RNA:protein ratio in response to acute temperature change, and rates of protein synthesis remained stable, showing temperature independence. Winter collected animals showed no change in either RNA activity with increasing temperature or in the RNA:protein ratio. Winter

collected *Ligia* acclimated at 15°C showed no change in their RNA:protein ratios, but significant increases in RNA activity from the mean values at 5°C occurred as temperatures rose. Foster *et al.* (1992), showed similar fractional rates of protein synthesis between cold and warm acclimated juvenile cod. These authors suggested that increased tissue RNA concentrations and reduced RNA activity in cold-acclimated juvenile cod were compensatory mechanisms for maintaining rates of protein synthesis at lower temperatures. Winter acclimatised *Ligia* were characterised by an increase in protein synthetic capacity which maintained rates of protein synthesis at levels close to those found in summer acclimatised animals, despite their lower RNA activities. As temperature increased, RNA activities increased. As RNA levels remained fairly constant over the temperatures tested, the increase in protein synthesis rates must have been attributable to the increase in the RNA capacity. Therefore, *Ligia* demonstrated an ability to make seasonal adjustments in k_s, but demonstrated no ability to make short-term adjustments in k_s in response to acute temperature change.

Ligia collected during the summer showed a 2.1°C increase in their critical thermal maxima (CTMax), established by loss of the righting reflex (Lagerspetz and Bowler, 1993), compared to animals from the winter (36.3°C *versus* 34.2°C). Similar observations have been made in other invertebrates where acclimation or acclimatisation to a higher temperature led to an increase in the CTMax (Edney, 1964a; Cossins and Bowler, 1987; Layne *et al.*, 1987; Lagerspetz and Bowler, 1993). The thermal plasticity of the CTMax has previously been demonstrated on other terrestrial isopod species by

Edney (1964a), who acclimated *Porcellio laevis* and *Armadillidium vulgare* at 10 and 30°C. The CTMax of both species increased, from 37.4°C to 41.6°C in *P. laevis* and from 38.3°C to 41.6°C in *A. vulgare* at the higher acclimation temperatures, showing that thermal tolerances change with season, as examined in the following chapter.

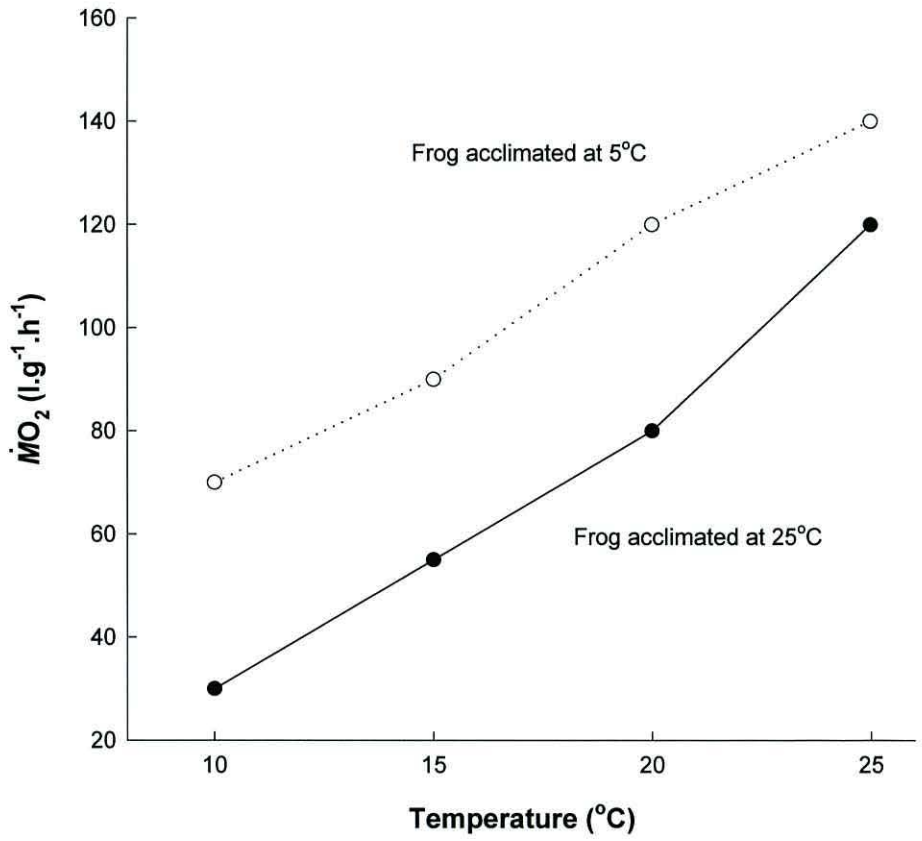


Figure 3.1. The classic effects of acute temperature change on metabolic rate in a typical warm- and cold-acclimated temperate ectotherm. Adapted from Randall *et al.* (1997).

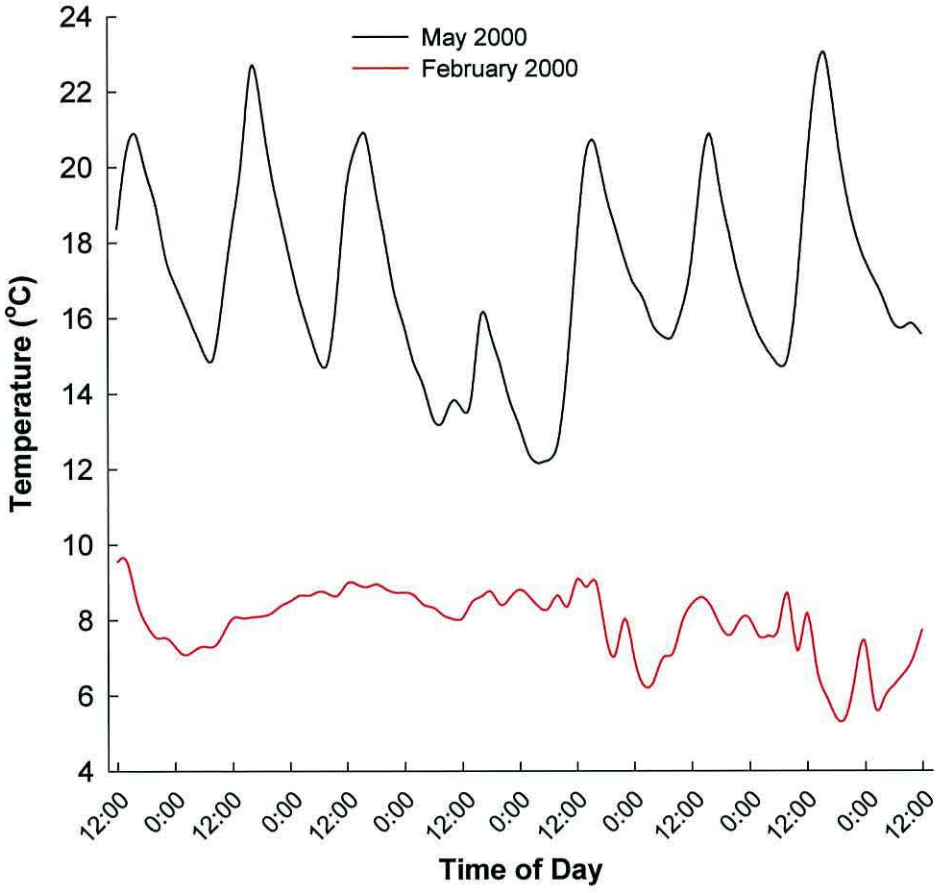


Figure 3.2. Variation in diel temperatures at Rhosneigr, Anglesey. The mean temperature over this period in February was 7.91°C ± 0.10, and 17.06°C ± 0.28 in May. Maximum and minimum recorded temperatures were 9.55°C and 5.40°C, respectively, in February, and 23.00°C and 12.15°C, respectively, in May. Temperatures were measured using a Hugrun 'Seamon Mini' Temperature Data Logger. The data logger was buried in the shingle where isopods were known to aggregate and left for two, 7 day periods. Once in February 2000, and again in May 2000.

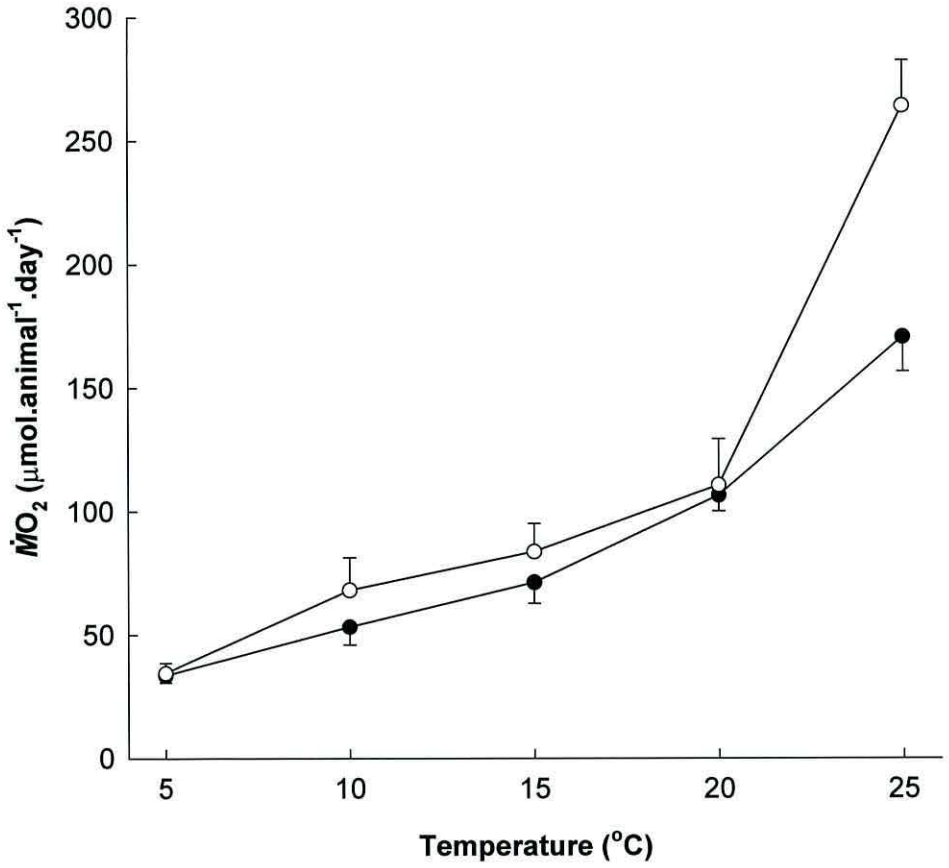


Figure 3.3. Relationship between acute temperature change and whole-animal rates of oxygen uptake ($\dot{M}O_2$) in winter acclimatised (white circles) versus summer acclimatised (black circles) *Ligia oceanica*. n = 6 for each data point except for winter animals at 25°C where n = 5. Values are expressed as means ± SE.

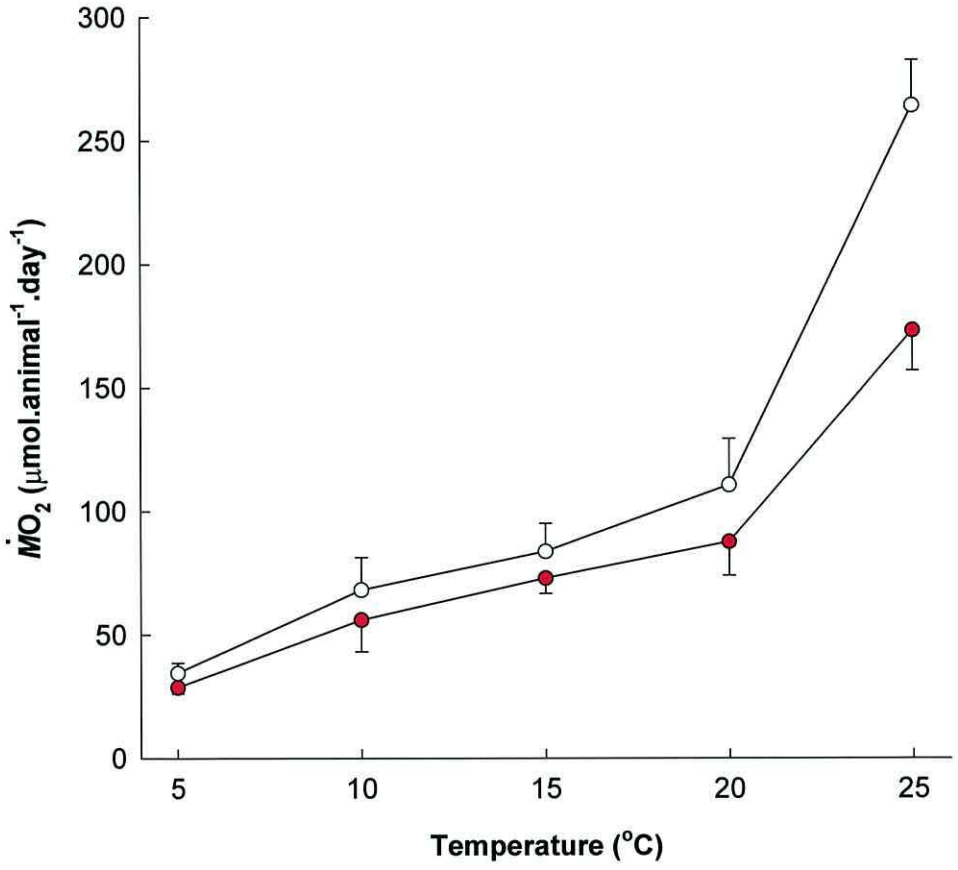


Figure 3.4. Relationship between acute temperature change and whole-animal rates of oxygen uptake ($\dot{M}O_2$) in winter collected, 15°C acclimated (red circles) versus winter acclimatised (white circles) *Ligia oceanica*. For acclimatised animals, n = 6 except at 25°C where n = 5. For acclimated animals, n = 6, except at 15°C where n = 5 and at 20 and 25°C where n = 4. Values are expressed as means ± SE.

Figure 3.5. **(a)** Whole-animal fractional rates of protein synthesis (k_s) and associated variables of **(b)** RNA activity (k_{RNA}) and **(c)** RNA:protein ratios in acclimatised *Ligia oceanica* collected in the summer (black circles) and winter (white circles) and exposed to acute temperature changes. n = 6 for all data sets, except at 10°C where n = 5. n = 5 for each summer data set, except at 5°C where n = 4, and 25°C where n = 3. Values are means ± SE.

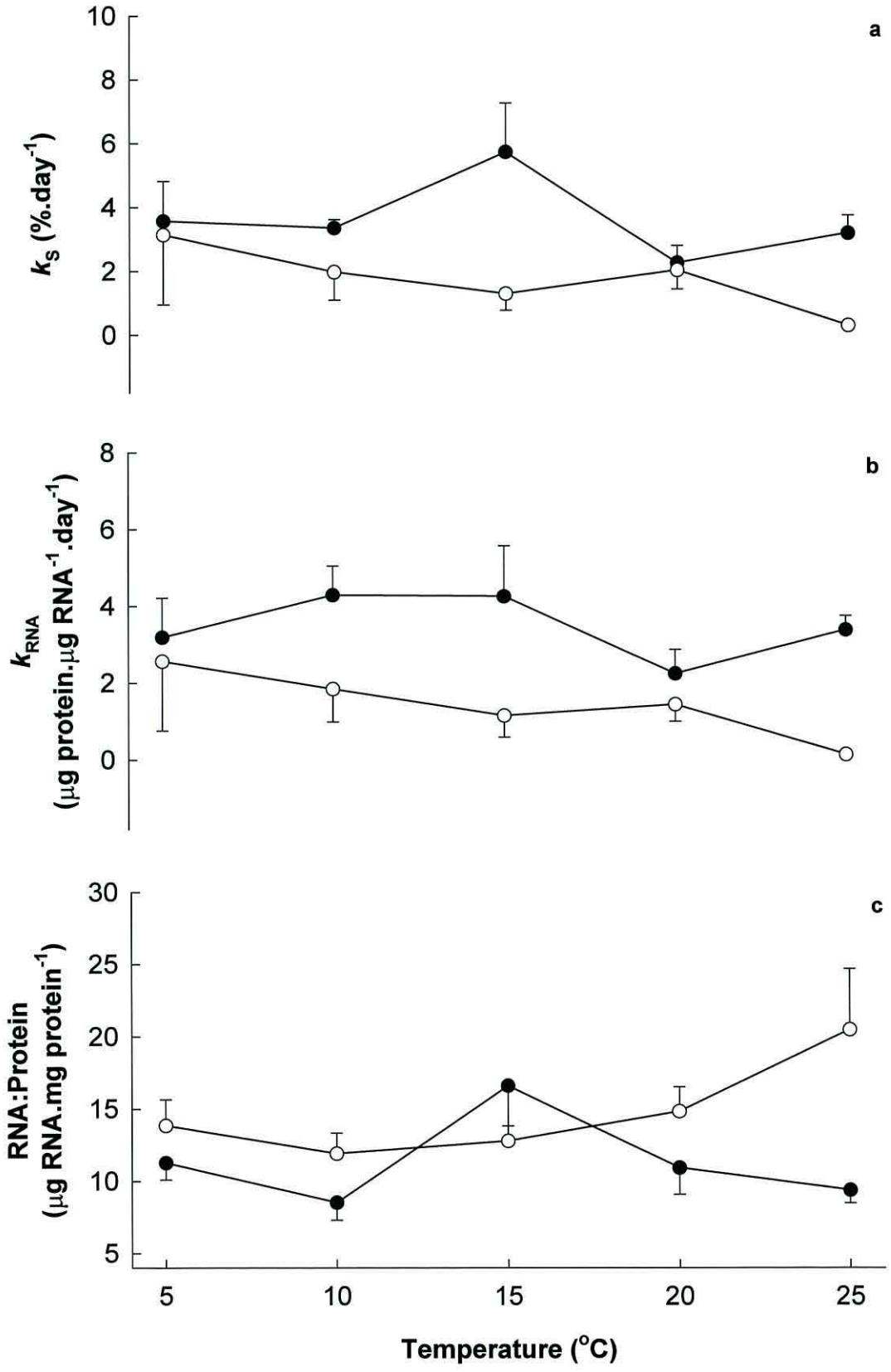
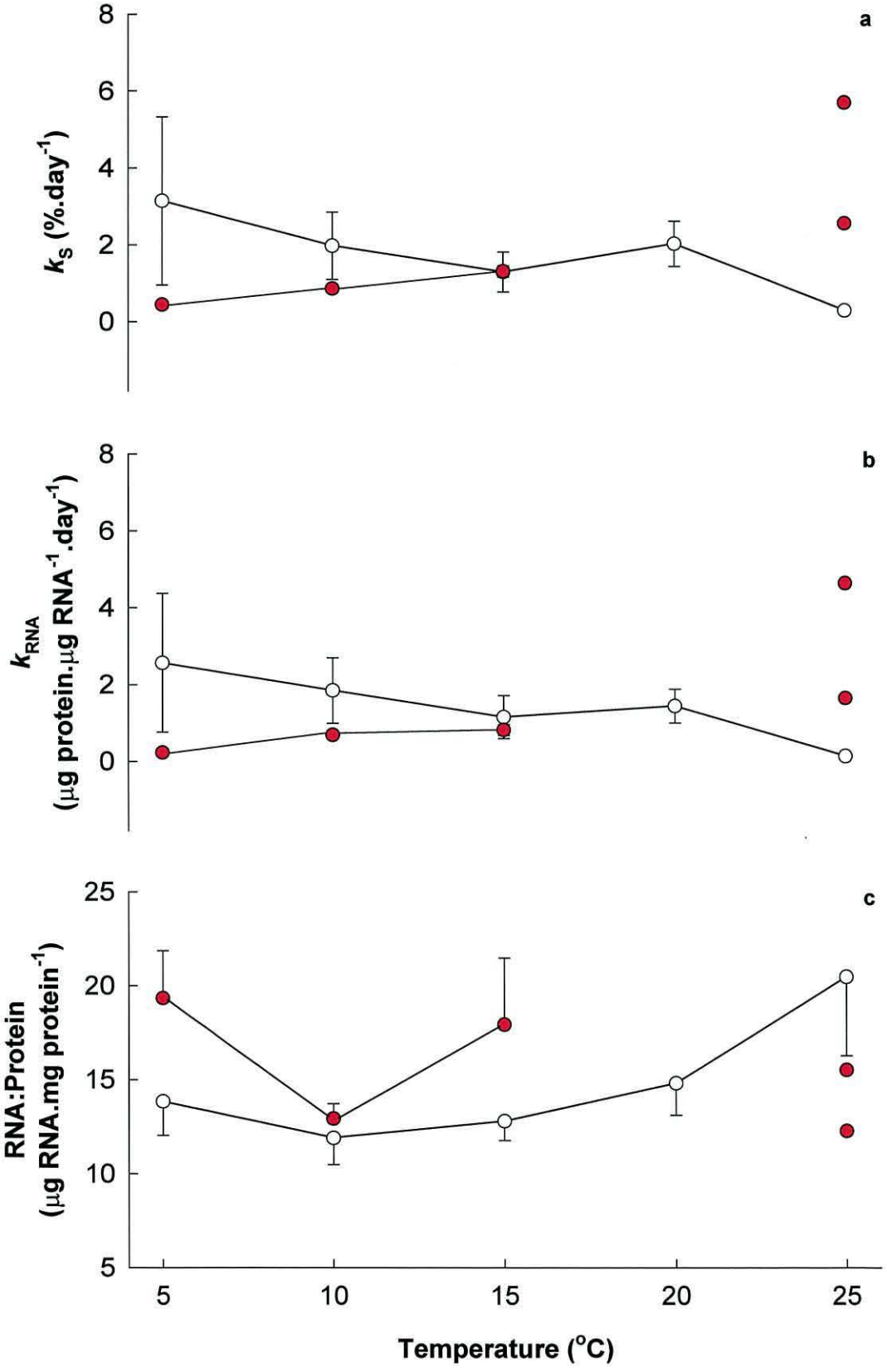

a
b
c
k_S (%.day^{-1})
k_{RNA} (µg protein.µg RNA^{-1}.day^{-1})
RNA:Protein (µg RNA.mg protein^{-1})
Temperature (°C)
10
8
6
4
2
0
30
25
20
15
5

Figure 3.6. The effect of acute changes in temperature on **(a)** whole-animal fractional rates of protein synthesis (k_s) and the associated variables of **(b)** RNA activity (k_{RNA}) and **(c)** RNA:protein ratios in *Ligia oceanica* collected in the winter and either acclimated at 15°C (red circles) or used directly from the shore (white circles). For 15°C acclimated animals, n = 6 for each data set except at 25°C where individual data points are plotted. For acclimatised animals, n = 5 for each data set, except at 5°C where n = 4 and 25°C where n = 3. Values are means ± SE.


a
b
c
k_S (%.day^{-1})
k_{RNA} (µg protein.µg RNA^{-1}.day^{-1})
RNA:Protein (µg RNA.mg protein^{-1})
Temperature (°C)
8
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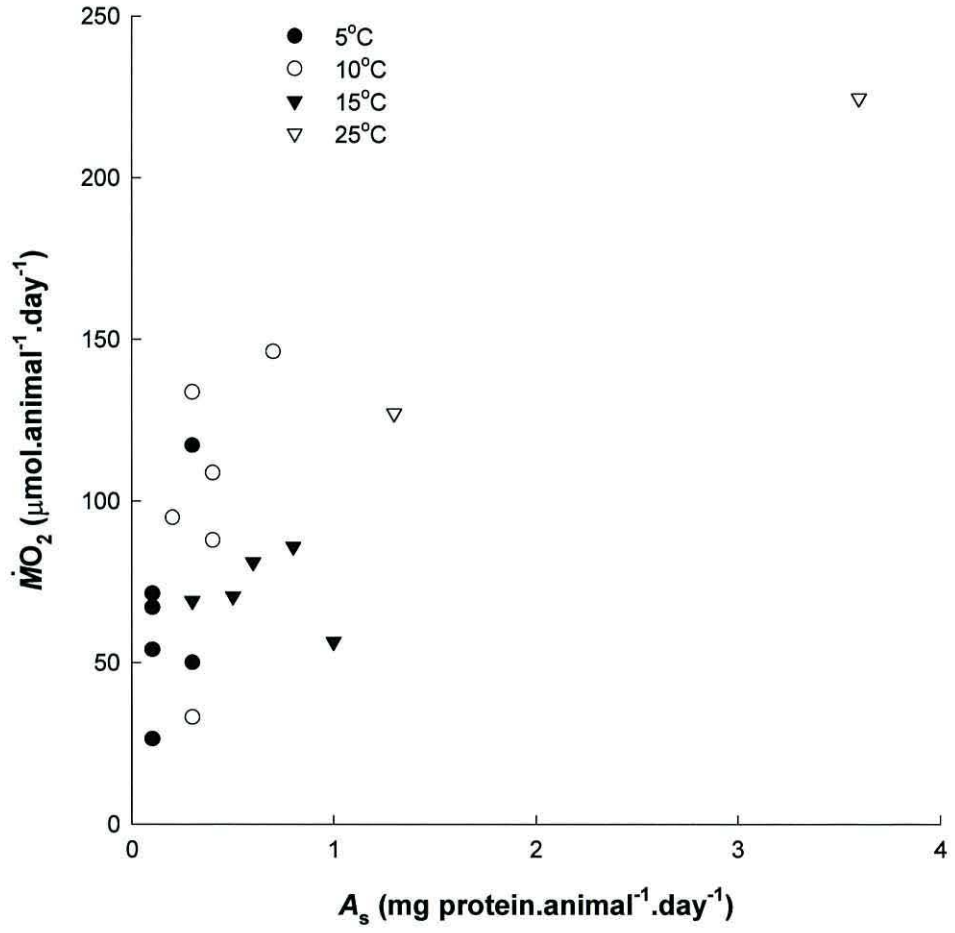


Figure 3.7. The relationship between absolute rates of protein synthesis (A_s) and whole animal rates of oxygen uptake ($\dot{M}O_2$) in winter collected, 15°C acclimated *Ligia oceanica* at a variety of acute temperature challenges. Data have been scaled to represent an animal of one gram wet weight, using the weight exponent 0.7 (Whiteley *et al.*, 1996).

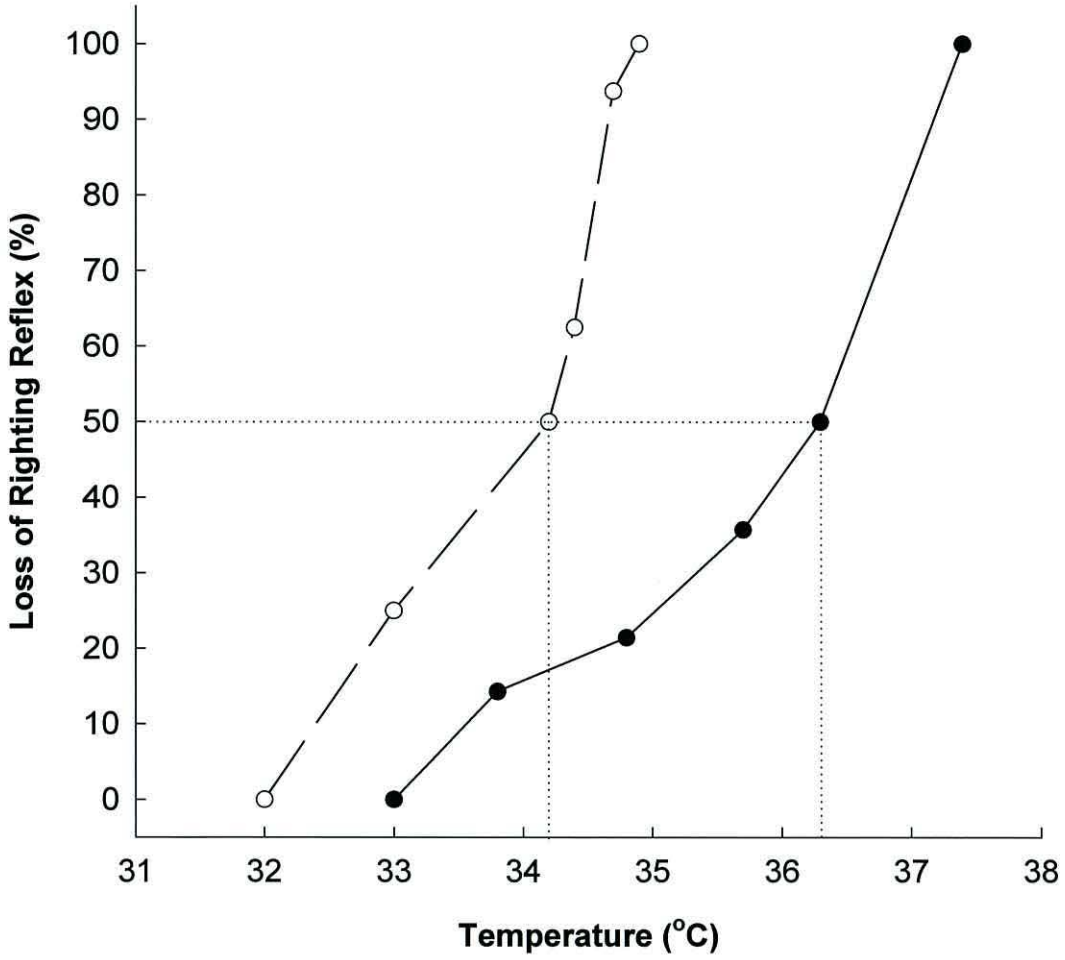


Figure 3.8. Comparison of the temperature at which 100% loss of the righting reflex occurred in winter (white circles, dashed line, n = 14) and summer (black circles, solid line, n = 16) acclimatised *Ligia oceanica*. The dotted line indicates the temperature at which 50% of animals lost the righting reflex.

