Adaptive responses to temperature and photoperiod in neuromuscular transmissionin carcinus maenas (l)

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ADAPTIVE RESPONSES TO TEMPERATURE AND PHOTOPERIOD IN NEUROMUSCULAR TRANSMISSION IN CARCINUS MAENAS (L).

By

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DECLARATION

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

Suhaila Qari.

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ABSTRACT

Neuromuscular synaptic transmission in the ectothermal crab *Carcinus maenas* was used to assess the extent to which photoperiod affected the attainment of temperature acclimation. A separate series of experiments were carried out to determine thermotolerance to high temperature (CTMax) and its dependence on prior heat shock and heat shock proteins.

Dactylopodite closer muscle resting potentials and the characteristics of evoked excitatory junction potentials in response to stimulation of the excitatory tonic axon were recorded with intracellular microelectrodes and the extent of acclimation to temperature under different photoperiodic regimes was measured. Animals were acclimated for at least two week to either 8°C or 25°C with short day (8hr: 16hr, L/D) or long day (16hr: 8hr, L/D) photoperiods.

Photoperiod differences had a marked effect on acclimation to temperature as measured by muscle resting potential and excitatory junction potential amplitude and facilitation. In short day photoperiods there was an acclimatory shift of muscle resting potential in crabs acclimated to 25°C compared with those acclimated to 8°C. Such an acclimatory shift of muscle resting potential was not seen in animals from long day photoperiods. By contrast facilitation showed acclimatory changes to temperature in long day but not in short day photoperiods. Excitatory junction potential amplitudes were increased in cold acclimation; this increase was larger from long day acclimated groups compared with short day groups. Furthermore photoperiod had a large effect on closer muscle tension in animals acclimated at low temperatures but little effect on animals acclimated at high temperatures.

Heat shock reduced the amplitude of EJPs recorded in the closer muscle but enabled the EJP to be maintained for much higher temperatures. Heat shock also resulted in an increase in thermotolerance as measured by an elevation of CTMax from 33.04°C to 34.17°C.

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Chapter One

General Introduction

The external environment has many physical variables, the abiotic environment and the biotic environment such as animals and plants. Some of the major abiotic variables are temperature, water availability and salinity, air humidity, light intensity, photoperiod, ambient pressure, gaseous composition of air. Temperature is an important environmental parameter which limits the distribution of living organisms and is an important determinant of their activity, particularly significant for animals whose body temperature fluctuates with changes in environmental temperature (Prosser and Heath, 1991). Environmental temperature variation will have a profound effect on rate processes of their biochemistry and physiology, and will result in changes at different levels of organization from the molecular to the behavior of the whole organism (Hochachka and Somero, 1984; Somero 1997). Life activities occur within a range of ~0 to +40°C; most animals live within much narrower limits. But the range of temperatures on earth is greater than the range within which life is possible, air temperatures range from -70°C in polar regions to +85°C in hot deserts. Whereas surface waters range from -1.82°C in Arctic and Antarctic waters to +30°C in tropical landlocked bodies of water (Prosser and Heath, 1991). Although regions of relative constancy are the deep oceans and polar seas where temperature is 2-4°C at all times (Prosser and Heath, 1991), animals living at depths greater than fifty meters may experience almost constant temperatures over an annual period (Nicol, 1964). Polar environments both terrestrial and aquatic are less varied and species diversity is less than in the tropic or temperate zones (Prosser and Heath, 1991).