Chapter Four

Characterising the Heat-Shock Response in *Ligia oceanica*: Effects of Season and Acclimation Temperature

4.1 *Introduction*

Over the past 10 years there has been growing interest in the cellular mechanisms underlying changes in thermotolerance. For example, Buckley *et al.* (2001), postulated that an intracellular 'thermostat' is present to trigger protective responses during thermal stress. Much evidence points to the involvement of heat-shock proteins as a means of sensing the temperature elevation and in determining heat tolerance of ectotherms. Collectively, studies have shown that: hsps are rapidly induced following heat-stress, for example, in *Drosophila melanogaster* (Parsell and Lindquist, 1993; Dahlgaard *et al.*, 1998); there is a correlation between hsp expression and increased thermal tolerance (Li and Werb, 1982; Parsell *et al.*, 1993; Dahlgaard *et al.*, 1998; Feder and Hofmann, 1999); removal of genes in encoding hsps leads to a decrease in thermotolerance in mutant *Drosophila* (Sanchez and Lindquist, 1990; Sanchez *et al.*, 1992); a lack of hsp-encoding genes in closely related species results in differences in thermotolerance (Bosch *et al.*, 1988); developmental stages unable to express hsps are unusually sensitive to thermal stress (Heikkila *et al.*, 1985); and activation of hsps by ectothermic animals occurs towards their upper thermal limits (Hofmann and Somero, 1995; Roberts *et al.*, 1997; Tomanek and Somero, 1999).

The heat-shock protein response, which serves to protect the cell from lethal temperatures (typically an elevation in temperature between 5 and 10°C (Lindquist, 1986)) involves an increase in the activity or level of a heat-shock

factor specific for heat-shock genes (Bonner, 1985; Craig and Gross, 1991). Although termed the heat-shock response, it is unclear whether it is simply the increase in temperature, or the accumulation of thermally denatured proteins, which triggers the induction of hsps (Ananthan *et al.*, 1986). Subsequently, transcription of specific heat-shock genes increases, resulting in increased concentrations of a variety of intracellular heat-shock proteins (hsps). In addition, constitutively expressed forms of hsps which are not induced by stress, known as heat-shock cognates (hscs), help in the translocation of unfolded proteins across intracellular membranes until they reach their destination, whereby they are re-folded (White *et al.*, 1994). Stress-induced forms of the hscs, the hsps, play a similar role, and many hsps broadly function as molecular chaperones, assisting with the correct re-folding of unfolded polypeptides and aggregations of unfolded proteins (Dietz and Somero, 1993; Morimoto, 1993; White *et al.*, 1994; Somero, 1995).

The heat-shock response described above, is an almost universal cellular response to a variety of environmental stressors, including heat, anoxia and heavy metals (Morimoto *et al.*, 1990), and the unusually high degree of conservation of hsp genes between species, and the universality of their expression suggests an important role for these proteins during stress responses (Bosch *et al.*, 1988; Dietz, 1994). To date, the heat-shock response has been found in most organisms. Only a very few have not induced hsps on exposure to thermal stress, for example, the Antarctic teleost, *Trematomus bernacchii* (Hofmann *et al.*, 2000), and a species of hydroid, *Hydra oligactis* (Bosch *et al.*, 1988). *Trematomus* is a cold-adapted stenotherm which lives in

the cold (-1.86 to +0.3°C) and stable Antarctic waters, and therefore does not experience heat-stress in its natural environment. *Hydra oligactis* also inhabits a thermally stable environment, although a congener from a fluctuating thermal environment, *H. attenuata*, did exhibit the hsp response indicating that thermal histories have an important influence on the ability to synthesise heat-shock proteins.

Positive correlations between normal body temperatures and hsp induction temperatures in a variety of species suggest that the heat-shock response is not genetically 'hard-wired', but is subject to acclimation or acclimatisation (Dietz and Somero, 1992; Somero, 1995). Indeed, seasonal differences appear to affect hsp expression in at least two ways: by altering the levels of endogenous hsps within cells, and by shifting the threshold induction temperatures (Hofmann, 1999). For example, in two species of eurythermal goby fish, *Gillichthys mirabilis* and *G. seta*, both induction temperatures for hsp synthesis and endogenous hsp levels were higher in animals seasonally acclimatised to summer conditions (Dietz and Somero, 1992). In addition, Fader *et al.* (1994), demonstrated seasonal variation of endogenous hsp levels in four species of riverine teleost with changes in season, with the lowest hsp levels occurring in the winter for all four species. Quantities of ubiquitin conjugates, important in the regulation of proteolysis (Lindquist, 1986) and hsp70, were also found to be greater in gill tissues from the mussel, *Mytilus trossulus*, collected in the summer, suggesting expression of hsp70 was not enough to stop all irreversible protein damage (Hofmann and Somero, 1995). Differences in thermotolerance are also evident in congeneric

organisms from the intertidal zone, where species from the more thermally variable high intertidal habitats often display more rapid induction of hsps, higher endogenous levels of hsps and better thermotolerance than low-intertidal or subtidal congeners, for example, the marine snail, *Tegula spp.* (Tomanek and Somero, 2000), and the bivalve, *Mytilus trossulus* (Hofmann and Somero, 1995).

To date, few studies have characterised the heat-shock response in organisms from their natural environment. Rocky intertidal shores in temperate locations show large and rapid variations in temperature on a daily basis, especially during the summer months. As shown in Figure 3.2, invertebrates such as *Ligia oceanica* can experience dramatic microclimatic variations within this highly changeable environment. *Ligia*, therefore, serves as an excellent study organism with which to examine hsp expression (both constitutive and *de novo*) in response to both acclimatisation and acclimation. Characterisation of the heat-shock response in summer and winter animals may also explain the seasonal differences in thermotolerance observed in Chapter 3.

4.2 *Materials and Methods*

4.2.1 *Collection and Maintenance of Animals*

Ligia oceanica were collected from the shores of Rhosneigr and Cemlyn Bay on Anglesey in June 2000 and January 2001. Animals for acclimatisation experiments were held overnight without food at control temperatures approximating the ambient air temperature recorded at the time of collection (15°C for animals collected in June, and 10°C for animals collected in January). Note that these temperatures differed from those of the previous chapters. In an effort to expose *Ligia* to the minimum of stress, the *in situ* temperatures at the time of collection were considered to be more suitable than using the temperatures of collection from previous years. Animals for summer and winter acclimation experiments were held at either 5, 10, 15 or 20°C for at least 4 weeks in incubators set to a light/dark cycle of 12/12 (0600 to 1800 hours light), and were fed potato shavings and *Fucus spp.* fronds twice weekly.

4.2.2 *Metabolic Labelling*

Ligia were labelled by injecting a solution of [^{35}S] methionine and cysteine (TRAN^{35}S-LABEL™, ICN Biomedicals Inc., sp. act. 1175 Ci.mM^{-1}) diluted in crab saline (1 µl label:3 µl saline) at a dose of 1 µl.100 mg^{-1} wet weight. This delivered a specific radioactivity of 2.5 µCi.µl^{-1} of injected solution. The probability of labelling newly synthesised proteins with the metabolic label was increased by the use of a methionine and cysteine mixture over a single

amino-acid metabolic label. Animals were injected through their dorsal surface at the pereon-pleon junction, just to the right or left of the alimentary canal. Prior to injection, a small amount of haemolymph was taken up into the syringe to ensure the solution containing the metabolic label was delivered into the haemocoel of the animal. The syringe was left in place for 10 seconds before removal to ensure adequate circulation of the injected solution. Unless otherwise stated, isopods were left for one hour at the appropriate treatment temperature before being metabolically labelled, and left for a further 2 hours before being decapitated, eviscerated, and snap-frozen in liquid N_2. Removal of the gut prior to protein preparation resulted in higher resolution of the protein bands. Samples were stored at -80°C prior to processing.

4.2.3 *Experimental Regime*

Ligia were removed from their incubators and individually placed in experimental chambers (lidded glass jars of approximately 60 cm^3) containing a piece of filter paper soaked in seawater. The seawater maintained a high relative humidity in the containers to prevent the animals from becoming desiccated at the higher incubation temperatures. The chambers were submerged in water to the level of the lids using a water bath (Grant Y22) maintained at the appropriate treatment temperature for the relevant amount of time, as outlined below. After heat-shock treatment, animals were snap-frozen in N_2 and patterns of newly synthesised proteins were detected by SDS-PAGE, followed by autoradiography.

4.2.3.1 *Time-Course Experiments*

A series of time-course experiments were carried out to characterise the temporal differences in patterns of newly synthesised protein expression in *Ligia* collected in the summer straight from the shore (acclimatised) and after acclimation at 15°C for 4 weeks. The animals were heat-shocked at 25°C, *i.e.* 10°C above the *in situ* temperature on the shore and 10°C above the acclimation temperature.

To examine the time taken to initiate a heat-shock response in *Ligia* straight from the shore, animals were either heat-shocked at 25°C (n = 12), or kept at 15°C (n = 12) for either one, 2 or 3 hours before being processed. Animals held for one and 2 hours were injected with the metabolic label at the start of incubation, whereas animals held for 3 hours were injected after one hour of incubation. An identical experiment was carried out on *Ligia* acclimated at 15°C for 4 weeks.

In addition, patterns of recovery were examined by following the patterns of newly synthesised protein expression for 2, 4, 6, 12 or 24 hours post heat-shock in 15°C acclimated animals. Animals were either held at the control temperature of 15°C (n = 10), or to the heat-shock temperature of 25°C (n = 10) for 3 hours before being injected with the [^{35}S] metabolic label. Animals were left for either 2, 4, 6, 12 or 24 hours at 15°C before being processed.

4.2.3.2 *Characterising the Threshold Temperatures for Heat-Shock Protein Induction in Ligia oceanica acclimatised in the Summer and Winter*

Ligia collected in June 2000 were held overnight at 15°C. The following day, animals were exposed to a temperature change which included either a control temperature of 15°C (*i.e.* no temperature change), or four heat-shock temperatures of either 25, 27, 29 or 31°C for 3 hours before being snap-frozen. In each case, two animals were exposed to each temperature change. *Ligia* collected in January 2001 and held overnight at 10°C were exposed to identical conditions to those of the summer acclimatised animals, apart from the temperature. Three animals were exposed to each temperature. Winter animals did not survive more than one hour at an incubation temperature of 31°C.

4.2.3.3 *Characterising the Threshold Temperatures for Heat-Shock Protein Induction in Summer and Winter Ligia oceanica Acclimated to Different Temperatures*

Ligia acclimated at 5, 10, 15 or 20°C in the summer were incubated at temperatures of either 15, 25, 27, 29 or 31°C for 3 hours before being snap-frozen (n = 20, one sample per temperature treatment). The 5°C acclimated individuals did not survive for one hour at 31°C. In a similar set of experiments, *Ligia* collected in the winter were acclimated at the same temperatures as the summer acclimated animals, and exposed to the same incubation temperatures for the same length of time. Additionally, there were four control groups at incubation temperatures of 5, 10, 15 and 20°C. Winter acclimated animals were exposed to the treatment temperatures for 3 hours

before being snap frozen (n = 46, 2 samples per temperature treatment). None of the winter acclimated *Ligia* survived longer than one hour at 31°C.

4.2.4 *Protein Separation and Analysis*

4.2.4.1 *Preparation of Proteins for SDS-PAGE*

Whole bodies were homogenised to a fine powder using a pestle and mortar pre-cooled in liquid N_2. A lysis buffer and protease inhibitor solution (300 µl 32 mM Tris-HCl (pH 6.8) 21 µl 2% SDS and 3 µl 100 mM phenylmethylsulfonylfluoride (PMSF)) was added to the homogenate and then boiled for 5 minutes. Insoluble material was removed by centrifugation at 6000 x g for 15 minutes. The supernatants were removed, aliquoted, and stored at –80°C prior to the determination of radioactivity by liquid scintillation using a Wallac WinSpectral 1414 scintillation counter and Optiphase 'Hisafe' scintillant.

4.2.4.2 *Gel Electrophoresis and Fluorography*

Proteins were separated using one-dimensional discontinuous SDS-PAGE according to Laemmli (1970), in a 12% acrylamide matrix at 48 mA for approximately 1.5 hours. Equal quantities of sample and sample buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.01% bromophenol blue) were boiled for 3 minutes. Proteins were loaded according to equivalent amounts of radioactivity (3 kBq or 18 x 10^4 dpm) per well. Each gel was simultaneously calibrated using a [^{14}C]-labelled molecular weight marker (Rainbow™ [^{14}C] methylated protein molecular weight markers, 14300 – 220000 Da, Amersham) according to the manufacturers instructions.

Gels were fixed in isopropanol:water:acetic acid (25:65:10) for 30 minutes, and impregnated with the autoradiographic enhancer, Amplify™ (Amersham) for 20 minutes before being dried and exposed to pre-flashed Hyperfilm™ MP (Amersham) for 48 hours at -80°C.

4.2.4.3 *Fluorogram Analysis*

Fluorograms were digitised using a Kodak DC120 digital camera and Kodak Digital Science ID Scientific Imaging software, and analysed using one-dimensional electrophoresis gel analysis software (Quantity One, v. 4.2.2, Bio-Rad). Variations in labelling intensity between gels were accounted for by normalising the newly synthesised protein bands using the [^{14}C]-labelled molecular weight marker (66 kDa) as a constant reference, enabling comparisons of optical density between different gels. Although the resultant optical density is expressed in arbitrary units and therefore does not give an indication of the absolute levels of newly synthesised protein, it does enable the relative characterisation of the heat-shock response, both in terms of the time-course for induction, and, upon cessation of heat-stress, for the patterns of recovery. Another reason for using normalised data was to account for any potential temperature effect (Q_{10}) on the rate of newly synthesised proteins (Roberts *et al.*, 1997).

4.3 *Results*

4.3.1 *Time-Course Experiments*

Initial studies on summer animals after 2 hours heat-shock showed the general pattern of heat-shock response in *Ligia*. The 70, 68, 60 and 53 kDa proteins corresponded in molecular weight to some of the major classes of heat-shock proteins (Figure 4.1). Densitometric analysis showed that summer *Ligia* acclimated at 15°C for 4 weeks and incubated at 25°C for 2 hours showed a consistent and approximate doubling in optical density of proteins of size classes 70, 68, 60 and 53 kDa over a control animal.

4.3.1.1 *Characterisation of the Time Taken to Induce a Heat-Shock Response in Ligia*

Patterns of heat-shock protein synthesis in summer acclimatised *Ligia* incubated at 25°C are shown in Figure 4.2. Size classes of proteins in the 77 and 64 kDa range were observed in addition to those outlined above. In general, for all size classes of hsp, induction was at its highest level after one hours incubation at 25°C. The greatest increases in relative intensity at one hour were shown by the 70 and 60 kDa proteins, which increased 4.8- and 5.8-fold from control levels, respectively. The levels of expression of the 77 kDa protein had almost returned to control values after 2 hours, whereas relative levels for all other protein size classes remained elevated at this time interval. By 3 hours, hsp expression for all size classes had returned to those for control animals.

Summer *Ligia* acclimated at 15°C showed levels of hsp expression at or below relevant control values for all protein classes after one hours incubation at 25°C, except for the 60 kDa protein, which had increased in relative intensity by 1.4-times the level at 15°C (Figure 4.3). After 2 hours at 25°C, expression of the 60 and 53 kDa proteins was at its highest relative to control values, as the relative intensities were 1.9- and 1.5-fold higher, respectively. Levels of expression of the 70 and 68 kDa proteins were at their highest after 3 hours. For example, the 68 kDa protein showed the greatest increase in relative intensity for any protein size class, with a 2.6-fold increase over control values by 3 hours.

4.3.1.2 *Comparison Between the Time-Course of Acclimatised and Acclimated Heat-Shock Responses*

The temporal patterning and intensity of the heat-shock response varied between acclimatised and 15°C acclimated summer *Ligia*. The rate at which hsp expression was initiated appeared to be more rapid in the acclimatised animals, as the highest levels of expression occurred after just one hour at 25°C for all class sizes of protein. In contrast, the heat-shock response peaked between 2 to 3 hours in the acclimated animals. Relative absolute levels of 70, 68 and 60 kDa protein expression were higher in acclimated animals, although the greatest increases in intensity relative to the control values were found in the acclimatised animals. Comparisons between the acclimated and acclimatised control groups revealed that levels of expression of the 70, 68 and 60 kDa proteins were higher in the control acclimated *Ligia*, with values 2.7-, 3.0- and 3.2-times higher, respectively.

4.3.1.3 *Characterisation of the Heat-Shock Response in Ligia: Recovery from Heat-Shock*

Summer *Ligia* acclimated at 15°C showed the highest expression of all size classes of hsp 2 hours after cessation of the heat-shock (Figure 4.3). After 2 hours, a general decline in labelling intensity was observed up until 12 hours into the recovery period for the 70, 68 and 60 kDa proteins, although levels remained elevated from control values. After 24 hours, a second peak in the expression of the 70 and 68 kDa proteins was observed, where levels were similar to those recorded after 6 hours of recovery. In contrast, the 53 kDa protein returned to control levels after 6 hours of recovery, but after 24 hours, labelling intensity increased to the levels recorded after 2 hours recovery. The relative changes in hsp expression between animals during recovery from heat-shock and the control animals is shown in Figure 4.4. The 68 kDa protein was consistently the most strongly expressed protein over the 24 hour recovery period compared to control values.

4.3.2 *Characterising Threshold Temperatures*

4.3.2.1 *Effect of Seasonal Acclimatisation*

Ligia acclimatised in the summer showed relatively high levels of hsp expression at 27°C for all size classes (Figure 4.5). However, peak labelling intensity was observed at 25°C for the 60 kDa protein, with a 3.4-fold increase over the control values at 15°C. At 29 and 31°C, expression of all proteins had returned to levels similar to the control values, apart from the 60 kDa protein, where a second peak in labelling intensity was observed, with levels 2.5-fold higher than control levels.

In winter acclimatised *Ligia*, the peak in labelling intensity for most hsp size classes also occurred at 27°C (Figure 4.6). The 70 and 68 kDa proteins showed the highest expression at this temperature, with 25.6- and 230-fold increases over control levels, respectively. Elevated expression of the 60 kDa protein was consistent, with an 11.6-fold increase over control values at both 25 and 27°C. At 29°C, the only protein which maintained a relatively high labelling intensity was the 60 kDa protein, where a 7.4-fold increase over control values was observed.

Constitutive expression of all protein size classes in control animals was higher in summer acclimatised *Ligia* than the winter animals. Comparisons between the 70 and 60 kDa control values of summer and winter acclimatised *Ligia* show that summer levels were 15.6- and 11.8-fold higher, respectively. Winter animals did not express the 68 and 53 kDa proteins. However, winter acclimatised *Ligia* showed the greatest relative changes in protein expression for all size classes, although the absolute levels of expression were higher in summer animals (see Figure 4.7 for changes in the expression of the 60 kDa protein). This figure shows that constitutive expression of the 60 kDa protein was 10-times higher in the summer control incubated at 15°C than the winter control incubated at 10°C. Indeed, the labelling intensity of the summer controls was almost as high as the peak expression recorded at 27°C in winter *Ligia*. For both summer and winter acclimatised animals, the 60 kDa protein was consistently the most dominant protein expressed at heat-shock temperatures. Summer *Ligia* showed a second peak in labelling intensity of

the 60 kDa protein at 31°C. In contrast, winter acclimatised *Ligia* incubated at 31°C failed to survive for one hour at this temperature.

4.3.2.2 *Effect of Acclimation Temperature – Ligia From the Summer*

Ligia acclimated at 5°C showed relatively high expression of hsps from all size classes at both 15 and 25°C (Figures 4.8 and 4.10). The labelling intensity of all proteins was at its lowest level at 27°C. At 29°C there was a second peak in hsp expression, dominated by the 60 kDa protein, where a 4-fold increase over expression at 27°C was observed. *Ligia* acclimated at 5°C did not survive more than one hour at the 31°C incubation temperature. *Ligia* acclimated at 10°C showed an upwards shift in the temperature of peak hsp induction to between 25 and 27°C. Again, expression of the 60 kDa protein was dominant over the other protein size classes. At 25 and 27°C, the relative labelling intensity of the 60 kDa protein was 5.2- and 5.5-fold higher than values at 15°C. A second peak in protein synthesis was not observed at 29 or 31°C in 10°C acclimated animals.

In animals acclimated at 15°C, the highest levels of hsp expression were observed at 25°C. Synthesis of the 53 kDa protein was dominant, showing a 2.4-fold increase in synthesis over the levels at 15°C. Expression of all protein size classes was relatively low at both 27 and 29°C. A small second peak in expression levels, similar to those of the control animal at 15°C, was observed. At the highest acclimation temperature of 20°C, there appeared to be constitutive expression of hsps from all size classes at 15°C, with little or no expression detected at either 25 or 27°C. A small peak in hsp expression

was evident at both 29 and 31°C, where levels of all protein size classes were similar to those observed at 15°C. Overall, *de novo* synthesis of hsps was less at the higher acclimation temperatures (15 and 20°C), with the strongest labelling intensity for all protein size classes occurring at 5°C acclimation. The strongest expression of the 60 kDa protein was observed in *Ligia* acclimated at 10°C.

4.3.2.3 *Effect of Acclimation Temperature – Ligia From the Winter*

In the winter, *Ligia* acclimated at 5°C showed a peak in labelling intensity at 25°C for all hsp size classes (Figures 4.9 and 4.11). Expression of the 60 kDa protein was dominant over the other size classes, and levels at 25°C were 1.6-fold higher than those of the control animal. Thereafter, labelling intensity of all proteins declined steadily as incubation temperature increased. The highest observed labelling intensity in winter animals at all heat-shock temperatures occurred in *Ligia* acclimated at 10°C, where peak expression of most proteins was observed at 27°C. Again, the 60 kDa protein was the most strongly expressed. *Ligia* acclimated at 15°C also showed peak hsp expression at 27°C. Levels of the 60 kDa protein were 2.3-fold higher than those of the control animal at 15°C. Expression of all protein size classes remained at control levels despite heat-shock at 25 and 29°C. No clear hsp induction temperature was evident in *Ligia* acclimated at 20°C. The highest protein expression occurred in the control animal at 20°C, suggesting constitutive hsp expression at higher acclimation temperatures.

4.3.2.4 *Comparison Between Winter and Summer Heat-Shock Responses in Acclimated Animals*

In *Ligia* acclimated at 5°C, hsp expression was higher in summer animals than it was in winter animals, although peak labelling intensity was observed at 25°C in both cases. Both summer and winter animals acclimated at 10°C showed a strong heat-shock response. The peak response occurred at 25 and 27°C in summer animals, and at 27°C in winter animals. There was a much stronger heat-shock response in winter *Ligia* acclimated at 15°C than for summer animals, with a clear peak in hsp expression evident at 27°C. The hsp response in summer animals at 15°C was relatively subdued, but the peak in labelling intensity occurred at 25°C. Both summer and winter *Ligia* acclimated at 20°C showed a subdued heat-shock response. Peak expression was at or below that of the control animals, suggesting constitutive hsp expression at this acclimation temperature.

Overall, patterns in the hsp response for summer *Ligia* were characterised by a double-peak in the expression of hsps and the ability to survive 3 hours at 31°C, the highest incubation temperature. The strongest expression of heat-shock proteins occurred in 5 and 10°C acclimated animals. Winter *Ligia* were characterised by a single-peak in hsp expression, with a steady rise and fall in labelling intensity occurring either side of the peak. The strongest expression of proteins was in animals acclimated at 10 and 15°C. Winter *Ligia* were unable to survive the highest incubation temperature of 31°C for more than one hour. In winter *Ligia*, constitutive heat-shock protein expression in control

animals increased as incubation temperature increased (see Figure 4.12). Unfortunately, there is no corresponding data for summer *Ligia*.

4.4 *Discussion*

It must be emphasised that the results are based on low sample numbers (between one and 3 samples per treatment temperature), and as such, do not take into account variability between individuals, which is likely to exist. Interpretations of these results therefore, are based on the overlying trends displayed by individuals, and as such, the results of these experiments can only offer tentative answers regarding the differences between summer and winter *Ligia*, and between acclimated and acclimatised animals.

4.4.1 *Acclimatised Animals*

The trend for *Ligia oceanica* acclimatised to summer conditions (Figure 4.5) was to express both higher endogenous levels of hsps, and when heat-shocked, the ability to mount a stronger heat-shock response than *Ligia* acclimatised in the winter (Figure 4.6). In summer animals, there was also a trend for peak hsp induction temperature for most hsp size classes to occur at 27°C. Moreover, there were two peaks of hsp induction for the 60 kDa protein, one occurring at 25°C, and another at 31°C.

The trend in the winter was for peak induction of most hsp size classes at 27°C, particularly for the 70 and 68 kDa proteins. In contrast, the 60 kDa

protein was strongly expressed at 25, 27 and 29°C which was the upper limit of thermal tolerance for these winter animals. Comparisons of hsp expression between the summer and winter controls revealed that overall, the constitutive hsp expression, was higher in the summer than in the winter animals. For example, constitutive expression of the 70 and 60 kDa proteins was 14- and 10-fold higher, respectively; peak expression of the 60 kDa protein was 3.6-fold higher at 25°C; and expression of the 70 and 60 kDa proteins was 1.8- and 1.9-fold higher, respectively at 27°C. The advantages of relatively high endogenous levels of hsps include the maintenance of native protein structure and processing of partially unfolded proteins as suggested by Dietz and Somero (1992). In addition, the variable thermal environment experienced by the summer *Ligia* appears to have induced higher constitutive hsp expression in preparation for the increased likelihood of heat-shock in the summer. There is probably little need for such high endogenous hsp levels during the winter, as temperature-induced protein denaturation is less likely. When winter animals acclimatised in the field were heat-shocked, expression of hsps peaked at 27°C before quickly falling at higher incubation temperatures. Similarly, *Drosophila* reduce the costs involved in stress resistance by only synthesising hsps on exposure to stress, and for only a short time (Krebs and Loeschke, 1994a). In this study, winter animals were not able to survive incubation at 31°C, showing that their limits of thermotolerance were reduced in the winter.

The trend for the attenuated heat-shock response shown by winter *Ligia* was probably modulated to a large extent by thermal histories. It would be highly

unlikely for winter animals to experience temperatures of between 25 and 29°C, and consequently, constitutive expression of hsps would place an unnecessary energetic burden on animals that were possibly already nutritionally limited. A further consequence of reduced endogenous hsp expression in winter animals may be to affect their translational capacity for hsp synthesis (Tomanek and Somero, 2000), as hsp70 may be involved in the binding and stabilisation of its own mRNA (DiDomenico *et al.*, 1982).

Higher summer constitutive hsp levels have been demonstrated in many organisms. Hofmann and Somero (1995) found that gill tissue from the mussel, *Mytilus trossulus*, had greater constitutive levels of hsp70 and ubiquitin when collected in the summer. This indicated both reversible and irreversible protein denaturation and consequently a greater need for molecular chaperones. The basic function of hsp70 is to stabilise and re-fold thermally denatured proteins. This hsp also binds to polypeptides translating on ribosomes preventing early folding and aggregation of nascent polypeptides (Frydman and Hartl, 1994). So, at higher environmental temperatures when rates of protein synthesis are usually higher, larger amounts of hsp70 are needed to chaperone increased amounts of ribosome-bound polypeptides. Relatively high constitutive levels of hsp70 and translationally-active heat-shock mRNA have also been found in all tissues examined from the eurythermal teleost, *Fundulus heteroclitus* (Koban *et al.*, 1991). This indicates that these fish are poised for a heat-stress, a prudent physiological state based on the 20°C fluctuation in diel temperature fluctuations that this species regularly experiences. In *F. heteroclitus*,

synthesis of hsps began just 15 minutes after exposure to a stress temperature and levels remained elevated for 24 hours. During hsp elevation, the hsps can either counter a subsequent temperature increase, or aid in protein recovery from thermal damage incurred on heat-stress. This suggests *F. heteroclitus* is poised for large and rapid temperature changes, a strategy which reduces the damage caused by the effects of thermal stress prior to the induction of the heat-shock response. A similar situation may be present in summer *Ligia*, where the ability to mount an effective and rapid response to a heat-shock, and the seasonal thermotolerance conferred by high constitutive levels of hsps, may be especially important due to the large and rapid diel temperature variations (Hofmann and Somero, 1995). The energetic costs of hsp expression are also likely to play a role in thermally-induced hsp induction, or whether hsps are expressed at all. Constitutive hsp expression, however, can be detrimental to an organism for a number of reasons: there is competition for the substrate and cellular machinery otherwise used for normal protein synthesis; It impedes the normal processing and degradation of non-native proteins; and it also competes in intra- and extra-cellular signalling pathways used for normal cellular function (Zatsepina *et al.*, 2001). In addition, over-expression of hsps is known to reduce fitness and imposes an extra and unnecessary energetic burden for winter animals, which are unlikely to experience heat-shocks in the range of 25 to 29°C. Krebs and Loeschke (1994a) demonstrated the deleterious effects of hsp expression on fitness by maintaining *Drosophila* at 25°C, or exposing them to a heat-stress of 36°C for 75 minutes either once, twice or three times. Fecundity, a measure of fitness, was reduced in heat-conditioned females, with further reductions in

fecundity being observed as the number of heat-shocks increased. In contrast, the highest survival rate at an acute heat-shock temperature (39°C for 100 minutes) was shown in flies which had been conditioned the most frequently. This demonstrates a clear temperature-dependent trade-off between fitness and survival and shows the flexibility of the heat-shock response and how it changes according to circumstances. A similar situation may exist in *Ligia*.

Changes in the reproductive state, initiated by hormonal changes may also play a role in determining any seasonal changes in constitutive hsp levels, as may photoperiod and any inherent seasonal rhythm (Feder and Hofmann, 1999). There may also be seasonal fluctuations in pollution levels, as pollutants which cause stress are known to induce hsp synthesis (Feder and Hofmann, 1999). Other abiotic factors which must also be considered when making comparisons between acclimatised and acclimated animals include nutritional status, desiccation stress, oxygen availability and ultra violet radiation (Tomanek and Somero, 1999).

4.4.2 *Acclimated Animals*

Most organisms studied to date strongly express hsp70 in response to heat-stress (Lindquist, 1986). However, there are several exceptions to this, and the preliminary results from this study may indicate that *Ligia* is another exception, as the marginally dominant hsp expressed by *Ligia* was a 60 kDa protein. However, this possibility will have to be supported using higher sample numbers in further studies. Dominant expression of hsp60 has also

been found in the freshwater coelenterate, *Hydra attenuata* (Bosch *et al.*, 1988; Bosch *et al.*, 1991). In *Hydra*, unlike *Ligia*, there was no evidence for the synthesis of a 70 kDa protein under stress conditions (Lindquist, 1986). The absence of hsp60 and any other response to stress in other species of hydra correlated with low resistance to heat-stress. When these results were extrapolated into the field, it was shown that *H. oligactis*, which does not synthesise hsp60, was more habitat restricted than *H. attenuata*, probably as a result of not being able to mount a heat-shock response. Similarly, Sanders *et al.* (1991), showed the importance of hsp isoform expression in the heat-tolerance of two limpet species, *Collisella scabra*, a high intertidal species, and *C. pelta*, an upper mid-tidal species. The high intertidal species had greater thermotolerance to acute heat-shock, and expressed an additional group of low molecular weight hsps and hsp60 isoforms not expressed by the upper mid-tidal species. In contrast, the freshwater isopod *Asellus aquaticus* dominantly expressed another molecular weight class of hsp other than hsp70. A 50 kDa protein was preferentially induced when *Asellus* was heat-shocked and regular protein synthesis was unaffected (Korhonen and Lagerspetz, 1996). The basic function of hsp70 is to catalyse protein folding and facilitate with inter-compartment transport, whereas hsp60 assists in the folding and assembly of enzyme-protein complexes of the mitochondrial inner membrane (Sanders *et al.*, 1991). Both may be involved in renaturing and disaggregating damaged proteins. Studies are beginning to show that thermal acclimation can affect the threshold induction temperatures for the heat-shock response (Lerman and Feder, 2001). In this study, there was a trend for the heat-shock response in the summer to be more pronounced at the lower

acclimation temperatures of 5 and 10°C. Peak hsp expression occurred at 25°C in summer animals acclimated at 5°C, and shifted to between 25 and 27°C in 10°C acclimated animals. *Ligia* collected in the summer and acclimated at 5°C died within one hour's incubation at 31°C. At all acclimation temperatures, except 10°C, there was a trend for a double-peak in the hsp response. In contrast, the strongest hsp response in winter *Ligia* occurred in animals acclimated at 10 and 15°C. In both cases, peak hsp induction occurred at 27°C. None of the winter *Ligia*, regardless of acclimation temperature, were able to survive incubation at 31°C for more than one hour. The acclimation temperature of 10°C coincided with *in situ* shore temperatures recorded at the time of capture. Constitutive hsp expression increased in winter animals for all protein size classes with increasing acclimation temperature (Figure 4.12). Increased sensitivity of the hsp response was apparent at the lower acclimation temperatures, at 5 and 10°C in summer-collected animals, and at 10 and 15°C in winter-collected animals.

A similar response was found in the eurythermal goby fish, *Gillichthys mirabilis* acclimated at 10, 20 and 30°C (Dietz, 1994). The induction of hsps 70 and 90 occurred at lower temperatures in those fish acclimated at the lower temperatures. This showed a correlation in the synthesis of hsps with the temperature of acclimation, and that hsp synthesis was subject to acclimatory manipulation. Similarly, an increase in the acclimation temperature from 13 to 23°C in two intertidal to subtidal temperate zone snail species, *Tegula brunnea* and *T. montereyi*, led to an increase in the temperature at which hsp70 was induced, from 24 to 27°C (Tomanek and

Somero, 1999). The temperature of peak induction also rose from 27 to 30°C. In contrast, expression of hsp38 was higher in animals acclimated to 13°C. Most of the changes in the induction and peak hsp temperatures occurred when acclimation temperature was increased from 13 to 18°C, and not from acclimation at 23°C, maybe because these species rarely encounter temperatures this high.

Winter *Ligia* acclimated at 5°C responded to heat-shock in a similar manner to winter acclimatised animals. Both showed a comparatively attenuated heat-shock response. When winter *Ligia* were acclimated at 10 and 15°C, constitutive hsp expression increased in control animals in preparation for future exposure. Animals acclimated at 20°C were characterised by relatively high constitutive hsp levels and did not respond to heat-shock *per se*.

The trend for summer *Ligia* acclimated at 5 and 10°C, was to show the strongest heat-shock responses compared to animals at the higher acclimation temperatures of 15 and 20°C, where expression of endogenous hsp levels were higher. Overall, a shift in the temperature sensitivity of the heat-shock response between summer and winter acclimated animals was apparent. *Ligia* collected in the summer were able to synthesise hsps at the lowest acclimation temperature of 5°C, whereas winter *Ligia* had a reduced heat-shock response at this acclimation temperature. In addition, there may be a switch in the patterns of hsp synthesis in both summer and winter *Ligia*. In summer animals, this switch occurred between 10 and 15°C, and in winter animals, between 15 and 20°C. Again, this preliminary observation requires

additional data before anything more definite can be ascertained. Chronic exposure to higher acclimation temperatures places greater reliance on high constitutive hsp expression and the subsequent ability to cope with a certain degree of heat-stress, without the additional and costly *de novo* synthesis of hsps over and above constitutive levels. Additionally, high constitutive hsp expression means *Ligia* may have the ability to deal with protein denaturation, whilst simultaneously synthesising normal proteins involved in growth and reproduction. This appears to be a trade-off between fitness and viability. Summer *Ligia* are probably in a better nutritional position than winter animals to address the energetic costs associated with expressing high constitutive hsp levels as food is readily available during the summer months. Enhanced hsp expression only occurs at the higher incubation temperatures, where constitutive hsp expression alone is not enough. This may account for the double-peak in hsp synthesis observed in summer animals and could be a 'last-ditch' attempt at minimising the damage caused by heat-stress.

Differences in the rate and intensity of hsp synthesis in two *Tegula* congeners were found by Tomanek and Somero (2000), a subtidal to low-intertidal species (*T. brunnea*), and a low- to mid-intertidal species (*T. funebralis*). Both were exposed to 30°C for 2.5 hours. Hsp synthesis was much more rapid in the low- to mid-intertidal species, as was recovery on cessation of heat-shock. Induction of hsps 90 and 77 didn't occur in the low-intertidal species until 14 hours and 12 hours, respectively, post heat-shock, but overall, stronger hsp synthesis was observed in this species. The low- to mid-intertidal species had the quicker response to heat-stress and may have been able to repair

damage incurred during emersion, with enough levels of hsp accumulation to cope with a similar heat-stress the following day.

There are tentative parallels to be drawn with acclimatised and acclimated *Ligia* in the aforementioned study on *Tegula*. *T. brunnea* and acclimated *Ligia* were both at more uniform temperatures and both showed slower induction of heat-shock response compared to *T. funebralis* and acclimatised *Ligia*. *T. brunnea* and acclimated *Ligia* also showed overall greater hsp induction which suggests they both suffered greater damage on heat-shock. This demonstrates both the nature of the relationship between the heat-shock response and an organism's thermal history, and the adaptability of the heat-shock response.

In contrast, acclimatised *Mytilus californianus* collected in the winter strongly expressed hsp70 at temperatures greater than 20°C, but the hsp70 response of animals collected in the summer was subdued (Roberts *et al.*, 1997). So in a paradoxical situation, induction of hsp70 was lowest when the temperature during emersion was highest. High constitutive levels of hsps may affect the threshold induction temperature of an organism (Buckley *et al.*, 2001), so when experiencing a mild heat-stress, there may be no need to induce heat-shock genes and the subsequent *de novo* synthesis of hsps, as the accumulation of hsps during acclimation may buffer against heat-stress. For instance, during emersion in sessile organisms such as mussels (Roberts *et al.*, 1997).

A similar result was found by Zatsepina *et al.* (2001), in a *Drosophila* strain from sub-equatorial Africa. This species was highly tolerant of exposure to chronically high temperatures (30°C), but paradoxically, did not show strong expression of hsp70. It is thought this strain has evolved to suppress deleterious phenotypes of hsp70 which reduce fitness. By way of an example, other strains of *Drosophila* cultured at abnormally high temperatures suffer the detrimental consequences of hsp70 expression, but not the benefits, as they never encounter temperatures high enough (Lerman and Feder, 2001; Zatsepina *et al.*, 2001). In another study by Goto and Kimura (1998), a cool temperate species of *Drosophila* was shown to have better heat- and cold-tolerance limits compared to two sub-tropical species. These authors concluded that it is important for the temperate species to withstand heat, as temperate summers can be hot, whereas thermal stability in sub-tropical regions is greater. The lower heat-tolerance of the sub-tropical species was a consequence of the costs involved with the production of hsps in a climate where hsps are not required, and hence not synthesised to the extent that they are in the temperate species. Organisms adapted to chronically high temperatures may have cellular components apart from hsp70 which respond to selection, so higher temperatures which are stressful for non-adapted organisms are not stressful for suitably adapted organisms (Lerman and Feder, 2001).

4.4.3 *Comparisons Between Acclimatised and Acclimated Animals*

The temporal patterning of the heat-shock response was very different between summer- and winter-collected *Ligia*, and laboratory acclimated *Ligia*.

The trend for all protein size classes in field acclimatised *Ligia* was that the peak hsp response was at its highest during the first hour of the heat-shock. In contrast, the peak response in acclimated animals came during the second hour of the heat-shock for the 60 kDa protein, and during the third hour for the 70 and 68 kDa proteins. The trend therefore, was that hsp induction was slower in animals returned to the laboratory and acclimated at different temperatures, probably as a result of the stable thermal regimes these animals were kept under.

The intensity of expression of different protein size classes also varied between acclimatised and acclimated *Ligia*. The 60 kDa protein was most strongly expressed in acclimatised animals, whereas the 68 and 60 kDa proteins were both strongly expressed in acclimated *Ligia*. Again, this observation was merely a trend in the preferential patterns of hsp synthesis noticed in *Ligia*, and needs to be reinforced with higher sample numbers before anything more emphatic can be ascertained. On cessation of heat-shock, the pattern of labelling in acclimated *Ligia* was similar for all protein size classes, with peak hsp induction occurring after 2 hours of recovery at 15°C. Differential expression of hsp size classes was also evident in the copepod *Eurytemora affinis*, when raised at different temperatures (Hakimzadeh and Bradley, 1990). Copepods raised at 4 and 15°C preferentially synthesised 79, 70, 58 ad 24 kDa proteins, whereas animals raised at 20°C expressed 109, 98, 82, 72 and 24 kDa proteins. The functional significance in the variation of patterns of hsp synthesis remains unclear.

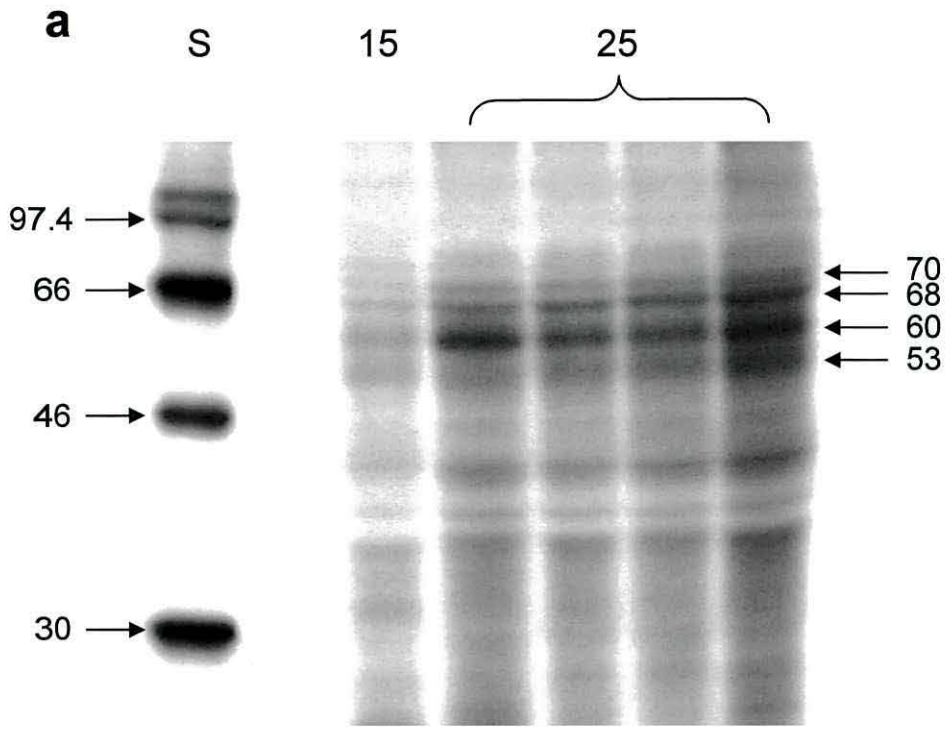
Acclimation at higher temperatures can be seen as a type of heat-hardening, a method of gradually introducing an organism to increased heat-stress. *Drosophila* cells exposed to a gradual temperature increase are able to withstand higher temperatures and recover normal protein synthesis much more quickly than cells exposed to rapid temperature increases (Lindquist, 1980). At lower acclimation temperatures, constitutive hsp expression is lower, as the chances of experiencing heat-shock temperatures will be much reduced. Consequently, there is a greater emphasis on the ability to mount a short, but effective heat-shock response, where the overall energetic costs involved in hsp expression are reduced in comparison to high constitutive hsp expression.

The costs and benefits of the stress response are dictated by the frequency of exposure to a stress. Thermotolerance in *Drosophila* embryos is correlated with hsp70 levels, and a subsequent loss of thermotolerance mirrors a loss of hsp70. Those cells which constitutively express hsp70 at normal temperatures show suppression of growth, but their viability remains unchanged (Feder *et al.*, 1992). There appears to be a direct correlation between the normal environmental temperatures a poikilothermic species experiences, and the levels of constitutively expressed hsp70 isoforms at non-stress temperatures (Ulmasov *et al.*, 1992). There are many other examples in the literature of animals demonstrating high constitutive hsp expression relative to season (Dietz and Somero, 1992), position on the shore (Sharp *et al.*, 1997), and latitude (Ulmasov *et al.*, 1992; Yu *et al.*, 1998; Zatsepina *et al.*, 2000).

An interesting point made by Dahlgaard *et al.* (1998), is that expression of hsp70 and the subsequent conferral of heat-resistance is probably of most consequence to *Drosophila* embryos and larvae, which by comparison with adult flies, are relatively immobile. This raises the question of whether highly mobile intertidal organisms such as *Ligia* are as critically reliant on the synthesis of hsps as are immobile mussels or limpets. Although *Ligia* are mobile, they are restricted to areas of high humidity. Unlike other terrestrial crustaceans, their permeable exoskeletons readily lose moisture through evaporative water loss leaving them prone to desiccation (Edney, 1961). Temperatures in the shingle microhabitat *Ligia* occupy during daylight can reach as high as 30°C in the summer months, close to the lethal temperature of *Ligia* for one hour, and the relative air humidity can be close to saturated (Edney, 1953). This leaves little margin for losing heat through evaporative cooling, so paradoxically, Edney (1953), claimed that *Ligia* can lose heat by moving to the shingle surface, where they may be directly exposed to insolation, but where the relative humidity is between 60 and 70%. It is unclear how frequently individual *Ligia* expose themselves on the shingle surface during daylight, as the risk of predation and desiccation will probably increase dramatically, so this method may be a last resort at bringing body temperatures down from the upper thermal tolerances for this animal. So it appears circumstances may arise where *Ligia* experience heat-stress close to their upper thermal tolerance, but where the increased thermal tolerance conferred by the expression of hsps allows them to reduce their body temperature subject to the energetic costs of hsp expression (constitutive and *de novo*) and increased desiccation stress.

Differences in hsp expression between seasons and acclimation regimes may be associated with energy budgets and the availability of energy resources. The costs of keeping the protein pool viable are a component part of living at high temperatures. Ultimately, this is important in habitat selection and biogeographic distribution. Patterns of hsp expression are probably related to the variability of the thermal habitat at different times of the year, for example, a more stable thermal habitat in winter compared to the summer, where considerable diel temperature variation is common. *Ligia oceanica* collected in the winter were less sensitive to heat-shock than summer animals as the thermal habitat during the winter months is more stable, reducing the need for costly hsp synthesis at a time when nutritional resources may be low. For similar reasons, animals acclimated to a constant temperature for 4 weeks were also less sensitive to heat-shock, although food was readily available. The magnitude and timing of the heat-shock response is dependent on: any acclimatory or seasonal effects (thermal history); the temperature of exposure; the rate at which this temperature is reached; the duration of the exposure; and the time after heat-stress when hsp synthesis is measured (Tomanek and Somero, 2000).

Figure 4.1. **(a)** Autoradiograph showing the pattern of newly synthesised proteins of 70, 68, 60 and 53 kDa in summer *Ligia oceanica* acclimated at 15°C for 4 weeks and heat-shocked at 25°C for 2 hours. The [^{14}C] molecular weight markers are shown in the left-hand lane (S). Temperature is expressed as degrees Celsius, and molecular weights are expressed as kDa. **(b)** Optical density of newly synthesised proteins of 70, 68, 60 and 53 kDa in control (15°C, n = one) and heat-shocked (25°C, n = 4) *Ligia oceanica*. Values at 25°C are expressed as means ± SE.


a
S
15
25
97.4
66
46
30
70
68
60
53

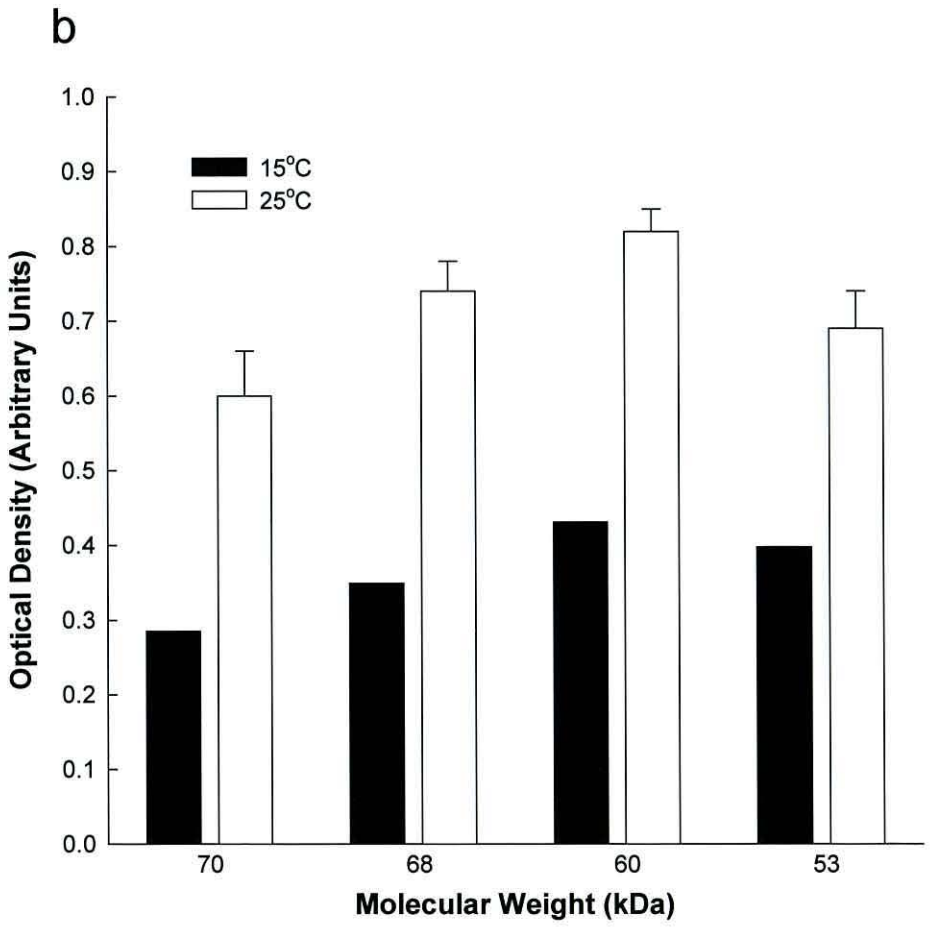
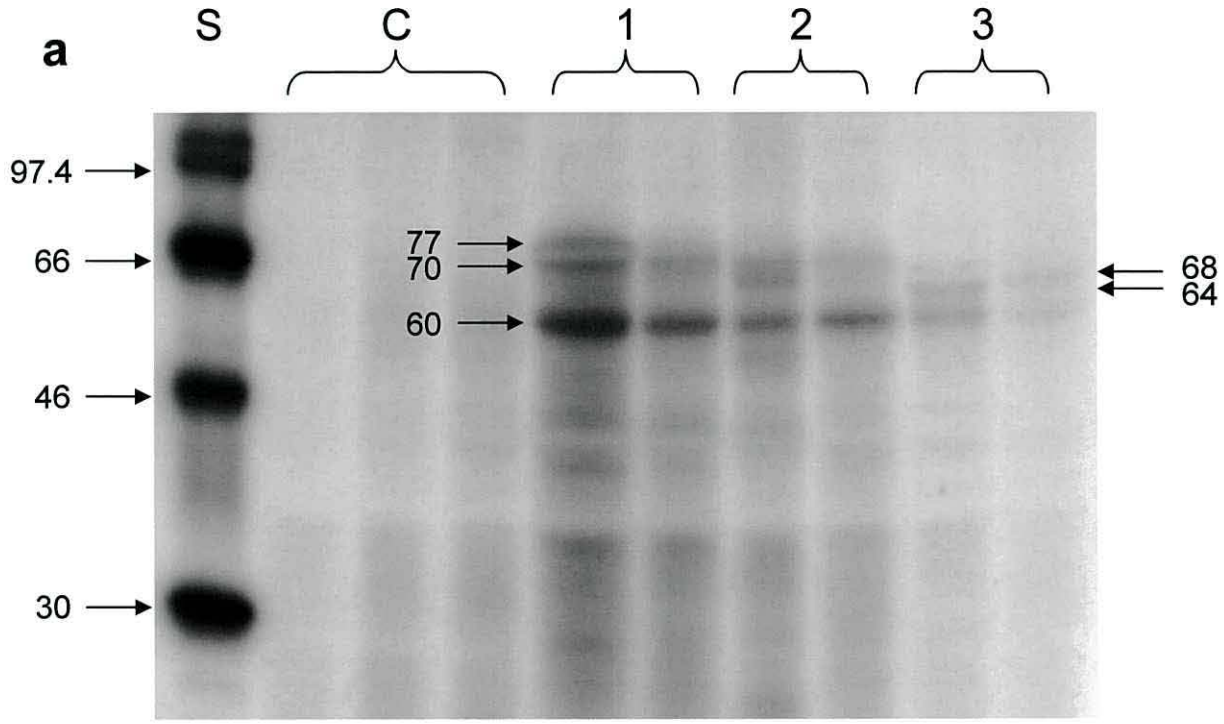

b
15°C
25°C
Optical Density (Arbitrary Units)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
70
68
60
53
Molecular Weight (kDa)

Figure 4.2. **(a)** Autoradiograph showing the incorporation of [^{35}S]-methionine into newly synthesised proteins of 77, 70, 68, 64 and 60 kDa in *Ligia oceanica* taken straight from the shore in summer, held at 15°C overnight and heat-shocked for 1, 2 and 3 hours at 25°C (n = 2 for each treatment). The far left-hand lane shows the molecular weight markers (S) and adjacent are the three control lanes (n = 3) (C). Molecular weights are expressed as kDa and time in hours. **(b)** Optical density of newly synthesised proteins of 77, 70, 68, 64 and 60 kDa after one, 2 and 3 hours of heat-shock at 25°C.