Many terms have been used to describe patterns of body temperature, one of which is cold blooded and warm blooded. But they are inaccurate terms as they provide no indication of the source or method of maintenance of body temperature. Desert snails for example, may have a body temperature of 40°C but is cold blooded whereas a hibernating

mammal with a body temperature of 5°C is warm blooded (Cossins and Bowler, 1987). Other definitions used are poikilotherm and homeotherm. Poikilothermic animals are thermal conformers, in which the body temperature (T_b) equals ambient temperature (T_a), and T_b rises or falls with *Ta.* In contrast, homeotherms are regulators and maintain relative internal thermal constancy in a changing environment. Such animals (birds and mammals) maintain their body temperature relatively constant by a variety of behavioural, circulatory, and metabolic activities over a range of ambient temperature. Both terms refer to variability or constancy of internal temperature, but they do not indicate whether there are regulatory mechanisms controlling body temperature. Crabs, frogs and snails for example, are poikilotherms whereas birds and mammals are homeotherms. But a fish that live deep in the ocean at a constant water temperature is a homeotherm although not in the same physiological sense that a mammal is a homeotherm (Prosser and Heath, 1991). Other terms are ectotherm and endotherm, which may be the most useful in describing the thermal status of animals. Animals whose thermal balance is predominated by external sources of heat are called ectotherms (i.e. solar radiation or a warm substrate); they gain heat from the environment for thermoregulation, whereas endotherms are animals whose thermal balance is predominated by their cellular metabolism. These animals produce heat within their own tissues to thermoregulate. Accordingly the terms ectothermy and endothermy refer to use of environmental and metabolic heat, respectively as sources of body temperature (Cowles, 1962).

Fluctuations in environmental temperature raise particular problems for ectothermic organisms, which generally lack mechanisms for physiological regulation of their body temperature, which is therefore determined by ambient temperature and the physical characteristics of the environment (Precht, *et al.*, 1973). Animals which survive over a wide range of seasonal or diurnal changes in temperature have developed several adaptations that enable them to survive these thermal conditions and are said to be eurythermal. In contrast animals which survive only over a more restrictive, narrow range of external temperature are said to be stenothermal. For example, the body temperature of an icefish, which lives in Antarctic seawaters is -1.9°C and might not vary over a year by more than 1°C (Withers, 1992). The eurytherm crayfish, on the other hand, shows normal

locomotion over the temperature range from 0°C to 26°C (Fischer and Florey, 1981). These abilities of some ectothermic animals to maintain normal behavior over such ranges of temperatures are believed to involve both metabolic and behavioral compensations and adaptations (Prosser and Nelson, 1981; Cossins and Bowler, 1987).

Behaviour.

Temperature is the most studied ecological factor and has profound effects on all biological processes of ectothermal animals. The direct effects of temperature on the various functions of organisms result compensatory effects, which are adaptive to maintain the relative constancy of the functions in spite of changes in the environmental temperature (Lagersptez, 1974). Changes in environmental temperature may result in two categories of response of organisms: behavioural and metabolic/ physiological (Prosser and Heath, 1991, Cossins and Bowler, 1987). In mobile animals, change in environmental temperature may result in behavioural migration or selection of more favourable thermal habitats; many poikilotherms show active selection of a preferred temperature when placed in a thermal gradient and so regulate their body temperature (Crawshaw and Hammel, 1974; Prosser, 1973). For example, bluegill sunfish select a T_b of about 26°C in a thermal gradient of water temperature from 6-34°C. This animal regulates behaviourally by selecting a preferred temperature, possible because it offers optimal conditions for function (Prosser and Heath, 1991). In some species the preferred temperature can be modified by acclimation or vary seasonally (Cossins and Bowler, 1987). Norris, (1963) noticed that intertidal fish *Girella* when acclimated to 11°C selected 13.7°C, but when acclimated to 23°C selected 20.7°C. whereas bullheads fishes (*Ameiurus nebulosus*) and green sunfish (*Lepomis cyanellus*), when left them for a long time in a thermal gradient, will select a temperature called the final preferendum which is the temperature at which acclimation temperature and selected temperature are the same (Fry, 1947). In general, a higher temperature is selected after exposure to higher acclimation temperature, but at extremes of the acclimation range preferred temperature may not change (Javaid and Anderson, 1967). In sessile organisms such a behavioural response is not used and therefore may have developed various physiological responses for example, metabolic, and cardiovascular in