a
S
C
1
2
3
97.4
66
46
30
77
70
60
68
64

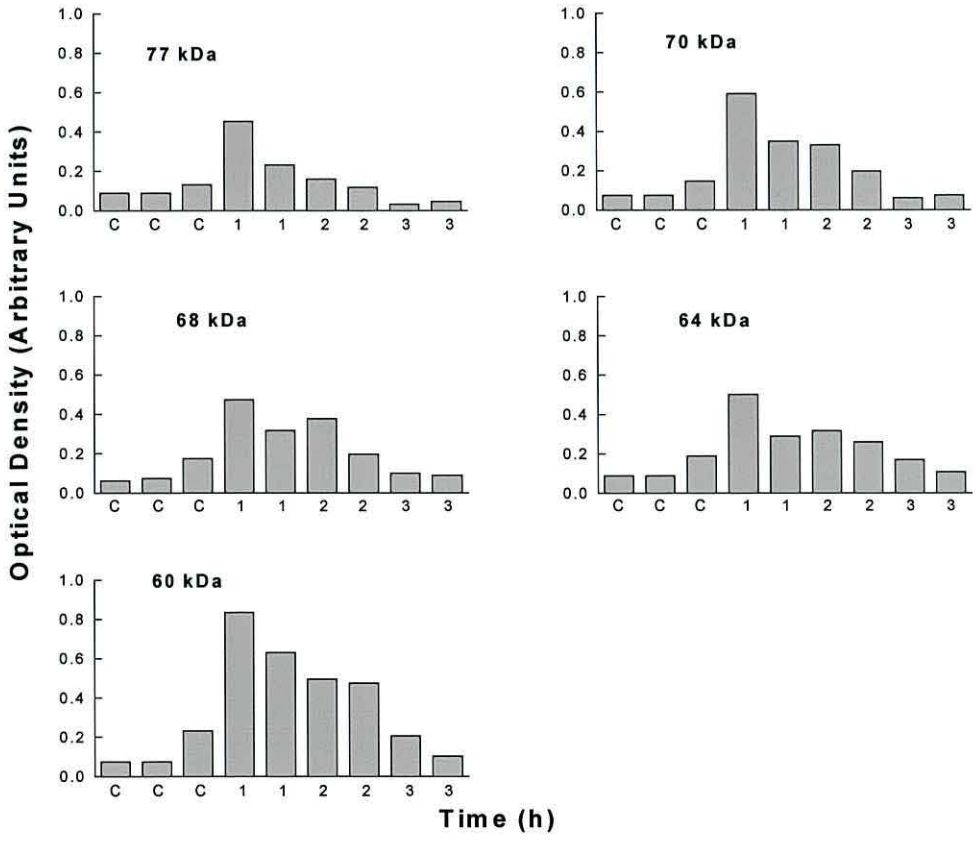
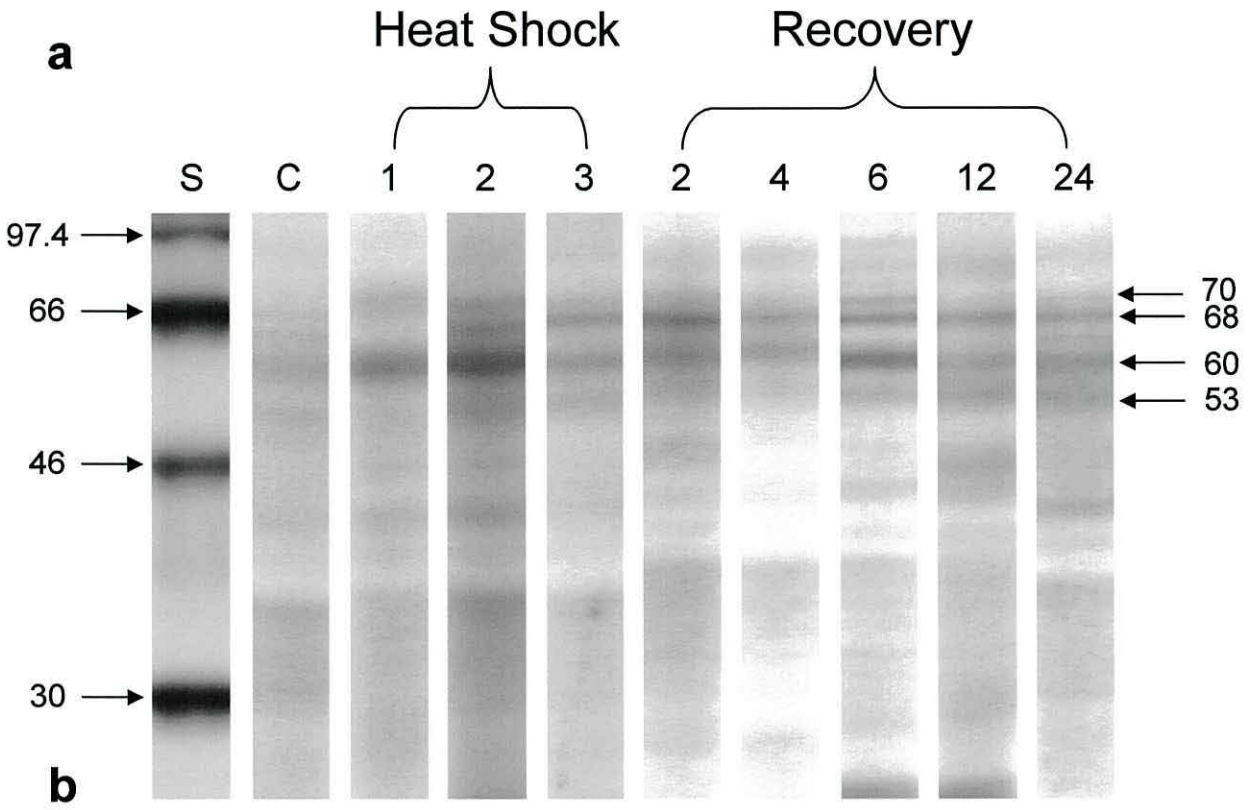
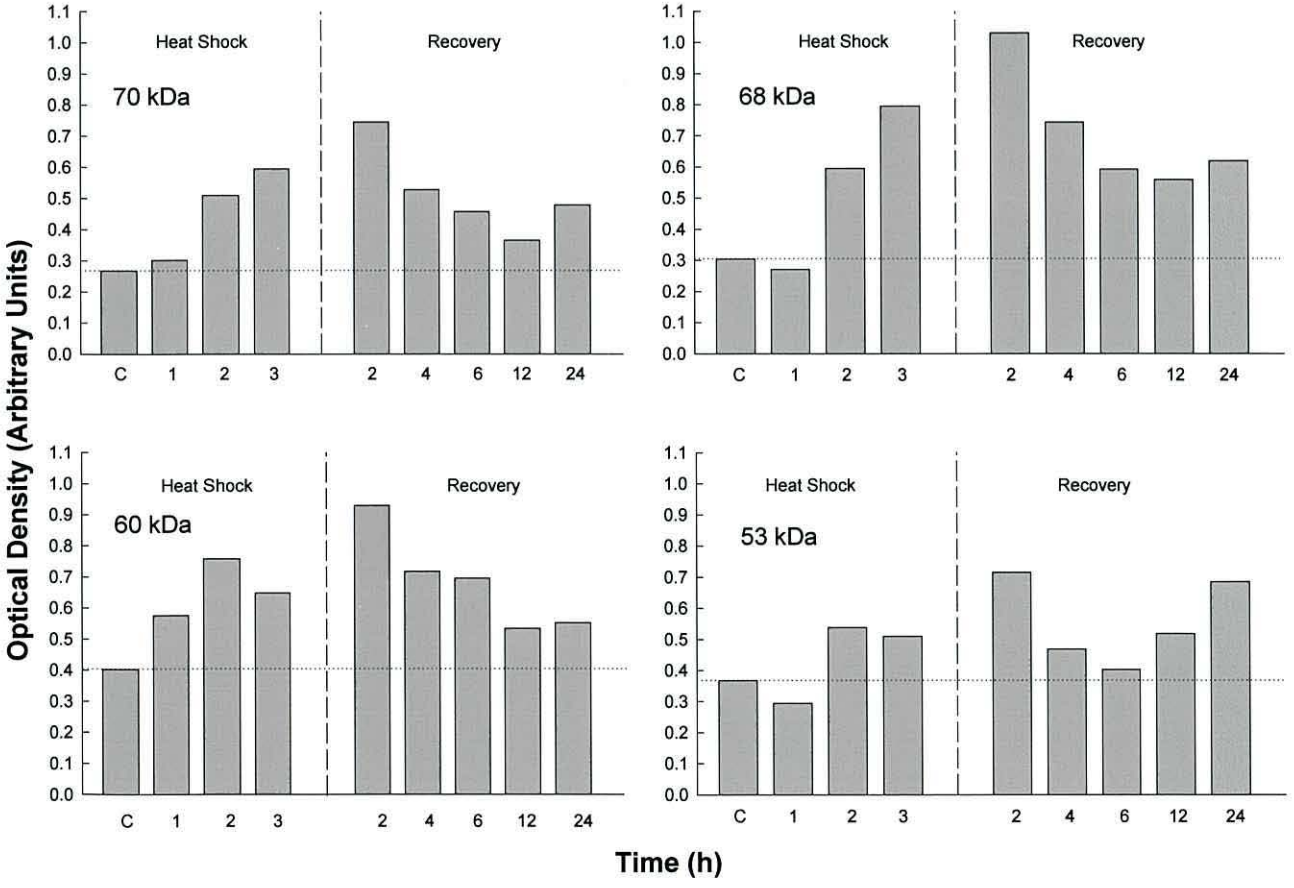

b
Optical Density (Arbitrary Units)
77 kDa
70 kDa
68 kDa
64 kDa
60 kDa
C C C 1 1 2 2 3 3
Time (h)

Figure 4.3. **(a)** Autoradiograph showing the time-course of [^{35}S]-methionine incorporation into newly synthesised proteins of 70, 68, 60 and 53 kDa in summer *Ligia oceanica* acclimated at 15°C. Animals were heat-shocked for one, 2 and 3 hours at 25°C and post heat-shock recovery periods were 2, 4, 6, 12 and 24 hours at 15°C. n = 1 for each treatment temperature. The [^{14}C] molecular weight markers are shown in the far left-hand lane (S) adjacent to the control lane (C) representing an animal left at 15°C (n = 1). All molecular weights are expressed as kDa. **(b)** Optical density for the newly synthesised 70, 68, 60 and 53 kDa proteins as a function of time (3 hours heat-shock and 24 hours recovery). The vertical dotted line separates those *Ligia oceanica* undergoing heat-shock at 25°C from those recovering from heat-shock at 15°C. The horizontal dotted line shows the optical density of the relevant controls held at 15°C.


a
Heat Shock
Recovery
S
C
1
2
3
2
4
6
12
24
97.4
66
46
30
70
68
60
53
b


Heat Shock
Recovery
70 kDa
68 kDa
60 kDa
53 kDa
Optical Density (Arbitrary Units)
Time (h)
C
1
2
3
2
4
6
12
24

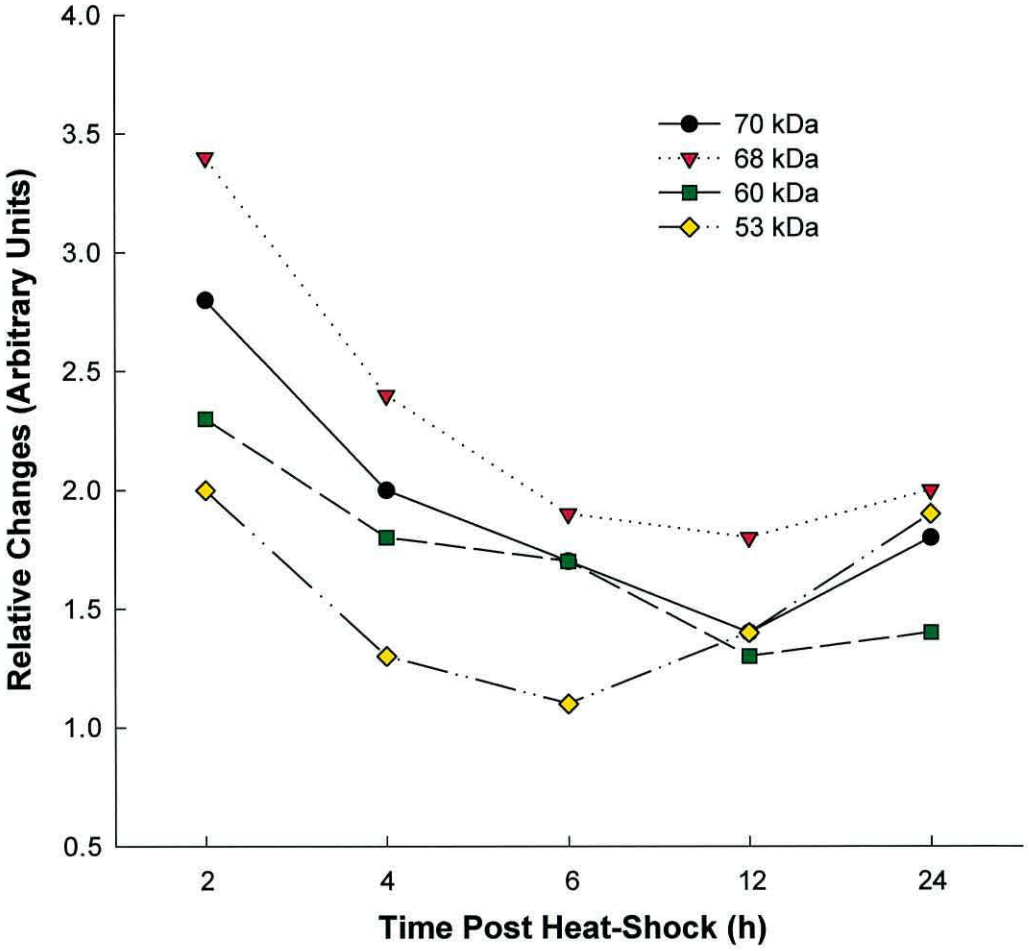
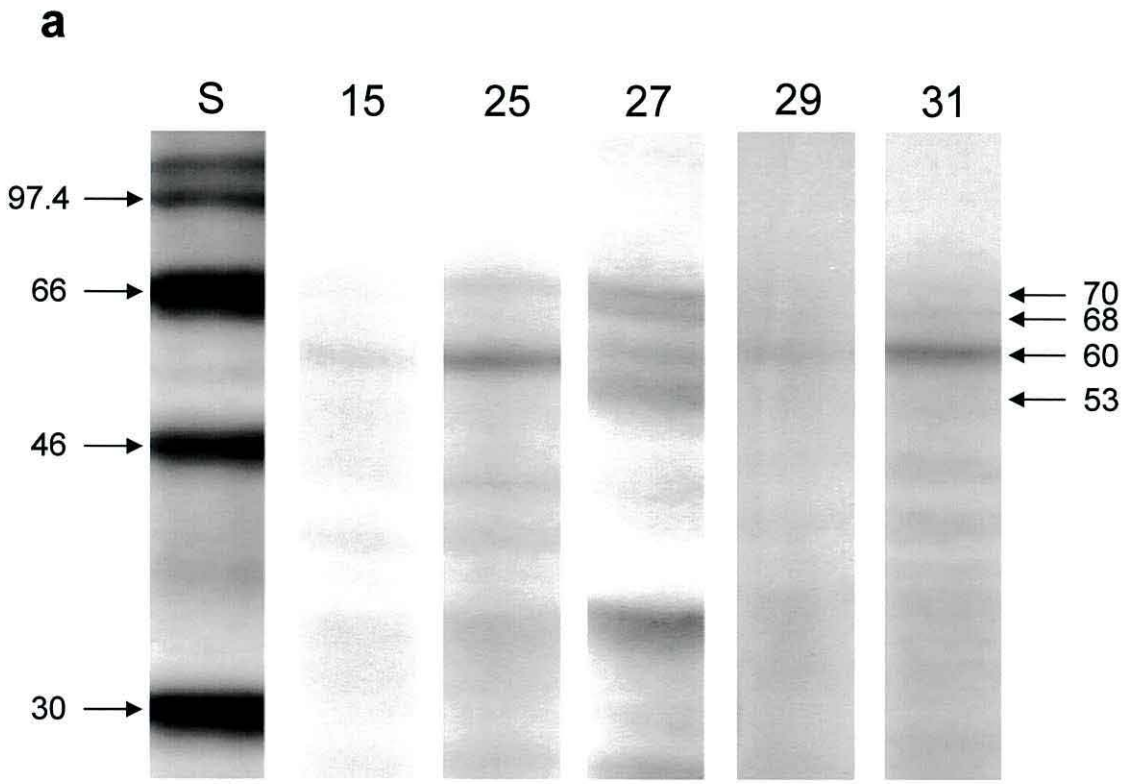


Figure 4.4. Relative changes in heat-shock protein expression in summer *Ligia oceanica* acclimated at 15°C for 4 weeks during recovery from a heat-shock (25°C). The optical density of samples at 2, 4, 6, 12 and 24 hours post heat-shock was divided by the relevant control value to give a factorial increase. n = 1 for each time period.

Figure 4.5. **(a)** Autoradiograph showing the incorporation of [^{35}S]-methionine into newly synthesised proteins of 70, 68, 60 and 53 kDa in *Ligia oceanica* taken straight from the shore in summer, held at 15°C overnight, and incubated at either 25, 27, 29 and 31°C for 3 hours (n = 1 for each treatment temperature including the control). Molecular weight markers are shown in the far left-hand lane (S). Molecular weight is expressed as kDa, and temperature as degrees Celsius. **(b)** Optical density of newly synthesised proteins of 70, 68, 60 and 53 kDa in *Ligia oceanica* heat-shocked at 25, 27, 29 and 31°C. The animal at 15°C is the control.


a
S
15
25
27
29
31
97.4
66
46
30
70
68
60
53

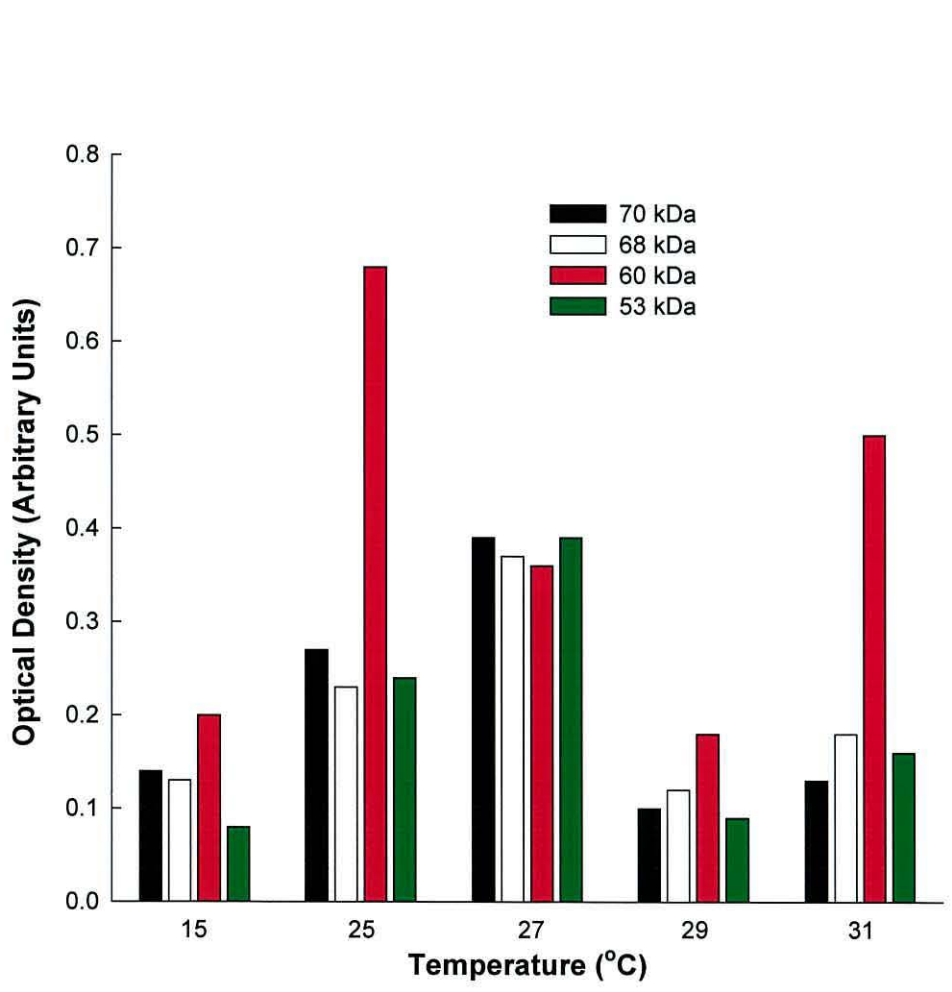
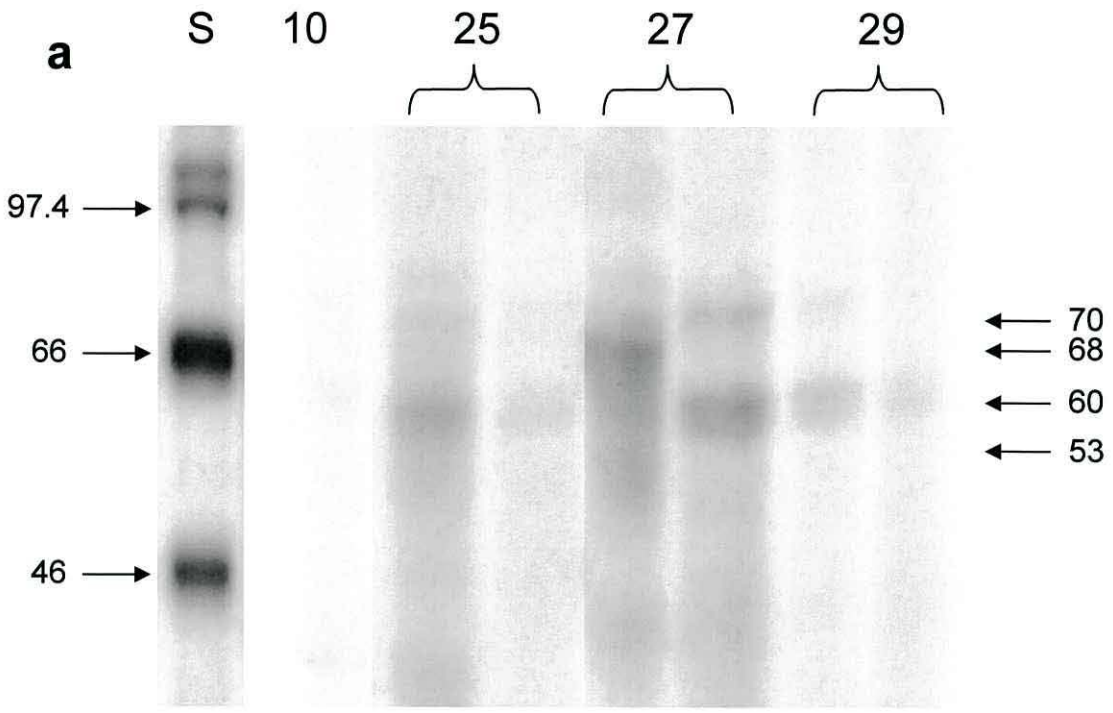
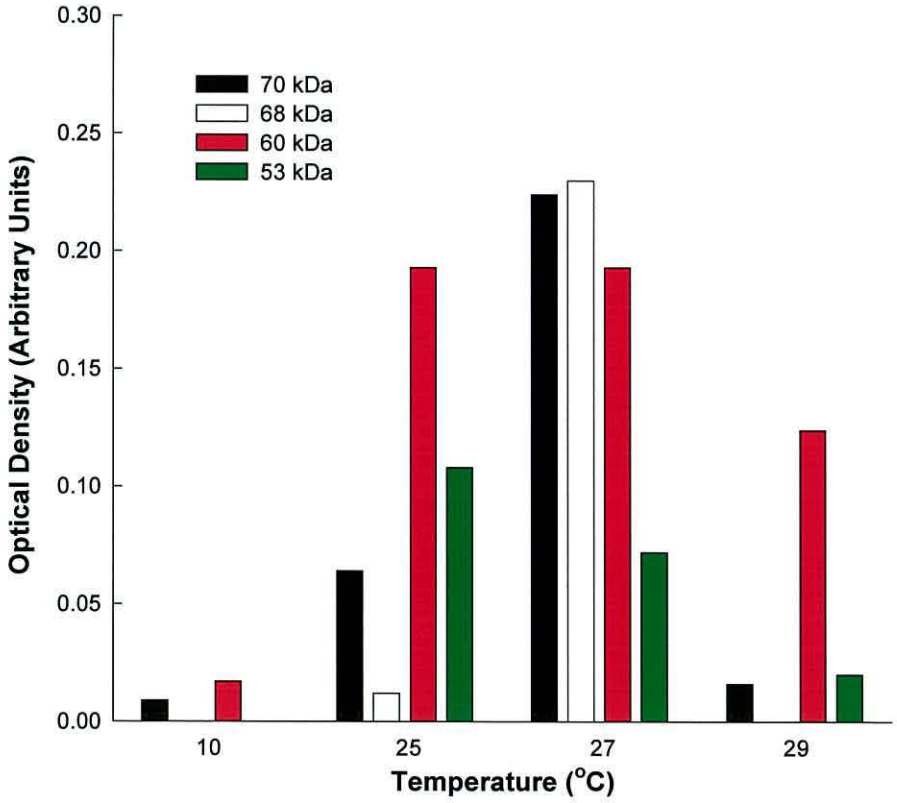

b
Optical Density (Arbitrary Units)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
70 kDa
68 kDa
60 kDa
53 kDa
15
25
27
29
31
Temperature (°C)

Figure 4.6. **(a)** Autoradiograph showing the incorporation of [^{35}S]-methionine into newly synthesised proteins of 70, 68, 60 and 53 kDa in *Ligia oceanica* taken straight from the shore in winter, held overnight at 10°C, and incubated at either 25, 27 and 29°C (n = 2). Molecular weight markers are shown in the far left-hand lane (S) adjacent to the control lane (n = 1) (10). Molecular weights are expressed in kDa, and temperature as degrees Celsius. **(b)** Optical density of the newly synthesised 70, 68, 60 and 53 kDa proteins in *Ligia oceanica* heat-shocked at 25, 27 and 29°C, relative to the control group at 10°C.


a
S
10
25
27
29
97.4
66
46
70
68
60
53


b
Optical Density (Arbitrary Units)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
70 kDa
68 kDa
60 kDa
53 kDa
10
25
27
29
Temperature (°C)

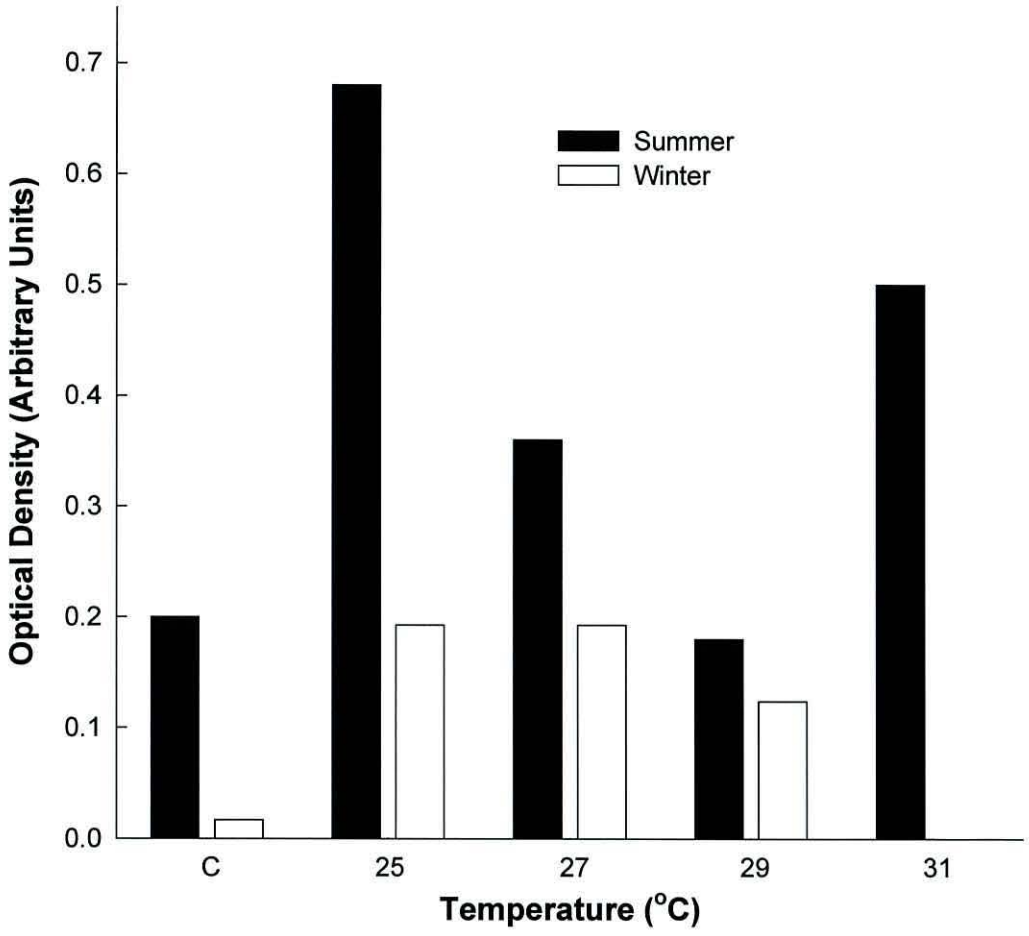


Figure 4.7. Relative optical density of newly synthesised 60kDa protein in summer (n = 1 for each treatment temperature) and winter (n = 1 for each treatment temperature) acclimatised *Ligia oceanica* incubated at 25, 27, 29 and 31°C for 3 hours. Incubation temperatures for the controls were 15°C for summer animals (n = 1) and 10°C for winter animals (n = 1).

Figure 4.8. Autoradiographs showing the incorporation of [^{35}S]-methionine into newly synthesised proteins of 70, 68, 60 and 53 kDa in summer *Ligia oceanica* acclimated at 5 **(a)**, 10 **(b)**, 15 **(c)** and 20°C **(d)** and incubated at 25, 27, 29 and 31°C for 3 hours. An attempt was made to incubate 5°C acclimated *Ligia oceanica* at 31°C, but none survived beyond the first hour. The [^{14}C] molecular weight markers are shown on the far right of each autoradiograph (S). Molecular weights are expressed as kDa and temperature as degrees Celsius. n = 1 for each treatment temperature.

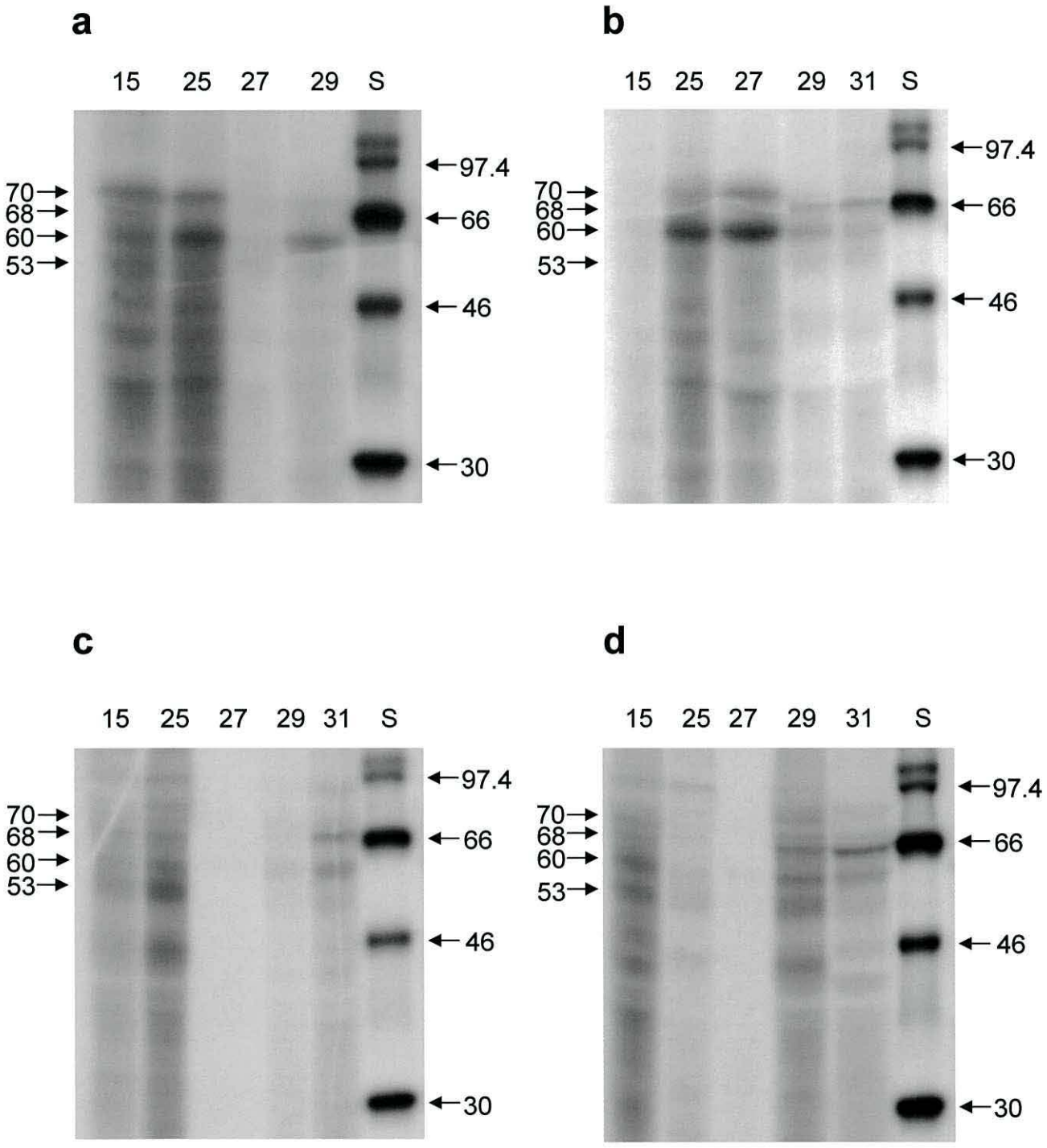

a
15 25 27 29 S
70
68
60
53
97.4
66
46
30
b
15 25 27 29 31 S
70
68
60
53
97.4
66
46
30
c
15 25 27 29 31 S
70
68
60
53
97.4
66
46
30
d
15 25 27 29 31 S
70
68
60
53
97.4
66
46
30

Figure 4.9. Autoradiographs showing the incorporation of [^{35}S]-methionine into newly synthesised proteins of 70, 68, 60 and 53 kDa in winter *Ligia oceanica* acclimated at 5 **(a)**, 10 **(b)**, 15 **(c)** and 20°C **(d)** and incubated at 25, 27 and 29°C for three hours. Molecular weight markers are shown in the far left-hand lane (S) and are expressed as kDa. In all cases, the lane adjacent to the molecular weight markers is the control lane for that acclimation temperature. Temperatures are expressed as degrees Celsius. n = 1 for each treatment temperature.

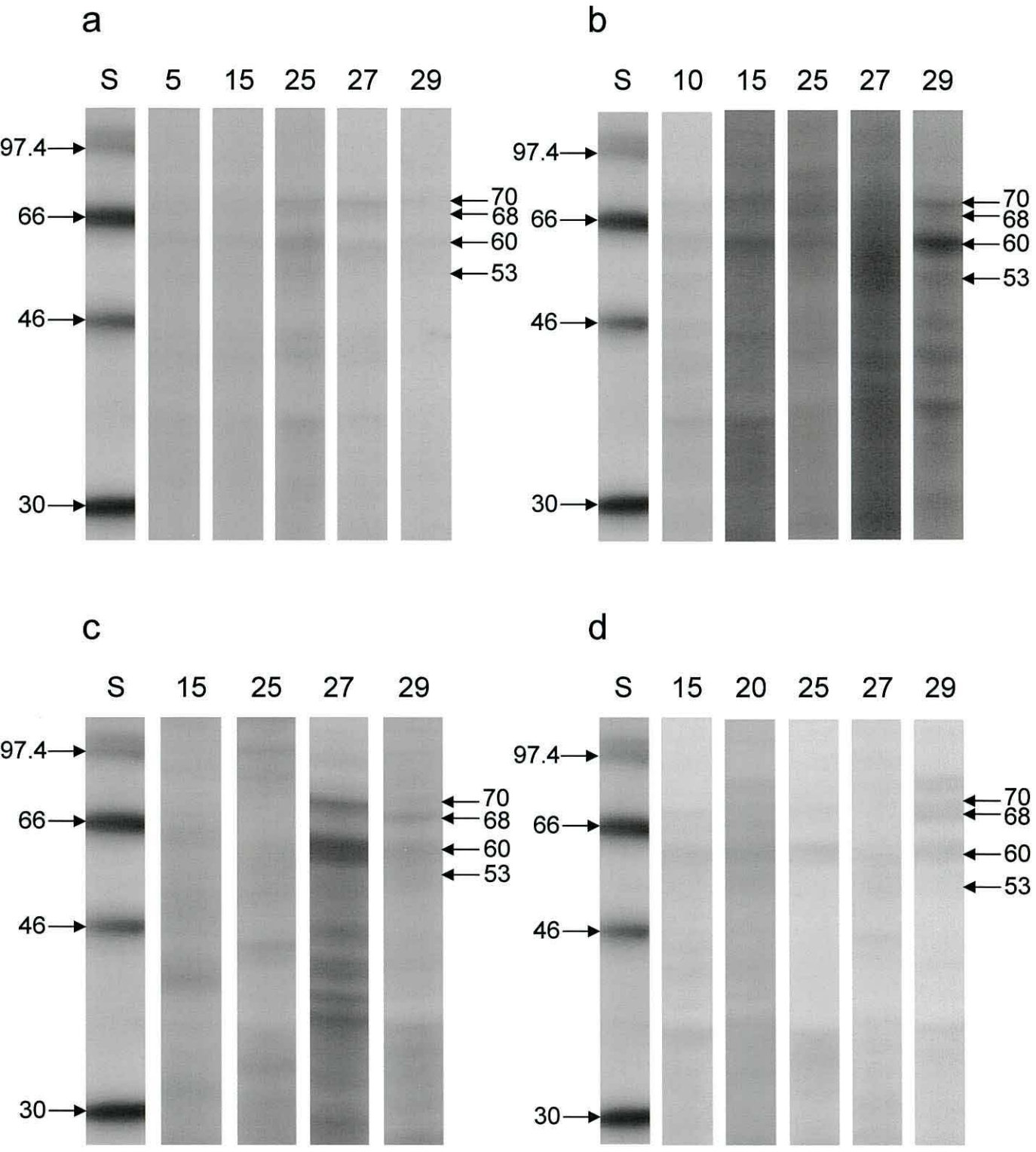
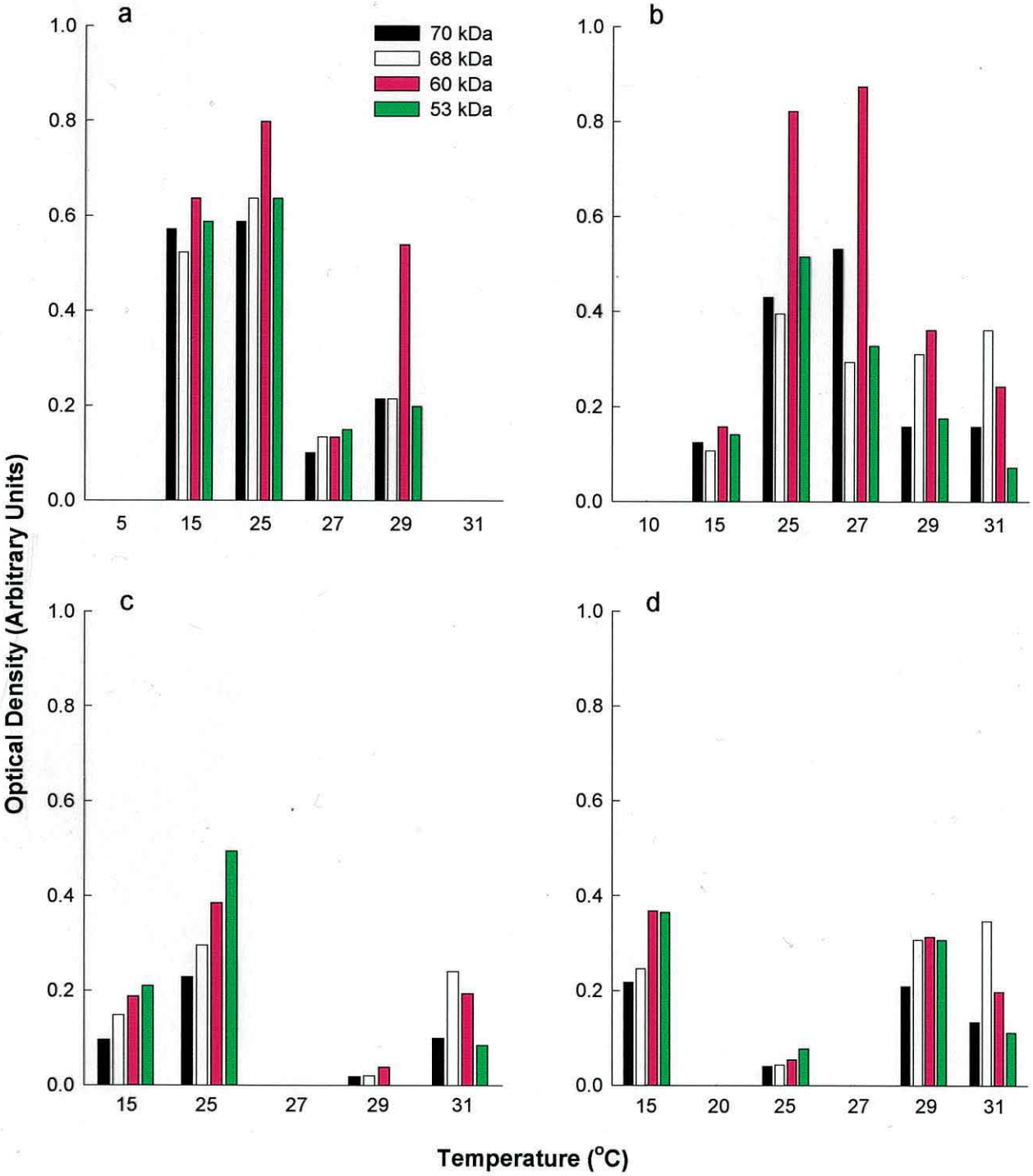
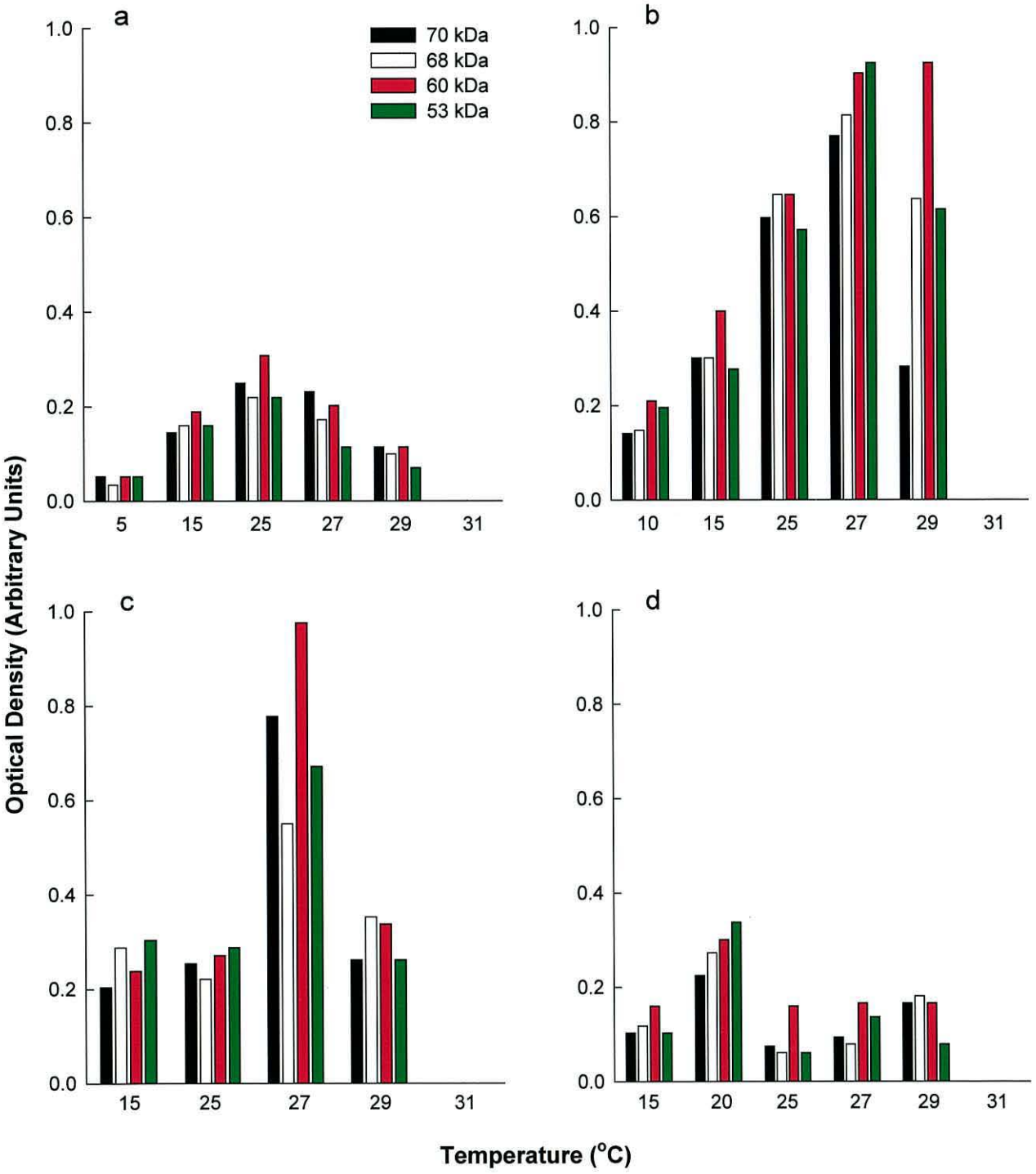

a
S
5
15
25
27
29
97.4
66
46
30
70
68
60
53
b
S
10
15
25
27
29
97.4
66
46
30
70
68
60
53
c
S
15
25
27
29
97.4
66
46
30
70
68
60
53
d
S
15
20
25
27
29
97.4
66
46
30
70
68
60
53

Figure 4.10. (Acetate overlay) Optical density of newly synthesised proteins of 70, 68, 60 and 53 kDa in summer *Ligia oceanica* acclimated at 5 **(a)**, 10 **(b)**, 15 **(c)** and 20°C **(d)** and heat-shocked at 25, 27, 29 and 31°C (except for the 5°C acclimated group where all animals died before 1 hour at 31°C heat-shock). n = 1 for each treatment temperature.

Figure 4.11. Optical density of newly synthesised proteins of 70, 68, 60 and 53 kDa in winter *Ligia oceanica* acclimated at 5 **(a)**, 10 **(b)**, 15 **(c)** and 20°C **(d)** and heat-shocked at 25, 27 and 29°C. Control animals are at incubation temperatures equivalent to their relevant acclimation temperatures. n = 1 for each treatment temperature.


a
b
c
d
70 kDa
68 kDa
60 kDa
53 kDa
Optical Density (Arbitrary Units)
Temperature (°C)
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
25
27
29
31


a
b
c
d
70 kDa
68 kDa
60 kDa
53 kDa
Optical Density (Arbitrary Units)
Temperature (°C)
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
25
27
29
31

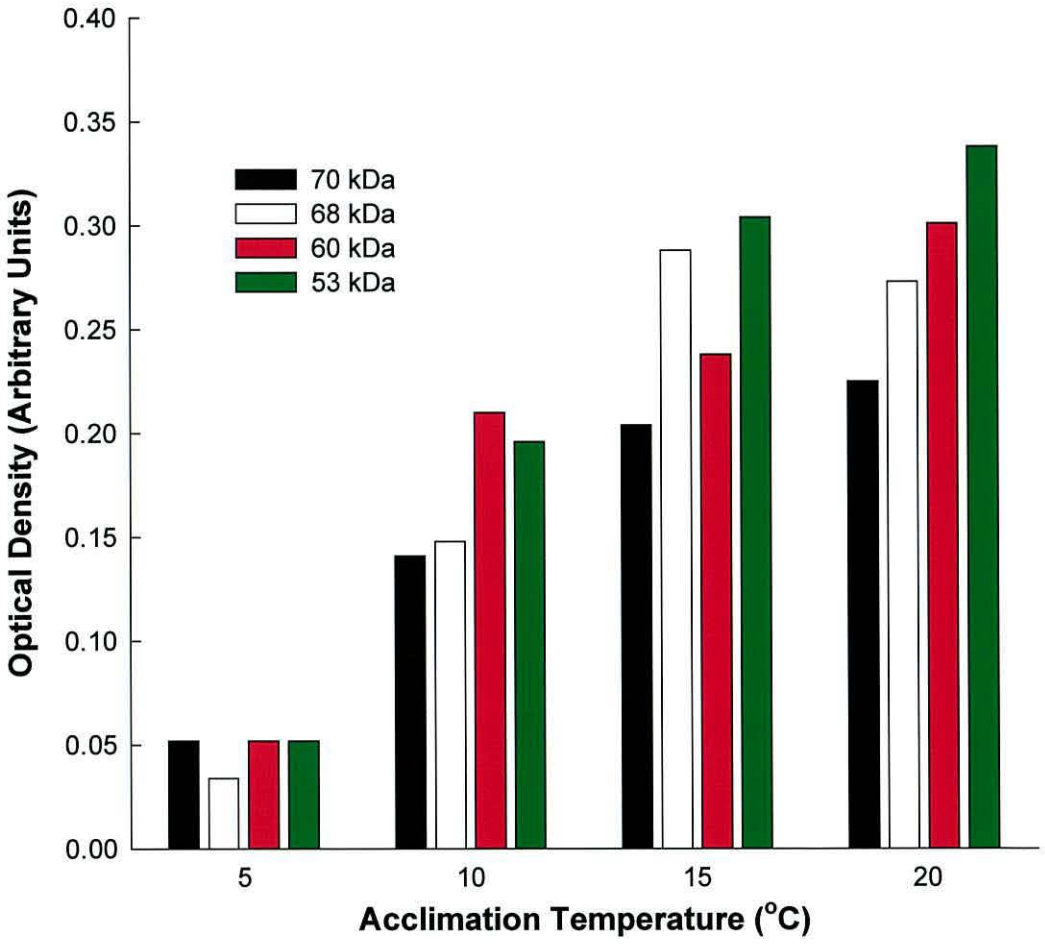


Figure 4.12. Optical density of constitutively expressed proteins of 70, 68, 60 and 53 kDa in winter *Ligia oceanica* acclimated at 5, 10, 15 and 20°C for 4 weeks. n = 1 for each treatment temperature.

Chapter 5

Metabolic Costs of General Rates of Protein Synthesis and Heat-Shock Protein Synthesis in *Ligia oceanica*

5.1 *Introduction*

The metabolic costs of general rates of protein synthesis have been studied in a variety of organisms, including chickens (Aoyagi *et al.*, 1988), teleosts (Houlihan *et al.*, 1993b; Houlihan *et al.*, 1995), and isopod crustaceans (Whiteley *et al.*, 1996). Collectively, these studies have revealed an inverse relationship between the rates of protein synthesis, and the metabolic costs of protein synthesis, *i.e.* as rates of protein synthesis increase, the metabolic costs of protein synthesis decrease. The reason why protein synthesis is more energetically costly at lower rates of synthesis is thought to be due to a fixed cost component, so at higher rates of synthesis, the fixed costs contribute to a lower proportion of the overall costs (Pannevis and Houlihan, 1992; Houlihan *et al.*, 1995). Variable energetic costs of protein synthesis have been demonstrated in rainbow trout hepatocytes, *Oncorhynchus mykiss* (Pannevis and Houlihan, 1992), and in the blue mussel, *Mytilus edulis* (Hawkins *et al.*, 1987), where the lowest costs of protein synthesis were associated with the highest rates of protein synthesis. Even though a number of studies have looked at the effects of various incubation temperatures on the metabolic costs of protein synthesis, none have examined possible dynamic changes in the metabolic costs in ectotherms exposed to seasonal changes in environmental variables. Seasonal changes in the energetic costs associated with protein synthesis could have marked effects on the ability of intertidal ectotherms, such as *Ligia*, to survive cyclical changes in environmental temperature.

The biological zonation that is so characteristic of rocky intertidal shores is known to be influenced by various physical factors, such as temperature, light intensity and desiccation (Nybakken, 1997). Temperature and desiccation in particular are proposed to be two of the main abiotic determinants of distribution of species on rocky shores (Hofmann and Somero, 1996b). However, more recent work has focussed on the role heat-shock protein expression may play in defining ecological distribution of species on the shore. Studies which have looked at heat-shock protein synthesis, have revealed positive correlations between hsp expression, and the ability to survive temperature stress (Lindquist, 1986; Morimoto *et al.*, 1990; Somero, 1995; Feder and Hofmann, 1999). The benefits of hsp expression during heat-stress appear obvious, however, a few studies have looked at the overall costs of hsp induction, which are thought, not only to be metabolically costly to synthesise (Sanchez *et al.*, 1992; Hofmann and Somero, 1995), but ultimately, may limit survival.

A number of workers have postulated that the relatively high costs of expression of heat-shock proteins may be due to a combination of factors, such as: the repression of normal cellular activity following heat-shock, as shown by *Drosophila* cell cultures (DiDomenico *et al.*, 1982); the increase in energy consumption following a heat-shock (Gething and Sambrook, 1992), (more specifically, the use of ATP for maintaining functional pools of molecular chaperones, the ATP-dependent degradation of irreversibly damaged proteins, and the *de novo* synthesis of proteins to replace thermally damaged proteins (Hofmann and Somero, 1996b)); and a possible energy

debt caused by the previous factors, resulting in increased vulnerability to further stress (Krebs and Loeschke, 1994b). For example, in the bivalve, *Mytilus trossulus*, Hofmann and Somero (1995) demonstrated that increased energy expenditure from the expression of hsps affected the allocation of energy resources for growth and reproduction. Similarly, a number of studies have revealed that over-expression of hsps can significantly decrease fitness in *Drosophila* (Feder *et al.*, 1992; Krebs and Loeschke, 1994a; Krebs and Loeschke, 1994b).

The previous chapter demonstrated both higher constitutive levels of hsp expression and a stronger heat-shock response in acclimatised *Ligia* from the summer compared to the winter. Evidence from previous studies on the effects of hsp expression on fitness parameters suggests that expression of hsps is only of benefit to organisms which regularly experience temperatures approaching their upper limits of survival, such as intertidal invertebrates during the summer. The continued expression of hsps by *Ligia* in the summer, would therefore suggest a trade-off between continued viability at heat-stress temperatures, and fitness parameters, measured by growth and fecundity.

This chapter aims to determine the metabolic costs of general rates of protein synthesis and heat-shock protein synthesis in both acclimated and acclimatised *Ligia oceanica* from the summer and the winter. The relationships between metabolic rate and both general rates of protein synthesis and heat-shock protein synthesis will be investigated using the protein synthesis inhibitor, cycloheximide. The energetic consequences of

both of these variables will be discussed in relation to season, recent thermal history, and with regard to the possible seasonal re-distribution of energy resources. Finally, the metabolic costs of protein synthesis in *Ligia* will be compared to those found in a polar isopod, and a temperate marine isopod, and the strategies used by cold-adapted ectotherms to limit energy expenditure will be discussed with regard to temperate ectotherms.

5.2 *Materials and Methods*

Ligia oceanica were caught from under rocks and in crevices at the high water mark of spring tides on the rocky shore at Rhosneigr (O/S grid reference: 231787, 372452) and Cemlyn Bay (O/S grid reference: 233075, 393820), either in January 2001 (winter animals) or in June 2001 (summer animals). Male intermoult animals were returned to the laboratory and either left overnight at *in situ* temperatures (acclimatised animals) or acclimated to a range of temperatures for at least 4 weeks. The experiments were carried out in two phases: firstly to investigate the metabolic cost of general rates of protein synthesis in *Ligia* with change in temperature; and secondly to investigate specific costs of heat shock protein synthesis. In each case, the metabolic costs of protein synthesis were estimated directly by simultaneous measurement of whole-animal rates of oxygen uptake and protein synthesis, with and without treatment with the protein synthesis inhibitor, cycloheximide (CHX).