order to survive such changes in temperature (Precht, 1958). The internal temperature of many ectotherms is close to the ambient temperature; the problem therefore, is one of retaining a degree of functional integrity in a thermally fluctuating environment. Some ectotherms overcome this problem by living in an environment that provides near constant thermal conditions. Hawaiian ghost crabs, *Ocypode ceratophthalma* for example, live in burrows and in ocean at temperatures around 26-28°C, and their nocturnal habits ensure that they do not overheat in the sun (Florey and Hoyle, 1976). On the other hand, some ectothems live in environments which fluctuate in temperature; in the intertidal goby fish *Gillichthys seta* for example, whose body temperature may range from 8 to 40°C (Dietz and Somero, 1992). In this fish compensatory mechanisms must be present that permit the animal to function over a wide range of temperatures (Stephens, 1985). In some cases, mobile ectotherms may not exhibit the behavioral responses when environmental temperature changes are too rapid, behavioural mechanisms will be inadequate to enable the animal to select different temperature habitat and the animals does not have any physiological mechanism for adaptation, then the temperature may have lethal effects (Prosser and Heath, 1991).

Acclimatization and acclimation.

Ectotherms, which experience seasonally varying temperatures, have a number of strategies; one mechanism is a change in cellular physiology and/or biochemistry. This phenomenon in nature condition is called acclimatization, which takes days or weeks to achieve. Seasonal acclimatization occurs in response to changes in several factors such as temperature, photoperiod, oxygen tension, food and water availability. If compensatory change is induced in the laboratory under the controlled conditions is called acclimation. Thermal acclimatization for example is change in the upper critical temperature of a species in response to seasonal changes in temperature; the direct effect of temperature will be to alter the rate processes of cellular biochemistry and physiological function to compensate for variation. Whereas if produced experimentally by a change in temperature alone, are called thermal acclimation (Cossins and Bowler, 1987). So, temperature acclimation and acclimatization are compensatory, reversible changes to physiology,

cytology and biochemistry of ectotherms that are adaptive and considered to contribute to increased fitness to new thermal conditions; ectotherms particularly display an impressive variety of such compensatory phenomena (Cossins and Bowler, 1987). Figure 1.1 shows a schematic diagram illustrating Precht's (1958) scheme, which consists of patterns of compensation, based upon the comparison of the rate of any process after direct transfer to a new temperate after acclimation. Solid line shows the normal rate temperature curve, this curve shows the increase in rate upon transfer from T_1 to T_2 (higher temperature). After a period of days or weeks, if at T_2 the rate is unchanged, then no adaptation has occurred. However if the rate is altered, then some adjustment has occurred. The broken lines represent the new rate versus temperature, if the rate at T_1 and T_2 are the same after the acclimation then complete acclimation or type (2) or has occurred, or partial acclimation or type (3) has occurred if the rate below the normal rate and above the complete rate (between points A and B). When the rate below point B then supraoptimal acclimation or type (1) has occurred, or elevated above point A then inverse acclimation or type (5) has occurred. In natural environment there are changes in more than one parameter, and so tissue responses may occur as directed by central nervous and or humoral factors.

Adaptation.

Ectotherms and some endotherm animals experience temperature fluctuations either on a daily or on a seasonal basis. Thermal acclimation may involve two forms of adaptive change in either capacity or resistance adaptation (Precht, 1958). Capacity adaptation is alterations that permit relatively normal function over a range of environmental conditions or over normal range (short term or daily) of temperatures, which are partly metabolic and partly behavioural (Prosser, 1973; Pretch, 1958; Prosser and Nelson, 1981). But the adaptive change in the temperature resistance or tolerance of some function, in which case it is called a thermal resistance adaptation, that permit continued function at environmental extremes (long term or seasonally) of temperatures (Lagerspetz, 1974; Prosser and Nelson, 1981). Adaptive change in a function is often based on an adaptive change in the system controlling that function; include the endocrines and the nervous system (Lagerspetz, 1974). The difference between two adaptations is that capacity adaptations are mostly