5.2.1 *Costs of general rates of protein synthesis*

General rates of protein synthesis and oxygen uptake were measured in acclimatised isopods at *in situ* temperatures, and in isopods acclimated at either 5, 10, 15 and 20°C, both in the winter and in the summer. For this set of experiments, animals were returned to the laboratory in January and in June and split into six groups. One group of six animals was left over night (10-16 hours) in individual respirometer chambers held in a temperature-controlled incubator at the *in situ* temperature, either 20°C in the summer, or 5°C in the winter, to represent animals straight from the shore (*i.e.* animals acclimatised to seasonal conditions). Each chamber contained filter paper soaked in sea water to maintain a high relative humidity. Isopods were held over night to prevent the animals from feeding, thereby avoiding the influence of specific dynamic action, which is known to influence whole-animal rates of protein synthesis (Carefoot, 1987; Houlihan *et al.*, 1990b; Brown and Cameron, 1991; Brown and J.N., 1991; Whiteley *et al.*, 2001). As it was only possible to take measurements from six animals in one day, one repeat visit to the shore was made to collect a further six animals for the acclimatisation experiments. The remaining animals were divided equally into five groups of twelve animals for acclimation to either 5, 10, 15, 20 and 25°C for at least 4 weeks. Animals were maintained at constant temperature as explained in Chapter 2. Prior to experimentation, six animals from each acclimation temperature were separated out from the main group and held in a separate container for 24 hours to deprive the animals of food. Faecal pellets were also removed to prevent the possible influence of the SDA response. Animals were then placed into individual respirometer chambers, containing filter paper soaked in

sea water, 24 hours before experiments commenced to allow the isopods to settle to the experimental conditions. The chambers were sealed with a lid to prevent dehydration, and held in the temperature controlled incubator at the appropriate acclimation temperature.

At the start of each experiment, for both acclimatised and acclimated animals, isopods were very carefully removed from their respective respirometer chambers and injected with cycloheximide (CHX) and crab saline at 1.8 μmol CHX.100 mg^{-1} wet weight, and replaced into the chamber. Injections were made into the haemocoel through the dorsal surface at the pereon-pleon junction, just to the right or left of the alimentary canal. Prior to injection, a small volume of haemolymph (1-2μl) was taken up into the syringe to ensure the CHX/crab saline solution was delivered into the haemocoel of the animal. The syringe was left in place for 10 seconds before removal to ensure adequate circulation of the injected solution. The CHX proved difficult to dissolve in crab saline, and the CHX/crab saline solution had to be thoroughly vortexed prior to each injection. Each chamber was then reconnected to the manometer block and the manometer left open so that the chambers were in contact with ambient conditions. Each respirometer was suspended in a water bath run at the appropriate temperature *i.e.* either the *in situ* temperature in the case of acclimatised animals (20°C in summer and 5°C in winter), or the acclimation temperature at which the animals were held at for 4 weeks. In each case the chambers were immersed to the lids for effective temperature equilibration. Initial experiments demonstrated that temperature equilibration between the chambers and the water bath took at least 30 minutes and that it

was important to keep the chambers in the water bath at all times to keep the equilibration time down to a minimum. After 2 hours, the animals were removed and injected with [^{3}H] phenylalanine at a dose of 0.10 μCi.100 mg^{-1} wet weight and replaced in the respirometer chambers for a further 60 minutes. Oxygen uptake readings were taken in the last 30 minutes of the incubation period. Consequently the total time for each experiment was 3 hours. At the end of the 3 hour period the animals were removed, decapitated and snap frozen in liquid nitrogen for the analysis of protein synthesis rates.

5.2.2 *Costs of heat shock protein synthesis*

In the second set of experiments, *Ligia* were collected from the shore in June 2000, and returned to the laboratory. The animals were held overnight in individual respirometry chambers at the *in situ* temperature of 15°C. The following morning, animals were heat shocked by increasing the temperature of the water bath to either 25, 27, 29, or 31°C. The experiment was repeated several times, as only six animals could be measured at once. A similar procedure was carried out as explained in the previous section (5.2.1) for the estimates of general rates of metabolism and protein synthesis. In summary, animals were left in the chambers for 2 hours, removed carefully for the injection of [^{3}H] phenylalanine and returned to the chambers for a further 60 minutes. Oxygen uptake measurements were taken in the last 30 minutes. In this set of experiments, animals were not injected with CHX.

In an attempt to estimate the specific costs of heat shock protein synthesis, preliminary experiments were carried out on animals acclimated at 15°C in the

summer and winter. Isopods were collected in January and June 2001, and held at 15°C for 4 weeks in incubators set to light/dark cycle of 12/12 (0600 to 1800 hours light), and were fed potato shavings and *Fucus spp.* fronds twice weekly. Prior to experimentation the animals were treated as described for the estimation of general rates of protein synthesis. In this case, animals were heat shocked after the injection of saline or cycloheximide by increasing the temperature of the water bath to 25°C. Again animals were left for 2 hours after the injection of CHX and then a further 60 minutes after injection of the radiolabel before processing. Oxygen uptake measurements were taken in the last 30 minutes. In addition, twelve animals were maintained at 15°C over the 3 hour period to act as a control.

The effect of CHX on the synthesis patterns of heat shock protein production was determined by metabolic labelling of newly synthesised proteins for observation by SDS-PAGE and autoradiography, as detailed in Chapter 4. Synthesis patterns of heat shock proteins were investigated before and after CHX treatment in animals acclimatised to summer conditions and in winter animals acclimated at 15°C. In each case, *Ligia* were either injected with saline, or a CHX/saline cocktail at a dose of 1.8 µmol CHX.100 mg^{-1} wet weight at the start of the experiment, and immediately placed in their respective respirometers for exposure to heat shock. The [^{35}S] metabolic label was administered one hour after the sham/CHX injection according to the methods described in Chapter 4, and animals were left for a further 2 hours before being snap-frozen in liquid nitrogen.

5.3 *Results*

5.3.1 *Metabolic Costs of General Rates of Protein Synthesis*

Tables 5.1 and 5.2 show the effects of the protein synthesis inhibitor, cycloheximide (CHX), on whole-animal rates of oxygen uptake ($\dot{M}O_2$) and absolute rates of protein synthesis (A_s) in *Ligia oceanica* straight from the shore and after acclimation to different temperatures in the laboratory. The effects of CHX on *Ligia* acclimated at 5, 10 and 15°C were unexpected, as treatment with CHX should inhibit protein synthesis at translation, resulting in concomitant reductions in rates of oxygen uptake. As such, the only sections of Table 5.1 where the metabolic costs of protein synthesis could be calculated were summer and winter acclimated *Ligia* at 20°C, and summer and winter acclimatised *Ligia* taken straight from the shore. See section 5.4 for a discussion of the problems associated with using CHX in this study.

5.3.1.1 *Acclimated animals*

Treatment with CHX had very little effect on whole-animal rates of protein synthesis and oxygen uptake in *Ligia oceanica* acclimated to 5 or 10°C, regardless of season. At the higher acclimation temperature of 15°C, there was a 53.1% reduction in A_s levels in the summer animals but no change in the winter. In contrast, animals acclimated at 20°C showed a reduction in both variables after treatment with CHX in both seasons. More specifically, *Ligia* acclimated at 20°C in the summer showed a 14.2% reduction in mean rates of

oxygen uptake, and a 74.1% reduction in mean A_s after treatment with CHX, giving a mean CHX-sensitive $\dot{M}O_2$ of 5.4 μmol.animal^{-1}.day^{-1}, and a mean CHX-sensitive A_s of 1.0 mg protein.animal^{-1}.day^{-1}. The proportion of oxygen uptake attributable to whole-animal absolute rates of protein synthesis, assuming 100% inhibition of protein synthesis was 19.2% (7.3 μmol animal^{-1} day^{-1}). In the winter, *Ligia* acclimated at 20°C showed a 24.0% reduction in mean rates of oxygen uptake, and a 23.3% reduction in mean A_s after treatment with CHX. This resulted in a mean CHX-sensitive $\dot{M}O_2$ of 7.8 μmol.animal^{-1}.day^{-1}, and a mean CHX-sensitive A_s of 0.1 mg protein. animal^{-1}.day^{-1}. The proportion of oxygen uptake attributable to whole-animal absolute rates of protein synthesis, assuming 100% inhibition of protein synthesis was 72.1% (23.5 μmol.animal^{-1}.day^{-1}). The resulting values can be used to estimate metabolic costs of protein synthesis, as outlined in section 5.3.2.

5.3.1.2 *Acclimatised Animals*

Unlike animals acclimated at 5°C for 4 weeks, *Ligia oceanica* collected in the winter and held overnight at 5°C experienced a reduction in both rates of oxygen uptake and protein synthesis. Cycloheximide treatment caused a 38.1% reduction in mean rates of oxygen uptake, and a 54.4% reduction in mean A_s, resulting in a mean CHX-sensitive $\dot{M}O_2$ of 17.6 μmol.animal^{-1}. day^{-1}, and a mean CHX-sensitive A_s of 0.4 mg protein.animal^{-1}.day^{-1}. The proportion of oxygen uptake attributable to whole-animal absolute rates of protein synthesis, assuming 100% inhibition of protein synthesis was 70.0% (32.3 μmol.animal^{-1}.day^{-1}). A cycloheximide-induced reduction in metabolism

and protein synthesis was also observed in the summer at an *in situ* temperature of 20°C (Table 5.1). In this case, isopods showed a 8.9% reduction in mean rates of oxygen uptake, and a 52.8% reduction in mean A_s after treatment with CHX, giving a mean CHX-sensitive $\dot{M}O_2$ of 3.6 μmol.animal^{-1}.day^{-1}, and a mean CHX-sensitive A_s of 1.0 mg protein.animal^{-1}.day^{-1}. The proportion of oxygen uptake attributable to whole-animal absolute rates of protein synthesis, assuming 100% inhibition of protein synthesis was 17.3% (7.1 μmol.animal^{-1}.day^{-1}).

5.3.2 *Estimates of Metabolic Costs*

The relationship between CHX-sensitive oxygen uptake and CHX-sensitive changes in absolute rates of protein synthesis can be used to calculate the energetic costs of whole-animal rates of protein synthesis. CHX-sensitive oxygen uptake was converted into millimoles of ATP using the assumption that one mole of oxygen is equivalent to 6 moles of ATP (Reeds *et al.*, 1985). The metabolic cost of protein synthesis for *Ligia* collected in the winter and acclimated at 20°C was 469.8 mmol ATP.g protein^{-1} synthesised. In contrast, the metabolic cost of protein synthesis rates in animals collected in the summer and acclimated at 20°C was 14.5-times lower at 32.5 mmol ATP.g protein^{-1}. The metabolic cost of protein synthesis for *Ligia* collected from the shore in winter was 245.2 mmol ATP.g protein^{-1}, and for animals collected in the summer, this value was 10.4-times lower at 23.5 mmol ATP.g protein^{-1}.

The highest metabolic costs of protein synthesis were found in *Ligia* collected in the winter and acclimated at 20°C for 4 weeks (Table 5.2). The unit cost of

one gram of protein synthesised was 1.9-times higher in these animals than it was in *Ligia* collected straight from the shore in winter. Metabolic costs of protein synthesis in both acclimatised *Ligia* and *Ligia* acclimated at 20°C in the summer were remarkably similar, the unit cost of synthesis for one gram of protein being marginally higher in acclimated animals. Costs of protein synthesis were between 10.4- and 14.5-fold higher in *Ligia* collected in winter than in animals collected in the summer. By relating absolute rates of protein synthesis with metabolic costs of protein synthesis on Fig. 5.1, it is possible to see that the metabolic costs of protein synthesis declined with increasing whole-animal A_s values. Note that two of the results are close to, or at the theoretical minimum metabolic costs of protein synthesis (Houlihan *et al.*, 1995; Marsh *et al.*, 2001).

5.3.3 *Metabolic costs of Heat-Shock Protein Synthesis*

5.3.3.1 *Patterns of Synthesis*

Saline-injected *Ligia* collected in the winter and acclimated at 15°C expressed hsps corresponding to 70, 68, 60 and 53 kDa size classes (Figure 5.2) when incubated at 15°C for 3 hours, although their expression was relatively weak. After treatment with CHX, all hsp synthesis was inhibited, apart from a small hsp60 response, which was similar in intensity to the levels found in saline-injected animals. Saline-injected animals incubated at 15°C and heat-shocked at 25°C showed greater hsp expression for all protein size classes compared to saline-injected animals incubated at 15°C. Labelling intensity of the 70, 68, 60 and 53 kDa proteins was 5-, 2.5-, 11- and 2-fold higher than the 15°C control values. Hsp expression in CHX-injected animals was inhibited at 25°C.

The effects of CHX treatment on *Ligia* collected in the summer and held overnight at 15°C are shown in Figure 5.3. In all cases, CHX had an inhibiting effect on hsp labelling intensity. The percentage reduction in labelling intensity after treatment with CHX for all protein size classes is shown in Table 5.3. The CHX-induced reductions in labelling intensity were greatest at the higher heat-shock temperatures of 29 and 31°C, and had least effect at 25°C. At 29°C, hsp synthesis was completely inhibited. Overall, the intensity of hsp expression was attenuated in winter animals relative to *Ligia* collected in the summer.

5.3.3.2 *Rates of Oxygen Uptake and Protein Synthesis*

The effects of heat shock on winter *Ligia* acclimated at 15°C and incubated at 25°C for 3 hours are summarised in Table 5.4 and Figure 5.4. In winter *Ligia* acclimated at 15°C, there were no significant differences in whole-animal rates of oxygen uptake or protein synthesis between saline-injected animals and CHX-injected animals. Unfortunately, the lack of corresponding summer data makes seasonal comparisons impossible. Exposure to heat-shock, however, was associated with a reduction in whole-animal rates of protein synthesis and oxygen uptake in summer *Ligia* collected straight off the shore (Table 5.5). In general, as heat-shock temperature increased, there was a greater decrease in A_s and $\dot{M}O_2$ levels. For example, mean A_s at 29°C was 2.3-times lower than values at 15°C, and mean $\dot{M}O_2$ values were 1.6-times lower.

5.4 *Discussion*

Many estimates of the costs of protein synthesis are based on theoretical calculations or on the correlations between whole-animal oxygen uptake and whole-animal protein synthesis (see Table 5.6). The problem with these estimates is that the results are highly variable, not only between these two methods, but also within them. For example, Waterlow and Millward (1989) compared the theoretical and correlative costs of protein synthesis in mammals and found a 5-fold difference between the two methods. In fish, Jobling (1985) found the theoretical costs of protein synthesis to be anywhere from 50 to 100 mmol ATP per gram of protein synthesised. Theoretical costs of protein synthesis in cod (*Gadus morhua*) ranged from 23 to 44% of total oxygen uptake (Houlihan *et al.*, 1988; Houlihan, 1991; Lyndon *et al.*, 1992), and between 11 and 22% of total oxygen uptake in the grass carp, *Ctenopharyngodon idella*, (Carter *et al.*, 1993). In invertebrates, whole-body rates of protein synthesis in the blue mussel, *Mytilus edulis*, were found to account for between 16% and 25% of basal metabolic rate (Hawkins, 1985; Hawkins *et al.*, 1986; Hawkins *et al.*, 1989). Metabolic costs of whole-animal protein synthesis in *Carcinus* were between 19 and 37% of measured oxygen uptake (Houlihan *et al.*, 1990b) and in *Octopus vulgaris*, protein synthesis rates accounted for even higher metabolic costs of between 33 and 51% (Wells *et al.*, 1983). All of these estimates are thought to be the absolute minimum energetic costs of protein synthesis (Reeds *et al.*, 1985; Waterlow and Millward, 1989; Hawkins, 1991). Other studies in which interspecific

relationships between protein synthesis and heat production have been compared indicate a minimal estimate of 20% for the metabolic costs associated with protein synthesis in a wide range of taxa, including bivalves, teleosts and mammals (Hawkins, 1991).

In contrast to the estimates described above, metabolic costs of protein synthesis can be measured directly using the protein synthesis inhibitor, cycloheximide (CHX), combined with the simultaneous determinations of protein synthesis and oxygen uptake rates. There are a number of potential problems associated with the use of CHX when attempting to inhibit protein synthesis. Dr. N.M. Whiteley (personal communication) has evidence that in crustaceans, or at least the shore crab, *Carcinus maenas*, there is the ability to recover from CHX treatment given sufficient time, which suggest the drug is being metabolised, and that additional oxygen is being used as a result. Personal observations of *Ligia* after administration of CHX showed that relative to sham-injected *Ligia*, those injected with CHX were more distressed (as noted by activity levels). It could be assumed that the oxygen uptake of active *Ligia* will be higher than that of inactive or less active individuals. Additionally, there were problems associated with the solubility of CHX in crab saline. These observation highlight the importance of constructing a time-course for the effects of CHX on *Ligia oceanica*, and also a dose/response study, as CHX may affect other cellular processes in addition to protein synthesis above certain concentrations leading to overestimates of the metabolic costs of protein synthesis (Fuery *et al.*, 1998; Wieser and Krumschnabel, 2001). These experiments were unfortunately unable to be

determined in this study due to time constraints, but some of these problems are currently being addressed. More experiments using CHX to ascertain the metabolic costs of protein synthesis in *Ligia* are currently being undertaken, with tissue samples waiting to be analysed. Additional studies using an alternative protein synthesis inhibitor actinomycin D, which inhibits the transcription of mRNA, will also be carried out at a future date.

The results for rates of oxygen uptake presented in this chapter are not directly comparable to $\dot{M}O_2$ results from previous chapters, as *Ligia* were treated differently prior to the measurements of oxygen uptake rates. For example, in Chapters 2 and 3, *Ligia* were placed into individual respirometry chambers and left for 12 hours prior to taking measurements, allowing sufficient time for the effects of handling stress to diminish. In this chapter, animals were injected with either CHX or crab saline and left for 2 hours for the drug to take effect. *Ligia* were then injected with [^{3}H] Phe and left for a further 30 minutes before respirometry measurements were recorded. Subsequently, the increased stress of handling and injecting may explain the unexpectedly high $\dot{M}O_2$ measurements presented in Table 5.1, specifically of the winter acclimatised control group.

Estimates of the metabolic costs of protein synthesis are made by relating CHX-sensitive oxygen uptake rates to CHX-sensitive protein synthesis rates, as protein synthesis is an important contributor to total energy expenditure in organisms (Hawkins, 1991). However, direct measurements of the metabolic costs of protein synthesis using CHX are few and far between, and a

summary of the data available in the literature can be displayed in a single table (Table 5.6). Using CHX, estimates of the metabolic costs of protein synthesis in trout hepatocytes (*Oncorhynchus mykiss*) at temperatures of between 5 and 20°C ranged from 666 to 3,498 mmol ATP per gram of protein synthesised (up to 79.7% of total oxygen uptake) at protein synthesis rates of between 1 and 9% per day (Pannevis and Houlihan, 1992). However, metabolic costs of 36 mmol ATP per gram of protein synthesised (31% of total oxygen uptake) were recorded for juvenile tilapia, *Oreochromis mossambicus*, at a protein synthesis rate of 30% per day (Houlihan *et al.*, 1993b). In endotherms, such as chickens, 72 mmol ATP were required per gram of protein synthesised (28.8% of total oxygen uptake) at protein synthesis rates of 29% per day (Aoyagi *et al.*, 1988). These results indicate an inverse relationship between the energy costs for protein synthesis and the rates at which the proteins are synthesised, as explained below.

The costs of protein synthesis are split into two components, a fixed or independent component, and a variable or dependent component (Pannevis and Houlihan, 1992; Houlihan *et al.*, 1995; Smith and Houlihan, 1995). Evidence for this comes from the observation that as rates of protein synthesis increase, the energetic costs of protein synthesis (measured in moles ATP) decrease (Houlihan, 1991; Pannevis and Houlihan, 1992; Houlihan *et al.*, 1995; Smith and Houlihan, 1995; Whiteley *et al.*, 1996; Smith *et al.*, 2000). For instance, in rainbow trout hepatocytes, 3,500 mmol of ATP per gram of protein synthesised are required at a growth rate of 0-1% per day, whilst 670 mmol ATP per gram of protein synthesised are required at a growth

rate of 7.4% per day (Pannevis and Houlihan, 1992). In addition, Hawkins *et al.* (1987), found that when *Mytilus edulis* were transferred from 10 to 20°C, mussels with the higher rates of protein synthesis had lower daily oxygen uptake rates. Increased rates of protein turnover enabled the mussels to adapt to the higher temperature more quickly whilst simultaneously reducing oxidative expenditure. Thus, increased rates of protein synthesis and turnover are intimately linked with the ability of *M. edulis* to acclimate to temperature change.

The costs of protein synthesis are variable because of a fixed component, which is due to the activation of tRNA and production of rRNA (Pannevis and Houlihan, 1992). As rates of protein synthesis increase, so the fixed costs make a decreasing contribution to the aerobic costs (Houlihan *et al.*, 1995). The highest unit costs per gram of protein synthesised in *Ligia* were associated with the lowest absolute rates of whole-animal protein synthesis. *Ligia* collected in the winter and acclimated at 20°C had the lowest A_s values (0.3 mg protein.day^{-1}), but the highest energetic costs of protein synthesis (469.8 mmol ATP.g protein^{-1}). Conversely, *Ligia* taken straight from the shore in the summer had the highest A_s values (1.8 mg protein.day^{-1}), but the lowest energetic costs of protein synthesis (23.5 mmol ATP.g protein^{-1}). The fixed costs, therefore, contribute less to overall costs as rates of protein synthesis increase, as is the case in most animals studied to date (Pannevis and Houlihan, 1992; Houlihan *et al.*, 1995; Smith and Houlihan, 1995). Figure 5.5 compares the metabolic costs of whole-animal rates of protein synthesis in *Ligia* with the other study of isopod crustaceans by Whiteley *et al.* (1996),

whilst Figure 5.6 compares whole-animal rates of protein synthesis and their associated metabolic costs in a wide variety of organisms listed in Table 5.6. Correlative estimations have been derived from the slope of the line when relating A_s to $\dot{M}O_2$. The majority of the metabolic costs presented in the table have been estimated using the CHX inhibition of protein synthesis, but a few correlative estimations have also been included. The latter also follow the inverse relationship between energetic costs of protein synthesis and rates of protein synthesis. Some of the estimates of metabolic costs of protein synthesis detailed on Table 5.6 are less than the minimum theoretical costs of protein synthesis (48 mmol ATP.g protein^{-1} synthesised) (Houlihan *et al.*, 1995; Marsh *et al.*, 2001), which may be explained by the problems associated with using CHX as an inhibitor of protein synthesis, as discussed earlier. Subsequently, any results that fall below the minimum theoretical costs could be assumed to be at the minimum level. The large variation in the metabolic costs between the correlative and direct inhibition studies detailed on Table 5.6 may also be as a result of differences in experimental methodology.

Remembering the preliminary nature of these studies and the problems associated with the use of CHX evident in Table 5.6 (an increase in $\dot{M}O_2$ after CHX treatment rather than an expected decrease), the proportions of oxygen uptake attributable to whole-animal protein synthesis in summer *Ligia*, whether acclimated at 20°C, or taken directly from the shore, were very similar, at 19.2% and 17.3%, respectively. The same was true of *Ligia* collected in the winter, and either acclimated at 20°C, or used directly from the

shore, where whole-animal protein synthesis accounted for 72.1% and 70%, respectively, of control oxygen uptake. Therefore winter animals living at low temperatures on the shore have 10.4-times higher costs of protein synthesis, expressed as mmol ATP.g protein^{-1} synthesised, than summer animals. Consequently, 10.4-fold more ATP was required for protein synthesis in the winter *versus* the summer. Moreover, acclimation at 20°C had little effect on overall costs of general protein synthesis rates in *Ligia*, indicating an over-riding seasonal effect on metabolic costs. Therefore winter *Ligia* are not only faced with seasonal changes in ATP supply and 'turnover' but also changes in ATP demand to fuel protein synthesis, with winter animals experiencing an increase in ATP demand for the synthesis of a given quantity of protein. However, the expression of metabolic costs as mmol ATP per unit of protein synthesised may not be realistic or comparable to the whole-animal situation on the shore. Using the costs of protein synthesis outlined in this chapter, the actual metabolic costs of whole-animal rates of protein synthesis can be calculated. For example, in *Ligia* collected in the summer and winter, and acclimated at 20°C, the ATP costs were 4.5 and 113.1 μmol ATP.day^{-1}, respectively, scaled to represent an animal of one gram fresh weight using a weight exponent of 0.7 (Whiteley *et al.*, 1996) (data from Chapter 2). The costs in summer and winter acclimatised *Ligia* are dramatically higher, at 110.5 and 1190.8 μmol ATP.day^{-1}, respectively (data from Chapter 3) at their *in situ* temperatures of collection. Consequently, these data show that even though metabolic costs, expressed as mmol ATP.g protein^{-1}, are higher in the acclimated animals *versus* animals straight from the shore, estimates based on the absolute rates of protein synthesis, measured in Chapters 2 and 3,

show that ATP demand for protein synthesis is higher in acclimatised animals. The marked differences between summer and winter costs, however, remains, as the ATP required for the protein synthesised is 24.7- to 30.3-times higher in winter acclimated and acclimatised animals, respectively.

The higher metabolic demand of protein synthesis experienced by winter animals suggests that a larger proportion of the energy budget in the winter goes towards general rates of protein synthesis. Therefore, maintenance costs are considerably higher in the winter, and may result in a re-allocation of resources, perhaps from growth and reproduction, restricting these cyclical processes to the summer. Interestingly, in *Ligia*, despite the marked seasonal variations in metabolic costs for protein synthesis, *Ligia* have lower whole-animal rates of protein synthesis in the summer than in the winter. Again, this may be explained by a seasonal partitioning of the available energy budgets, with allocation of energy to other important, energy demanding processes during the summer, but not in the winter. This seasonal redistribution of energy resources may have consequences for the heat-shock response, and subsequently thermotolerance and survival. There are indications to suggest that winter animals are compromised in their ability to express hsps in response to heat-stress, which may be related to the increased costs associated with synthesising proteins during the winter, as described below.

The proportion of oxygen uptake attributable to protein synthesis in winter *Ligia* is remarkably similar to that found in the giant Antarctic isopod, *Glyptonotus antarcticus*, which lives permanently at low temperatures

(Whiteley *et al.*, 1996). Consequently, the relatively high costs associated with protein synthesis appears to be a feature of ectotherms living in low temperature habitats *per se*. Interestingly, the metabolic costs of protein synthesis in summer *Ligia* are similar to those of the temperate isopod species, *Idotea rescata*, which was also studied in the summer (Whiteley *et al.*, 1996). Unfortunately, no data is available for *Idotea* from the winter, and it may be that costs of protein synthesis in this species are also dependent on season. The seasonal effect on metabolic costs observed in *Ligia* is seen to persist in isopods with different thermal histories, and this raises interesting questions about ectotherms living permanently at low temperatures, such as polar marine invertebrates. For example, the stenothermal marine isopod, *Glyptonotus*, has a long history at living in the extremely cold and relatively thermally stable waters of the Antarctic, and yet the proportion of metabolism associated with protein synthesis is similar to that of the eurythermal isopod, *Ligia*, when measured in the winter. High metabolic costs of protein synthesis may be a driving force for protein stability at low temperatures (Clarke, 1987), and reduced protein turnover may represent an energy saving strategy (Hawkins, 1991). Low whole-body protein turnover appears to be characteristic of polar invertebrates (Clarke, 1998), but unfortunately, rates of protein degradation, which are difficult to study, are not known for any of the isopod species studied, and therefore these predictions remain speculative.

More recently, estimates on the costs of protein turnover in the embryos and larvae of the echinoderm, *Sterechinus neumayeri*, were calculated to be very low. Rates of protein turnover were, however, thought to be relatively high,

and comparable to rates in temperate sea urchin embryos (Marsh *et al.*, 2001). The similarity in protein turnover rates between the Antarctic and temperate sea urchin species was reported to be due to high cellular concentrations of RNA (up to 10-times greater than those found in a temperate species). An increase in protein synthesis rates in this Antarctic sea urchin would act to decrease costs of protein turnover, according to the assumption that there is a fixed cost of protein synthesis (Pannevis and Houlihan, 1992). Despite the observed compensation for protein turnover rates during early development in *S. neumayeri*, rates of development are slow, in keeping with other Antarctic marine invertebrates, as shown by Stanwell-Smith and Peck (1998). It is unclear how the increase in protein turnover rates seen in this Antarctic invertebrate relates to rates of growth and development in the embryos and larvae of *S. neumayeri*, and this species may be the exception rather than the rule. In the present study, rates of protein synthesis were marginally higher in winter rather than summer animals due to an increase in the RNA capacity. Despite these compensatory changes, whole-animal costs of protein synthesis were still higher in the winter compared to the summer.

Metabolic labelling of newly synthesised proteins in winter animals and consequent SDS-PAGE analysis (Figure 5.2) showed that hsps are synthesised on heat-stress, albeit at a reduced level compared to summer animals (Figure 5.3). In the winter, CHX treatment inhibited hsp synthesis, but had no effect on whole-animal rates of protein synthesis after 3 hours incubation at 25°C, even though $\dot{M}O_2$ levels decreased. In contrast, saline

injection at 25°C caused a slight, non-significant increase in $\dot{M}O_2$. As general absolute rates of protein synthesis were unaffected by CHX treatment, but hsp synthesis decreased or was inhibited altogether, it could be assumed that the decrease in $\dot{M}O_2$ after CHX treatment represents the energy requirements for hsp synthesis. Using this assumption, it is possible to calculate the energetic costs required for hsp synthesis in summer and winter animals from the data displayed in Table 5.4. It must be stressed that this is an assumption based on preliminary data, and more work needs to be done in this area before more emphatic statements can be made. Consequently, in *Ligia* acclimated at 15°C and heat-shocked at 25°C, the heat-shock response in isopods with a mean body mass of 400 mg required 160 µmol ATP in the winter, and 68 µmol ATP in isopods with a mean body mass of 434 mg in the summer. These data show that despite the weaker heat-shock response recorded in *Ligia* in the winter, the metabolic costs of hsp synthesis were 2.4-times higher in the winter animals than in the summer at a similar heat-shock temperature. Such estimates confirm that hsp synthesis is metabolically demanding and is more so in the winter than in the summer. Consequently, hsp synthesis will be restricted in the winter and may account for the failure to produce hsps at high temperatures. While these estimations provide useful information on the metabolic costs incurred during heat-shock, it is important to remember that the heat-shock response is triggered by increased protein misfolding and aggregation, associated with denaturation and degradation (Morimoto *et al.*, 1994; Somero, 1995). The metabolic costs associated with protein degradation are also considered to be relatively expensive, and the lack of

information on rates of degradation and therefore protein turnover during heat-shock in *Ligia*, remain an important area for further study.

In summary, costs of protein synthesis were found to be substantially more energetically expensive in winter compared to summer *Ligia*. Seasonal differences in metabolic costs remained after acclimation at 20°C for 4 weeks, and showed remarkable similarities between winter animals and data available for Antarctic marine invertebrates, and between summer animals and a temperate isopod species. Estimates on the metabolic costs of hsp synthesis indicate that hsp synthesis in winter *Ligia* is more energetically costly than hsp synthesis in the summer. The marked differences in metabolic costs of protein synthesis with season can explain the differences in thermotolerance observed in Chapter 3, and the different patterns of hsp synthesis in Chapter 4. These differences in the metabolic costs of protein synthesis may also explain the inability of winter *Ligia* to survive at 31°C for one week, unlike the summer animals, which were able to survive for 4 weeks at 25°C. Therefore, the metabolic costs of protein synthesis in these intertidal animals are fundamental to their survival in the face of temperature change.

Table 5.1. The effects of cycloheximide (CHX) on rates of oxygen uptake ($\dot{M}O_2$) and absolute rates of protein synthesis (A_s) in *Ligia oceanica* either taken straight off the shore (acclimatised) or acclimated to four different temperatures in the winter and in the summer. Values are expressed as means ± SE. $\dot{M}O_2$ is expressed as μmol.animal^{-1}.day^{-1}; A_s is expressed as mg protein.animal^{-1}.day^{-1}; $\dot{M}O_2$ and A_s are both scaled to represent an animal of one gram wet weight, using the weight exponent 0.7 (Whiteley *et al.*, 1996), temperature (T) is expressed as °C; animals collected in the winter are shown as W and animals caught in the summer as S; control animals injected with saline are shown as C; animals injected with cycloheximide are shown as CHX; the difference in both $\dot{M}O_2$ and A_s between control and CHX treated animals is shown as C-CHX. Asterisks indicate significant differences (single factor ANOVA plus Tukey, $P = < 0.05$) between control and CHX-treated animals.

Treatment	T		$\dot{M}O_2$					A_s				
			n	Controls	n	CHX	C-CHX	n	Controls	n	CHX	C-CHX
Acclimated	5	W	6	44.8 ± 9.3	6	47.8 ± 7.3	-3.1	6	0.1 ± 0.01	6	0.1 ± 0.02	-0.01
		S	3	13.9 ± 2.1*	5	42.0 ± 6.2*	-28.0	3	0.1 ± 0.01	5	0.2 ± 0.03	-0.1
	10	W	6	27.7 ± 2.5	5	47.5 ± 9.6	-20.0	6	0.14 ± .01	5	0.1 ± 0.01	0.02
		S	3	51.1 ± 6.2	3	51.8 ± 4.8	-0.7	3	0.3 ± 0.04	3	0.4 ± 0.1	-0.1
	15	W	12	38.2 ± 3.9*	12	60.6 ± 8.3*	-22.4	12	0.2 ± 0.02*	12	0.3 ± 0.1*	-0.2
		S	3	42.1 ± 15.5	3	50.4 ± 10.4	-8.4	3	1.8 ± 0.7	3	0.8 ± 0.2	1.0
	20	W	5	32.6 ± 6.5	4	24.8 ± 9.2	7.8	5	0.3 ± 0.1	3	0.2 ± 0.04	0.1
		S	3	38.0 ± 8.6	3	32.6 ± 10.1	5.4	3	1.4 ± 0.5	2	0.4 ± 0.3	1.00
Acclimatised	5	W	5	46.1 ± 13.3	3	28.6 ± 17.3	17.6	5	0.8 ± 0.4	3	0.4 ± 0.2	0.4
	20	S	4	40.7 ± 10.3	5	37.1 ± 7.6	3.6	4	1.8 ± 1.5	5	0.9 ± 0.4	1.0

Table 5.2. The effects of cycloheximide (CHX) on absolute rates of protein synthesis (A_s) and whole-animal rates of oxygen-uptake ($\dot{M}O_2$) in acclimated and acclimatised *Ligia oceanica*.

Temperature	A_s	$\dot{M}O_2$	Costs of A_s Assuming 100% Inhibition of Protein Synthesis	Control $\dot{M}O_2$	Percentage of Control $\dot{M}O_2$ Attributable to Protein Synthesis
*Summer 20	1.0	5.4	5.4	38.0 ± 8.6	19.2
*Winter 20	0.1	7.8	78.0	32.6 ± 6.5	72.1
†Summer 20	1.0	3.6	3.6	40.7 ± 10.3	17.3
†Winter 5	0.4	17.6	44.0	46.1 ± 13.3	70.0

* denotes laboratory acclimated *Ligia* and † denotes seasonally acclimatised *Ligia*. Values are expressed as means, A_s (CHX-sensitive absolute rates of protein synthesis) as mg protein.animal^{-1}.day^{-1}, and $\dot{M}O_2$ (CHX-sensitive rates of oxygen-uptake) as µmol.animal^{-1}.day^{-1}.

Table 5.3. The percentage inhibition of protein-labelling intensity in acclimatised *Ligia oceanica* collected in the summer at various incubation temperatures after treatment with the protein synthesis inhibitor, cycloheximide (CHX). The animals at 15°C were the control group.

Molecular Weight (kDa)	Incubation Temperature (°C)				
	15	25	27	29	31
70	80.9	51.8	79.0	100	83.6
68	80.0	30.9	67.3	100	87.9
60	79.7	70.8	59.1	100	95.6
53	67.1	16.4	62.2	100	85.9

Table 5.4. Changes in whole-animal absolute rates of protein synthesis (A_s) and rates of oxygen uptake ($\dot{M}O_2$) in heat-shocked *Ligia oceanica* collected in the winter and summer and acclimated at 15°C for 4 weeks. Animals were either sham-injected with saline, or with the protein synthesis inhibitor, cycloheximide (CHX). $\dot{M}O_2$ is expressed as μmol.animal^{-1}.day^{-1}, and A_s is expressed as mg protein.animal^{-1}.day^{-1}. There were no significant differences in A_s or $\dot{M}O_2$ between either saline-injected or CHX-injected winter animals when heat-shocked at 25°C for 3 hours. Values are means ± SE.

Temp (°C)		$\dot{M}O_2$			A_s			
	n	Saline	n	CHX	n	Saline	n	CHX
Winter 15	6	87.0 ± 13.4	6	105.1 ± 27.1	6	0.2 ± 0.1	6	0.4 ± 0.1
Winter 25	6	119.1 ± 21.6	6	58.0 ± 7.7	6	0.3 ± 0.03	6	0.4 ± 0.1
Summer 25	4	87.3 ± 14.7	8	59.4 ± 14.3	2	0.4 ± 0.1	2	2.0 ± 1.2

Table 5.5. The effects of various heat-shock temperatures on whole-animal rates of oxygen uptake ($\dot{M}O_2$) and absolute rates of protein synthesis (A_s) in acclimatised *Ligia oceanica* collected in the summer. $\dot{M}O_2$ is expressed as μmol.animal^{-1}.day^{-1}; A_s is expressed as mg protein.animal^{-1}.day^{-1}. $\dot{M}O_2$ and A_s values were compared with those of the saline-injected control animals (15°C). All values are means ± SE. Mean $\dot{M}O_2$ values at 25 and 27°C were significantly different from those at 15°C (single factor ANOVA plus Tukey. 25°C: *d.f.* = 1, F = 27.0, P = < 0.001; 27°C: *d.f.* = 1, F = 5.7, P = 0.03). Mean As values at 25, 29 and 31°C were significantly different from those at 15°C (single factor ANOVA plus Tukey. 25°C: *d.f.* = 1, F = 8.2, P = 0.01; 29°C: *d.f.* = 1, F = 8.6, P = < 0.001; 31°C: *d.f.* = 1, F = 17.4, P = < 0.001).

Temp (°C)	n	$\dot{M}O_2$	A_s
15 (Control)	10	333.4 ± 38.5	4.5 ± 0.6
25	10	121.8 ± 13.4	2.5 ± 0.4
27	10	236.5 ± 13.4	4.1 ± 0.6
29	10	252.9 ± 56.2	2.5 ± 0.4
31	8	261.9 ± 12.2	1.7 ± 0.3

Table 5.6. A summary table of rates of protein synthesis and their associated metabolic costs in a variety of organisms. Adapted from Houlihan *et al.* (1995). k_s, the fractional rate of whole-animal protein synthesis.

Species	k_s (%.day^{-1})	ATP (mmol.g protein synthesised^{-1})	Method	Reference
Teleosts	-	50 - 100	Theoretical	(Jobling, 1985)
Calf muscle	0.7 - 1.5	2,700	Theoretical	(Gregg and Milligan, 1982)
Pig muscle	1 - 6	948 – 3,450	Correlation	(Adeola *et al.*, 1989)
Trout hepatocytes	1 - 8.5	222 – 828	Correlation	(Pannevis and Houlihan, 1992)
Whole sheep	3 - 5	294	Correlation	(Harris *et al.*, 1989)
Blue mussel	3 - 8	150	Correlation	(Hawkins *et al.*, 1989)
Juvenile nase	14 - 25	150	Correlation	(Houlihan *et al.*, 1992)
Sheep hepatocytes	30 - 50	942	Correlation	(McBride and Early, 1989)
Ligia oceanica	0.14 - 5.7	264	Correlation	This study
Trout hepatocytes	1 - 9	666 – 3,498	CHX	(Pannevis and Houlihan, 1992)
Trout scale cells	0.5	1,302	CHX	(Smith and Houlihan, 1995)
RTG-2	1.1	798	CHX	(Smith and Houlihan, 1995)
Larval herring	3	588	CHX	(Houlihan *et al.*, 1995)
BF-2	9.1	66	CHX	(Smith and Houlihan, 1995)
Trout macrophages	2.9	276	CHX	(Smith and Houlihan, 1995)
Chickens	29	72	CHX	(Aoyagi *et al.*, 1988)
Juvenile tilapia	30	36	CHX	(Houlihan *et al.*, 1993)
Glyptonotus antarcticus	0.24	885	CHX	(Whiteley *et al.*, 1996)
Idotea rescata	0.9	237	CHX	(Whiteley *et al.*, 1996)
Ligia oceanica	1.2 - 8.2	24 – 470	CHX	This study

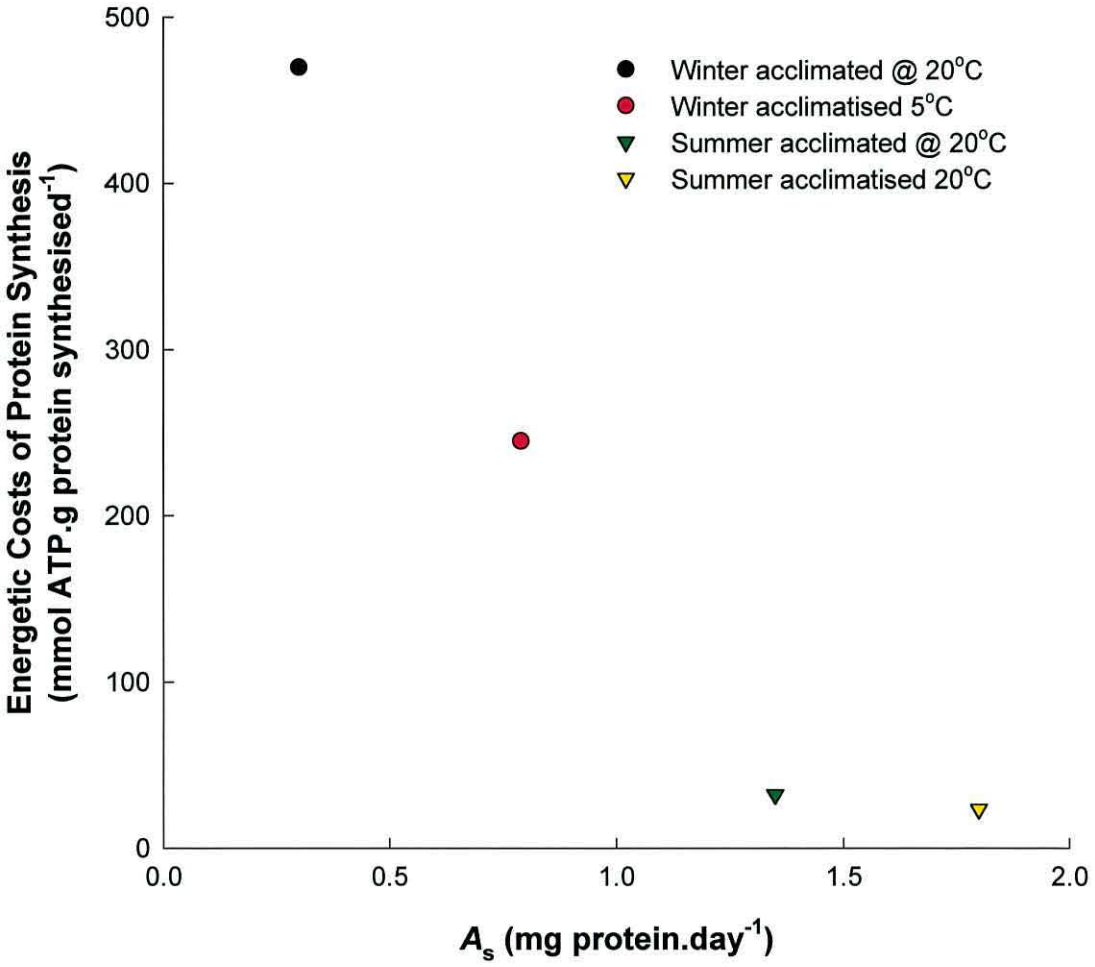
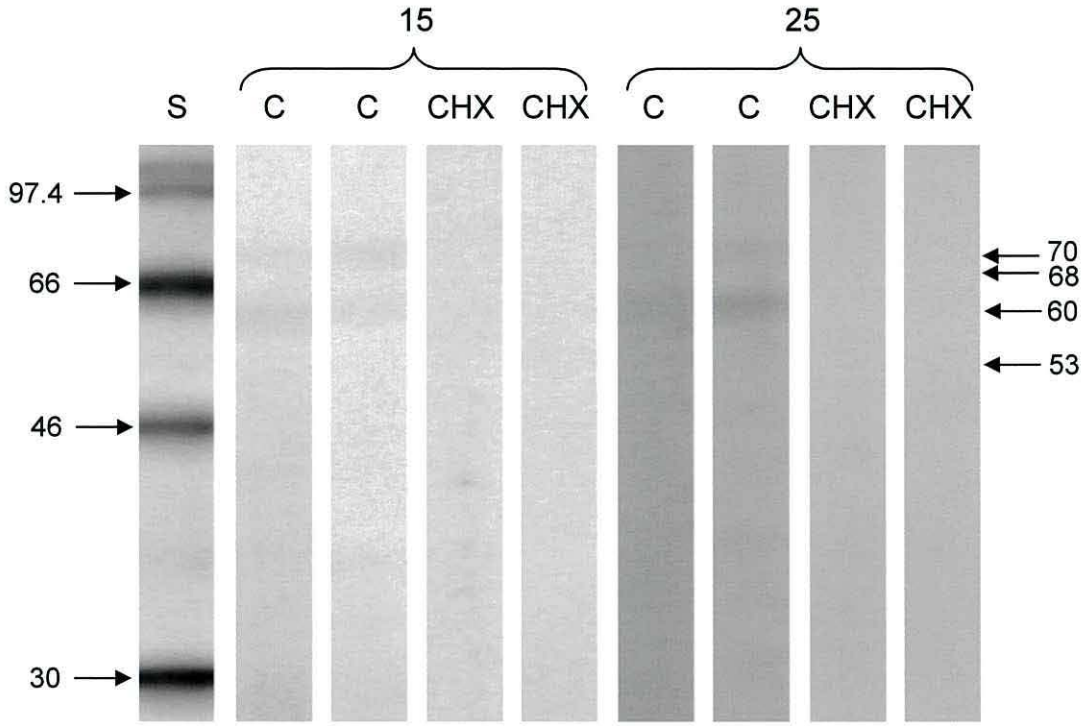


Figure 5.1. The relationship between whole-animal absolute rates of protein synthesis (A_s) and the metabolic costs of protein synthesis in acclimatised and acclimated *Ligia oceanica* collected in the summer and winter. Values are means.

Figure 5.2. **(a)** Autoradiograph showing the incorporation of [^{35}S]-methionine into newly synthesised proteins of 70, 68, 60 and 53 kDa in winter *Ligia oceanica* acclimated at 15°C, injected with either saline (n = 2, control) or cycloheximide (n = 2, CHX) and incubated at either 15 or 25°C for 3 hours. Molecular weight markers are shown in the far left-hand lane (S). Molecular weight is expressed as kDa and temperature as degrees Celsius. **(b)** Optical density of newly synthesised proteins of 70, 68, 60 and 53 kDa in *L. oceanica* injected with saline (C) or cycloheximide (CHX) and incubated at either 15 or 25°C for 3 hours.


15
25
S
C
C
CHX
CHX
C
C
CHX
CHX
97.4
66
46
30
70
68
60
53

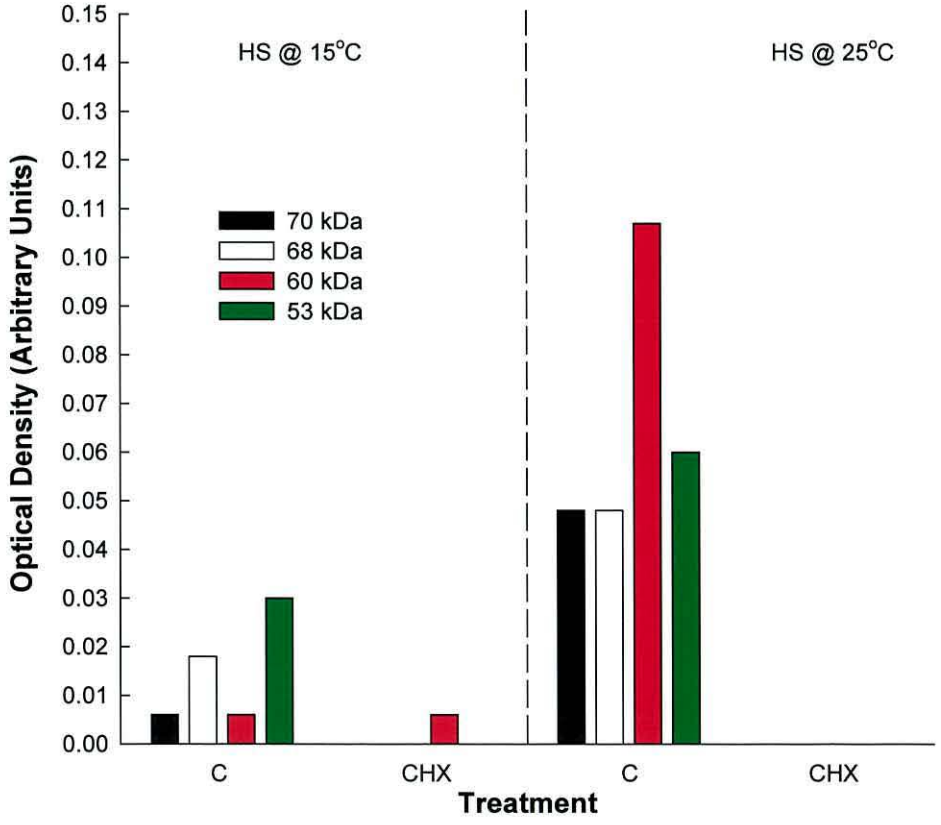
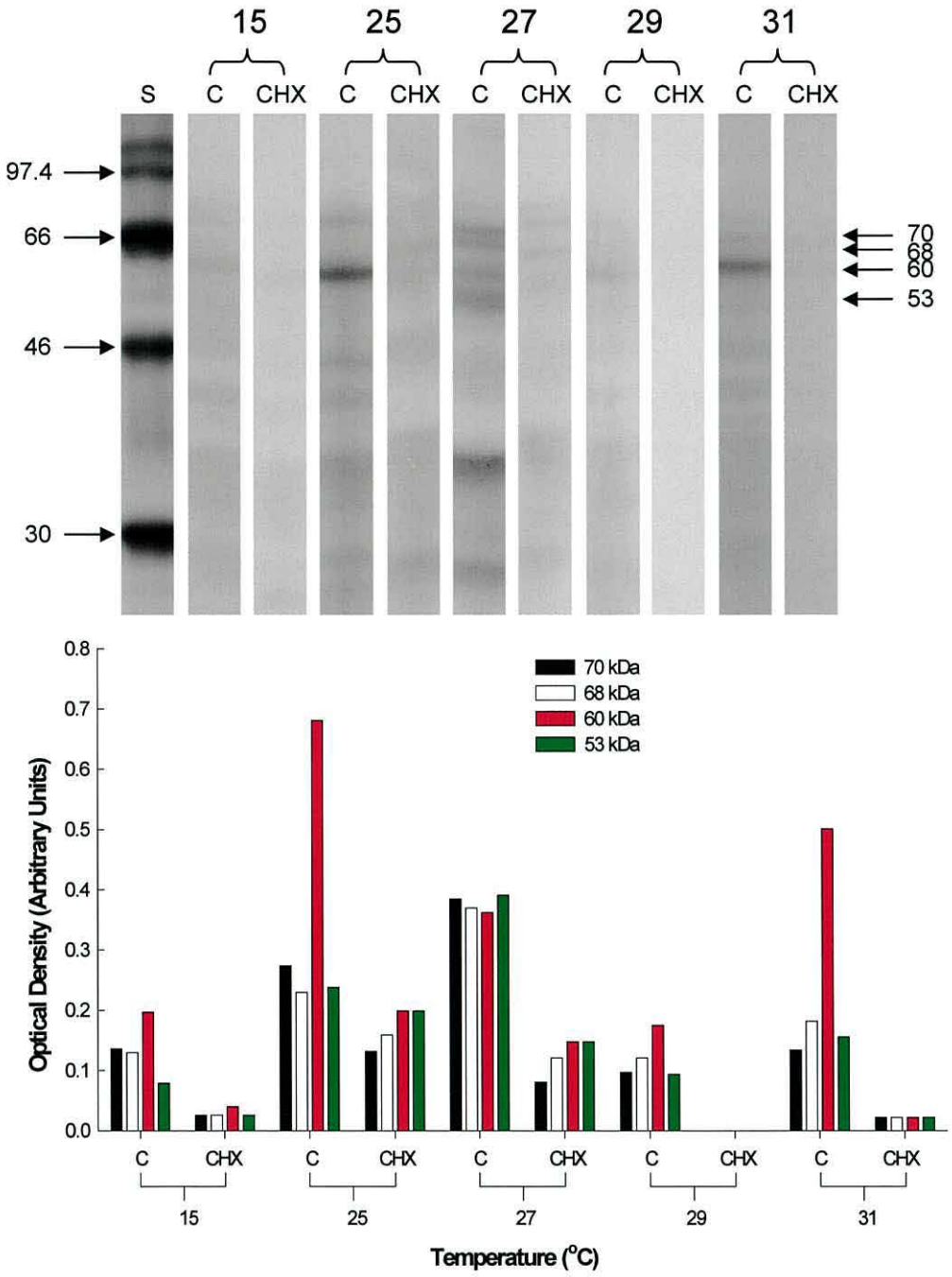

HS @ 15°C
HS @ 25°C
70 kDa
68 kDa
60 kDa
53 kDa
Optical Density (Arbitrary Units)
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
C
CHX
C
CHX
Treatment

Figure 5.3. **(a)** Autoradiograph showing the incorporation of [^{35}S]-methionine into newly synthesised proteins of 70, 68, 60 and 53 kDa in *Ligia oceanica* taken straight from the shore in summer, held at 15°C overnight, injected with saline (C) or cycloheximide (CHX) and incubated at either 15, 25, 27, 29 or 31°C for 3 hours. n = one for each separate treatment. Molecular weight markers are shown in the far left-hand lane (S). Molecular weight is expressed as kDa and temperature as degrees Celsius. **(b)** Optical density of newly synthesised proteins of 70, 68, 60 and 53 kDa in *L. oceanica* injected with saline (C) or cycloheximide (CHX) and incubated at 15, 25, 27, 29 or 31°C for 3 hours.


15
25
27
29
31
S
C
CHX
97.4
66
46
30
70
68
60
53
70 kDa
68 kDa
60 kDa
53 kDa
Optical Density (Arbitrary Units)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Temperature (°C)

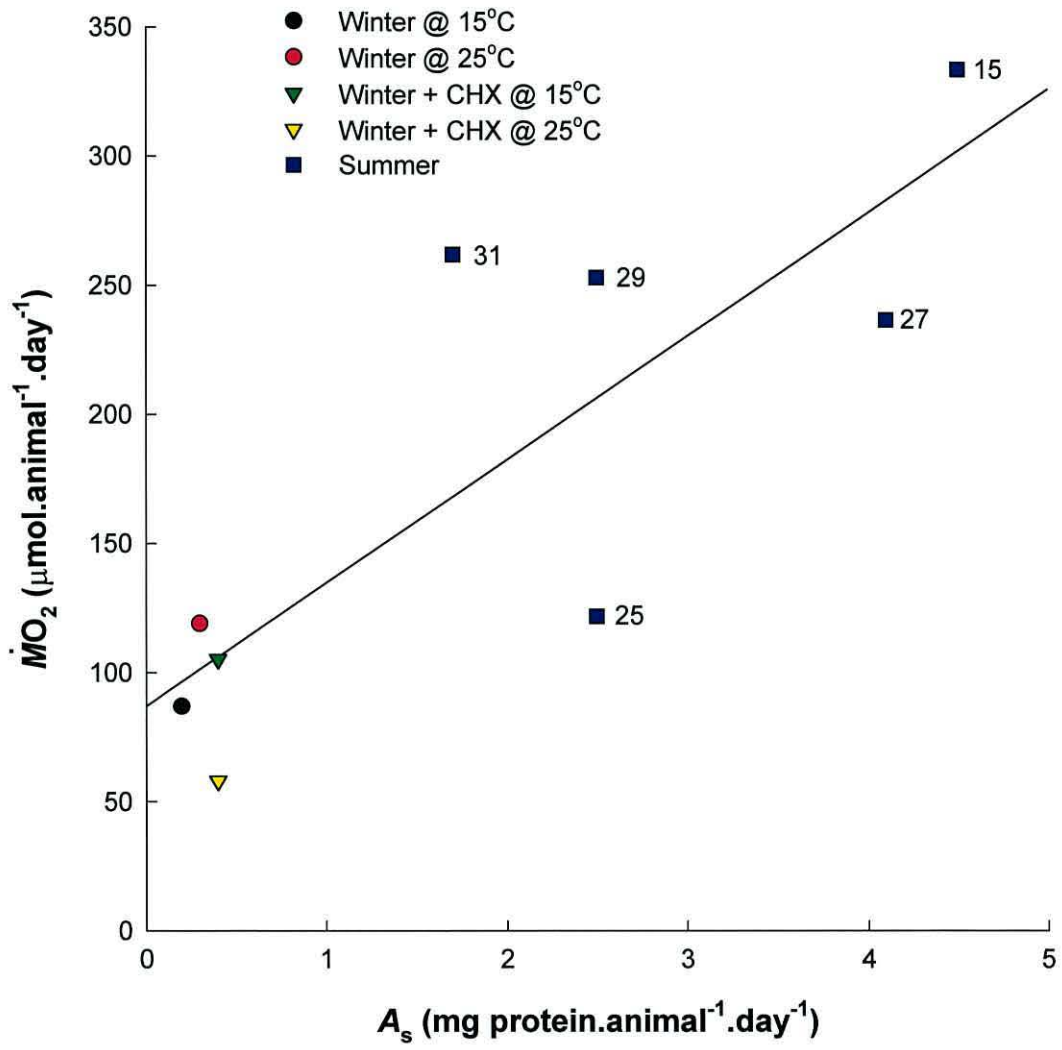


Figure 5.4. The relationship between absolute rates of protein synthesis (A_s) and whole-animal rates of oxygen uptake ($\dot{M}O_2$) in summer acclimatised and winter acclimated *Ligia oceanica* incubated at various temperatures and injected with either saline, or the protein synthesis inhibitor, cycloheximide (CHX). The relationship between A_s and $\dot{M}O_2$ is described by the following linear regression equation: $y = 87.0 + 47.8\ x$ ($r^2 = 0.7$, $F = 14.8$, $P = 0.006$).

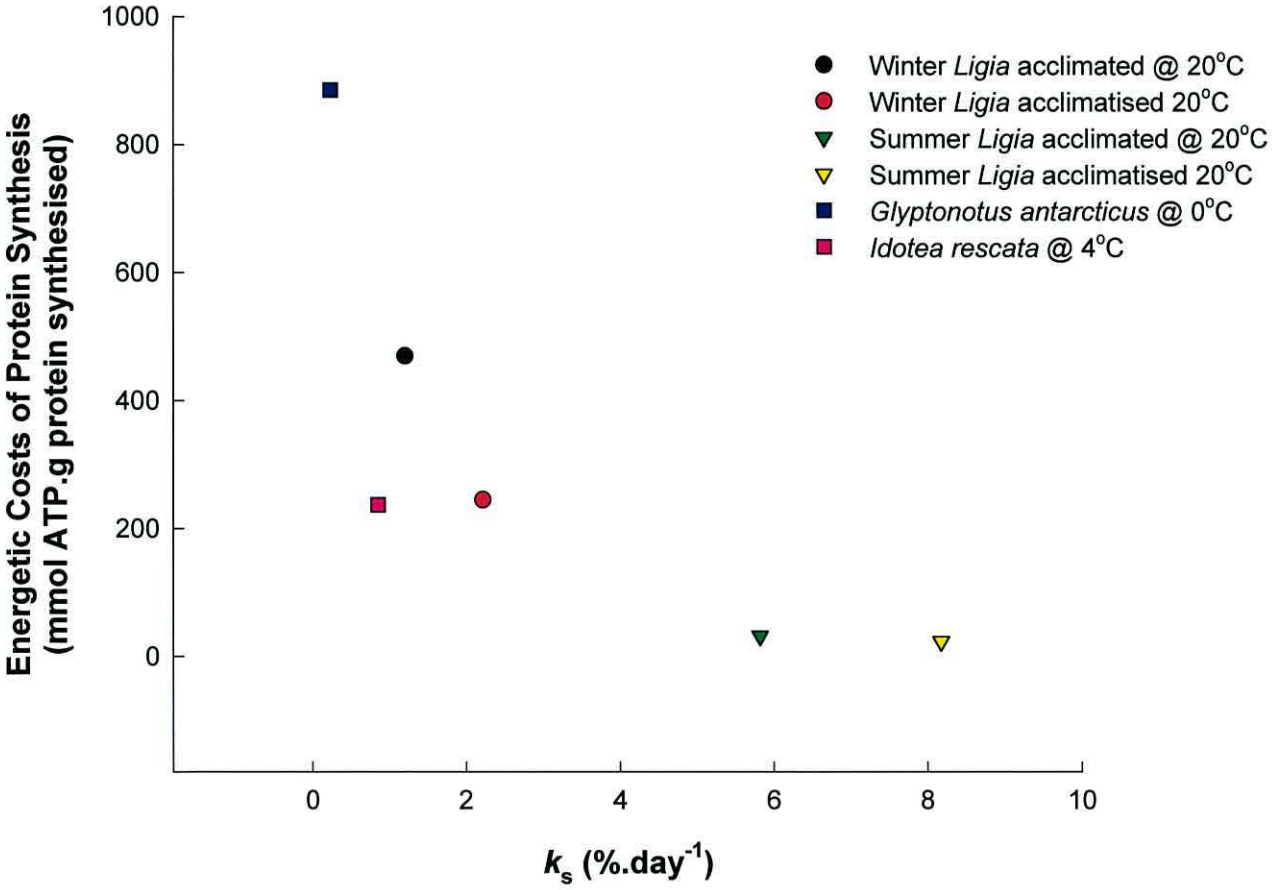


Figure 5.5. The relationship between whole animal rates of protein synthesis (k_s) and the metabolic costs of protein synthesis in two temperate isopod species (*Ligia oceanica* (this study) and *Idotea rescata* (Whiteley *et al.*, 1996)) and the giant stenothermal Antarctic isopod, *Glyptonotus antarcticus* (Whiteley *et al.*, 1996).

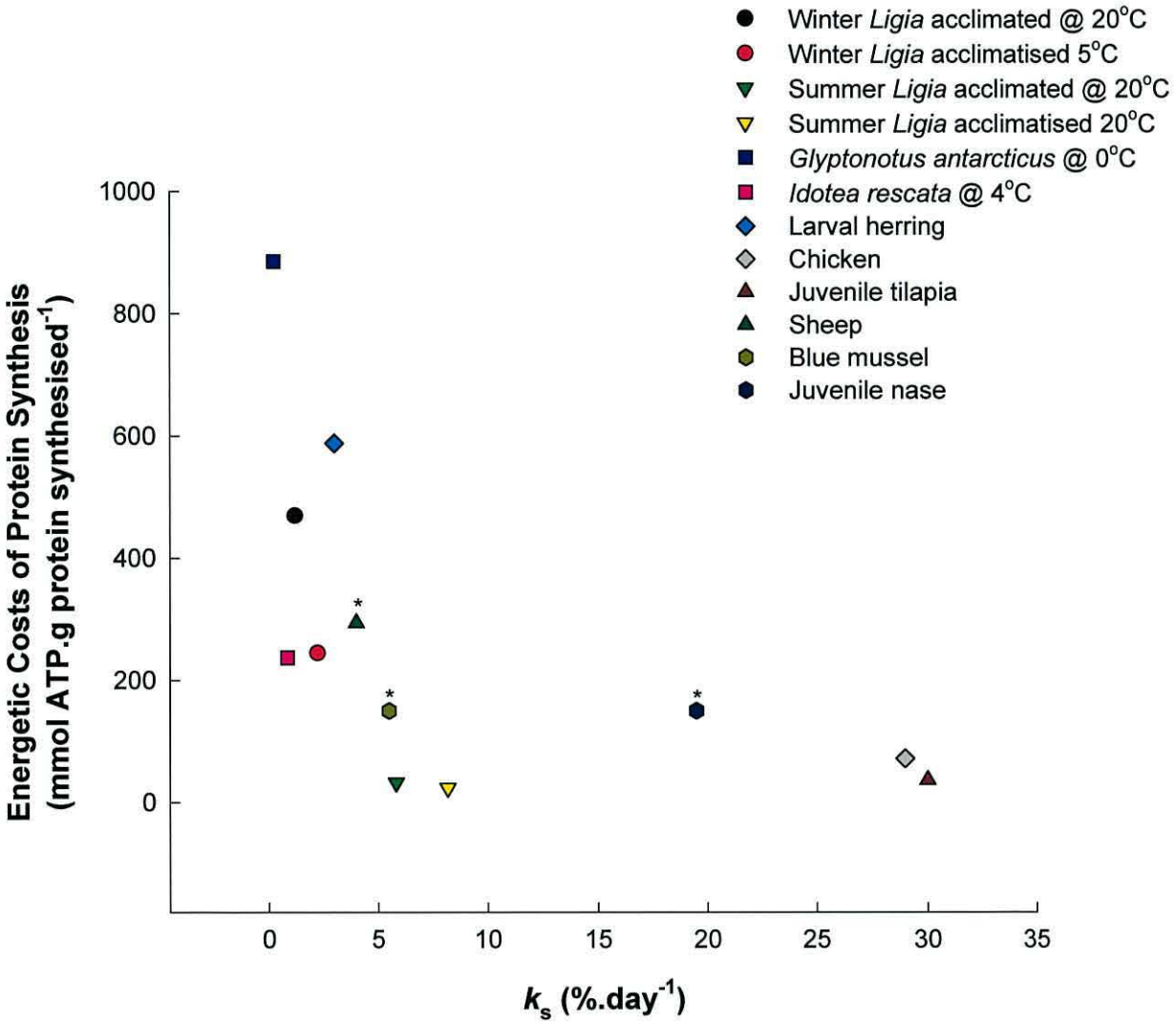


Figure 5.6. Whole-animal fractional rates of protein synthesis (k_s) and their associated metabolic costs in a variety of taxa detailed in Table 5.6. The data points marked with an asterisk have been estimated correlatively.

Chapter Six

General Discussion

6.0 *General Discussion*

Collectively, these studies have revealed that the temperate intertidal isopod, *Ligia oceanica*, experiences marked seasonal variations in whole-animal rates of metabolism, general rates of protein synthesis, and more specifically, patterns of hsp expression. Seasonal differences were observed in animals removed from the shore, *i.e.* acclimatised, and these differences persisted despite a period of temperature acclimation. More specifically, data from Chapters 2 and 3 showed that rates of metabolism increased in both summer and winter *Ligia* with increasing acclimation temperature. In general, $\dot{M}O_2$ levels were higher in *Ligia oceanica* collected in the winter than in the summer, at common acclimation temperatures, showing a degree of physiological adaptation by *Ligia* to the relatively cold temperatures experienced by these animals during the winter months. This is especially important in organisms which, like *Ligia*, remain active during the winter. Fractional rates of protein synthesis changed little with increasing acclimation temperature in both summer and winter *Ligia*, suggesting a degree of temperature independence of k_s. The largest changes in k_s occurred at the highest acclimation temperatures for both winter and summer animals, showing that k_s was more sensitive to temperature at the higher acclimation temperatures. Like $\dot{M}O_2$, k_s was generally higher in winter animals than in summer animals, because winter animals had higher capacities for protein synthesis (RNA:protein ratios), showing compensation for the relatively low RNA activities. A similar response to low temperature has been found in a

range of fish species (Foster *et al.*, 1992; Mathers *et al.*, 1993; McCarthy and Houlihan, 1997; McCarthy *et al.*, 1999). Interestingly, the capacity for protein synthesis was unaffected by temperature acclimation in both the summer and winter. The correlation between $\dot{M}O_2$ and k_s was poor in both summer and winter acclimated *Ligia*, as there was little change in k_s with increasing temperature. *Ligia* collected in the winter were unable to survive at 25°C for longer than one week, suggesting this temperature was close to the upper thermal limits for winter animals.

In Chapter 3, the metabolic responses of winter and summer *Ligia* to acute temperature change were studied in both acclimatised and acclimated animals. Similar to the situation in *Ligia* acclimated in the winter, winter acclimatised animals generally had higher $\dot{M}O_2$ values than summer animals in response to acute temperature change. This response follows the classic relationship between temperature and metabolic rate observed in a number of ectothermic animals, from invertebrates to vertebrates (Prosser, 1973; Cossins and Bowler, 1987; Randall *et al.*, 1997). In each case, the general increase in metabolic rate in winter or cold-acclimated animals, over their summer or warm-acclimated counterparts demonstrates partial compensation for the effects of low temperature. The degree to which animals can compensate for the effects of temperature however, vary, as described by Precht *et al.* (1955) and Prosser (1958). In this case, *Ligia* showed type II compensation *i.e.* translation. Again, acclimatised *Ligia* in the summer showed a different response to the winter animals when exposed to the higher temperature of 25°C. A dramatic increase in $\dot{M}O_2$ was evident in summer

Ligia at 25°C, where the $\dot{M}O_2$ for winter *Ligia* began to fall, suggesting that this temperature was approaching the upper limits of thermal tolerance for winter animals as described in the previous paragraph for winter acclimated *Ligia*. k_s in both winter and summer acclimatised *Ligia* was insensitive to acute temperature change, as were protein synthetic capacities and k_{RNA}. The higher RNA:protein ratios of winter acclimatised *Ligia* maintained k_s levels despite the lower k_{RNA} values, demonstrating seasonal changes to k_s values that remain unchanged during acute temperature change. Measurements of CTMax demonstrated an increase in the thermal tolerance limits of summer *Ligia*, which is a common feature of ectotherms from higher temperature environments (Edney, 1964a; Hallberg *et al.*, 1985; Cossins and Bowler, 1987; Layne *et al.*, 1987; Lagerspetz and Bowler, 1993). Differences in thermotolerances between the seasons meant that winter *Ligia* were unable to survive acclimation at 25°C or heat-shock at 31°C.

In Chapter 4, seasonal differences in the heat-shock response were investigated as changes in hsp induction temperature and hsp abundance, which have been shown to be correlated with changes in thermotolerance (Li and Werb, 1982; Parsell *et al.*, 1993; Dahlgaard *et al.*, 1998; Feder and Hofmann, 1999). Comparisons between acclimatised and acclimated animals revealed that, regardless of season, acclimatised *Ligia* had both higher endogenous levels of hsps and a more rapid and stronger heat-shock protein response than acclimated *Ligia*. The stable thermal regime of the acclimated animals may be responsible for the relatively attenuated heat-shock response compared to the acclimatised animals, where large diel temperature

fluctuations are apparent, especially during the summer months. The highest endogenous levels of hsps and the strongest heat-shock responses were found in summer acclimatised *Ligia*, where animals experienced two peaks in hsp expression: one at 25°C and another at 31°C. This second peak in hsp induction may be to reinforce the action of the high constitutive hsp expression characteristic of summer animals, where *de novo* hsp synthesis occurs towards the upper limits of thermal tolerance for *Ligia*. *Ligia* acclimated at 5°C displayed the strongest hsp response of any of the acclimated animals. Again, this observation suggests an over-riding seasonal component which cannot be altered by temperature acclimation. Constitutive hsp expression increased linearly with increasing acclimation temperature in winter animals, suggesting an increased requirement and subsequent energetic cost for the protective actions of hsps with higher acclimation temperatures. In addition to the costs of hsp expression, there may be additional costs associated with maintaining the viability of the existing protein pool at higher environmental temperatures. In all cases, hsp60 was the predominant heat-shock protein, as found in the freshwater coelenterate, *Hydra attenuata* (Bosch *et al.*, 1988; Bosch *et al.*, 1991). In addition, changes in hsp70 were also observed.

In order to explain the seasonal differences in thermotolerance observed in *Ligia*, and to relate these changes to metabolic demand, the investigations in Chapter 5 were designed to estimate the energetic costs associated with protein synthesis, as proteins are the most energetically expensive molecules to synthesise (Brafield and Llewellyn, 1982), but are by no means the only drain on metabolic reserves (for example: repair, the biosynthesis of other

complex molecules such as steroids and enzymes, mechanical work, cell division, ion transport, osmosis). To date, few studies have directly measured the metabolic costs of protein synthesis (Aoyagi *et al.*, 1988; Pannevis and Houlihan, 1992; Houlihan *et al.*, 1993b; Houlihan *et al.*, 1995; Smith and Houlihan, 1995), and none of these have examined whether the metabolic costs vary with season. In this respect, the results from Chapter 5 are extremely interesting, and provide a possible explanation for the attenuated heat-shock response and concomitant reduction in thermotolerance observed in winter *Ligia*, especially at higher temperatures. The main finding from Chapter 5 was that energetic costs of protein synthesis were considerably higher in winter *Ligia* compared to summer animals. Interestingly, this remains a seasonal effect, as the acclimation of winter animals at 20°C caused no change, with overall metabolic costs similar to those estimated in animals straight from the shore. For example, the proportions of oxygen uptake attributable to whole-animal protein synthesis in summer *Ligia*, whether acclimated at 20°C, or taken directly from the shore, were very similar, at 19.2% and 17.3%, respectively. The same was true of *Ligia* collected in the winter, and either acclimated at 20°C, or used directly from the shore, where whole-animal protein synthesis accounted for 72.1% and 70% of metabolic rate, respectively.

The data is even more interesting when comparisons are made with the data available in the literature for an Antarctic and temperate isopod species. Comparisons of the proportion of oxygen uptake attributable to protein synthesis between winter *Ligia* (acclimatised or acclimated at 20°C), and the

Antarctic isopod, *Glyptonotus antarcticus*, show a remarkable similarity, at around 70%. In contrast, summer animals show a closer similarity to the temperate isopod, *Idotea rescata*, with values at around 20%. These marked changes in metabolic costs can be explained by the assumption that protein synthesis costs consist of two components, one of which is a fixed cost due to RNA synthesis (Pannevis and Houlihan, 1992). Indeed, the data from the present study shows a strong relationship between costs in terms of mmol ATP.g protein^{-1} synthesised, and absolute rates of protein synthesis (Figure 5.1).

The implications of these relatively high winter costs are speculative, but do suggest that ATP demand is high at a time when ATP production may be limited. To maintain low maintenance costs, which does appear to be a characteristic of low temperature ectotherms, a reduction in protein turnover would be necessary, increasing protein stability and reducing the need for relatively energetically expensive protein synthesis (Hawkins, 1991; Whiteley *et al.*, 1996). Indeed, there is increasing evidence to suggest that low protein turnover rates are a characteristic of polar ectotherms (Clarke, 1998). Measurements of k_{RNA} and RNA:protein ratios from Chapters 2 and 3, show that some compensation for the temperature-related drop in k_{RNA} values takes place in winter animals, i.e. there is some ability to increase k_s levels, despite low temperatures. Therefore, k_s values are higher in winter animals than would otherwise be the case. Despite this seasonal ability to increase the protein synthetic capacity, protein synthesis rates remain low and are

therefore more costly in the winter, with small changes in k_s causing relatively large changes in ATP costs (see Figure 5.1).

In contrast to the winter animals, *Ligia* in the summer, with lower energetic costs of synthesis, showed little change in k_s values with an increase in acclimation temperature (Chapter 2). This observation suggests that energy allocation is being diverted to other energy-demanding processes, such as growth and reproduction. Such energetically costly processes may be severely limited in the winter with the associated high costs of protein synthesis, and therefore, ATP demand. This raises interesting questions about the allocation of energy to growth and reproduction in Antarctic ectotherms, where reduced protein turnover may play an important part in keeping maintenance costs relatively low and thereby ensuring longer survival through slower net loss of energy (Hawkins, 1991; Clarke, 1998).

Seasonal changes in the energetic costs of protein synthesis can also be used to explain differences in the hsp response between winter and summer *Ligia*. For example, estimates of the proportion of $\dot{M}O_2$ attributable to hsp synthesis, given in Chapter 5, show that the unit cost of protein synthesis is higher in the winter versus the summer. Therefore, hsps are relatively costly to synthesise, and even though there was a weaker heat-shock response in winter animals, inhibition by CHX accounted for 46.4% of control $\dot{M}O_2$ levels, compared to 28.7% in the summer. Therefore the ability to synthesise hsps in the winter is limited by the higher metabolic costs. In the natural environment, the necessity to respond to elevated temperatures is much reduced, and

therefore *Ligia* survive by not experiencing temperatures higher than 20°C. In the summer, the hsp response is more acute, with a double-peak in hsp60 synthesis, ensuring survival at 31°C. In addition, constitutive levels of hsps are higher in summer compared to winter animals, probably as a direct result of the differences in metabolic demand of hsp synthesis.

In summary, the higher metabolic costs of protein synthesis in winter *Ligia* can be used to explain their attenuated hsp response, their reduction in thermotolerances, and hence the inability of winter *Ligia* to survive elevated temperatures. Despite these marked seasonal changes in metabolic costs, there is little correlation between whole-animal rates of oxygen uptake and protein synthesis in *Ligia* during temperature change, with $\dot{M}O_2$ levels more sensitive to temperature than k_s levels. These latter observations show the importance of using protein synthesis inhibitors to directly measure the metabolic costs of protein synthesis, rather than relying on correlations, as other energy-demanding processes such as protein degradation may cause changes in $\dot{M}O_2$. Despite the unknown influence of protein degradation rates on $\dot{M}O_2$ levels, these studies are unique in showing that metabolic costs of protein synthesis, which are important in somatic growth, protection from heat-shock, and in the replacement of heat denatured proteins, are highly seasonal. Interestingly, winter *Ligia oceanica* are compromised by high metabolic costs of protein synthesis at a time when food resources, and therefore energy stores are likely to be limited, reducing their ability to synthesise heat-shock proteins, and ultimately, influencing their survival and distribution limits on the shore.

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Conference Abstracts

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The Society for Experimental Biology Annual Meeting
The University of Exeter 27th – 31st March 2000

Poster Presentation

A10.17 – Seasonal changes in the relationship between protein synthesis rates and metabolism in an intertidal crustacean.

L.S. Faulkner and N.M. Whiteley, School of Biological Sciences, University of Wales, Bangor, UK

Whole-animal rates of oxygen uptake and protein synthesis were measured in the semi-terrestrial, supralittoral isopod, *Ligia oceanica* (L.), collected from the shore in January and July. Animals were acclimated to five different temperatures: 5, 10, 15, 20 and 25°C for 8 weeks. Rates of oxygen uptake were determined by constant pressure respirometry, and rates of protein synthesis by the flooding dose technique. At 5 and 10°C, rates of protein synthesis were similar in both winter and summer animals (~ 0.28%.day^{-1}), but at 15 and 20°C, rates of protein synthesis in the winter increased significantly, to 0.67%.day^{-1} and 1.16%.day^{-1}, respectively, while summer values remained unchanged (~ 0.26%.day^{-1}). Subsequently, Q_{10} for protein synthesis rates in winter animals was 3.29 compared to a Q_{10} of 1.22 in summer animals. Rates of oxygen uptake were higher in winter *versus* summer animals regardless of temperature, and increased exponentially with temperature to give Q_{10} values of 2.06 in winter, and 2.37 in summer. These data suggest modifications in the relationship between oxygen uptake and protein synthesis rates in *Ligia oceanica*, with a decrease in temperature sensitivity in summer animals during acclimation.

Comparative Biochemistry and Physiology 126B – Milestones and Goals
The University of Cambridge, 30th July – 3rd August 2000

Oral Presentation

S104 – Temperature effects on protein turnover in crustacean muscle.

N.M. Whiteley and L. S. Faulkner, School of Biological Sciences, University of Wales, Bangor, UK

Temperature has been shown to have a direct effect on fractional rates of protein synthesis in the whole body and in the muscle tissue of a number of crustacean species. For instance, in the eurythermal isopod, *Idotea rescata*, protein synthesis rates in the ventral longitudinal muscle increases with a Q_{10} of 1.99 over a 10°C rise in acclimation temperature, accompanied by an increase in actin mRNA levels and an increase in RNA activities at constant RNA:protein ratios. Moreover, temperature related increases in protein synthesis rates in *I. rescata* are associated with an equivalent increase in metabolic rate, suggesting that the two variables are closely related.

More recently, it has been shown that temperature related changes in rates of protein synthesis and metabolism are modified by seasonal acclimatisation. In the winter, whole body rates of protein synthesis in the intertidal isopod, *Ligia oceanica*, acclimated to a range of temperatures, increased with a Q_{10} of 3.7, associated with an equivalent increase in oxygen uptake rates. In contrast, there was no change in protein synthesis rates in summer isopods with increase in acclimation temperature, even though oxygen uptake rates continued to rise. These data suggest that either the metabolic cost of protein synthesis changes with temperature in summer isopods, or that the contribution of other processes to overall energy demand, such as protein degradation, increases in the summer. As skeletal muscle is a major site of protein degradation, studies are currently underway to assess seasonal

effects of temperature on protein turnover in the ventral longitudinal muscles of *L. oceanica*. Assessment of protein turnover rates will allow us to establish the ability of crustacean muscle to adapt to seasonal changes in temperature, and the effects of these relationships on protein accretion and seasonal growth patterns.

The Society for Experimental Biology Annual Meeting
The University of Wales, Swansea 8th – 12th April 2002

Poster Presentation

A2/C5.25 – Do seasonal changes in protein metabolism influence thermal tolerances in an intertidal crustacean?

L.S. Faulkner, N.M. Whiteley, Biological Sciences, University of Wales, Bangor, UK

Temperature tolerances in the intertidal semi-terrestrial isopod, *Ligia oceanica*, vary seasonally with CTMax falling from 37.4°C in the summer to 34.9°C in the winter. Below these values, animals survive temperatures of 25-31°C by synthesising heat-shock proteins (hsps). Examination of hsp synthesis patterns in seasonally acclimatised *Ligia oceanica* showed a different response in summer versus winter. In the summer, animals synthesised a protein with a molecular weight of 60 kDa (hsp60) at two induction temperatures of 27 and 31°C. Animals collected in the winter, however, synthesised hsp60 at the lower induction temperature of 25°C, and continued to express hsp60 at 27 and 29°C. Unlike summer animals, winter animals did not survive heat-shock at 31°C, suggesting that survival of *Ligia oceanica* at elevated temperatures is linked to the ability to synthesise hsps. Metabolic costs of protein synthesis also vary between summer and winter acclimatised *Ligia oceanica*, which may explain differences in seasonal hsp synthesis patterns. Isopods collected from the shore in the winter were

characterised by high costs of whole-animal protein synthesis (40.9 µmol O_2.mg protein^{-1}), which accounted for 70% of mean oxygen uptake rates. In contrast, metabolic costs were 4.6-times lower in animals collected in the summer (3.9 µmol O_2.mg protein^{-1}), accounting for 17% of mean oxygen uptake. Consequently, hsp synthesis, and therefore, survival of intertidal isopods at elevated temperatures in the winter, may be restricted by the high costs of protein synthesis at a time when energy stores and food resources are limited.

The American Physiological Society Intersociety Meeting: Evolution, Integration and Application
San Diego, August 2002

Poster Presentation

Influence of thermal stress on rates of protein synthesis and metabolism in an intertidal crustacean

N.M. Whiteley and L.S. Faulkner, School of Biological Sciences, University of Wales, Bangor, UK

The semi-terrestrial isopod, *Ligia oceanica*, inhabits humid micro-habitats at the high water mark of Spring tides, where it experiences elevated temperatures in the summer months due to the radiant heating effects of the sun. SDS-PAGE and autoradiography of acclimatised animals demonstrated that *L. oceanica* survives periodic exposure to high temperatures (25, 27, 29 and 31°C for 3 hours) by synthesising heat shock proteins (hsps) with molecular weights of 70, 68, 60 and 53 kDa. In contrast, general rates of protein synthesis decreased significantly at each heat-shock temperature. For example, absolute rates of protein synthesis fell from 2.75 ± 0.37 (10) to 1.24 ± 0.23 (10) mg protein.animal^{-1}.day^{-1} after heat shock at 25°C for 3 hours. Despite the increase in temperature, whole-animal rates of oxygen uptake

also declined during heat shock, falling from 206 ± 26 (10) to 58 ± 6 (10) $\mu mol.animal^{-1}.day^{-1}$ between 15 and 25°C.

The relationship between whole-animal rates of protein synthesis and metabolism was further investigated by injecting heat-shocked animals with the protein synthesis inhibitor, cycloheximide. Such treatment inhibited hsp synthesis but had little effect on general rates of protein synthesis, while there was a slight decline in oxygen uptake rates. From this data it appears that the preferential synthesis of heat-shock proteins in *L. oceanica*, at least within 3 hours of heat-shock, are less metabolically costly than general rates of protein synthesis.