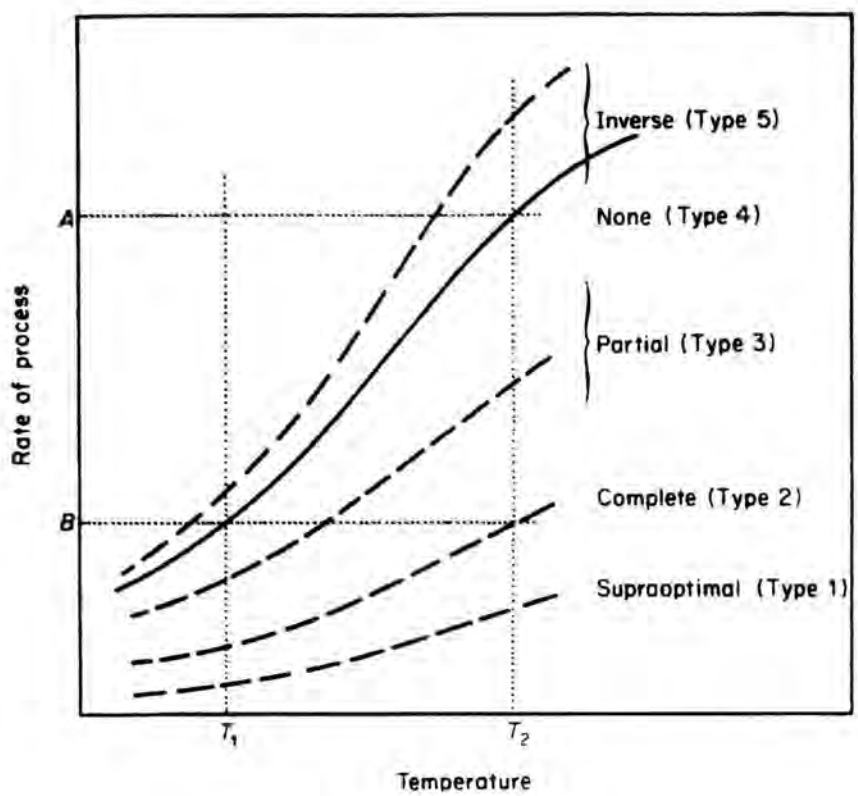


Figure 1.1: Schematic diagram to illustrate the terminology of Precht (1958) concerning rate process compensation following transfer of an animal from temperature T_1 to T_2. The solid line represents the increase in rate after transfer to the higher temperature. After a period of days or weeks at T2 (period of acclimation) the rate may alter to give a new rate temperature curve. The broken lines indicate the possible new rate temperature curves after the period of acclimation (Taken from Cossins and Bowler, 1987; after Precht, 1958)

phenotypic, whereas resistance adaptations may involve a genotypic adaptation, which is the basis for evolution and they gradually become fixed within a population over a large number of generations (Precht, 1958). But there is no clear or easily understood relationship between capacity and resistance adaptations. For example, there are reports of animals which display resistance adaptations but not capacity adaptations and vice versa (Cossins and Bowler, 1987). Some animals show resistance adaptation without capacity adaptation for extreme temperature, in frogs for example which muscle and liver metabolism is unaltered by acclimation but neuromuscular transmission and functioning of the central nervous systems are sensitive to temperature acclimation (Bishop and Gordon, 1976).

Capacity acclimation.

Precht (1958) defined the capacity adaptation as that which modifies the physiological performance of an individual or the rate of its vital processes over the normal range of temperatures, this phenomenon called physiological compensation. During acclimation, from many studies, the plasma membrane lipid makes good compensatory changes with respect to acclimation. Cuculescu (1996) for example, found that membrane lipid saturation was increased in 22°C acclimated *Carcinus maenas* and *Cancer pagurus* as compared with crabs at 8°C. In both species, it was also found that membranes from legs held at 8°C were more fluid than from legs held at 22°C in homothermally adapted crabs. On the other hand thermal acclimation has also been found to produce compensatory changes in the spontaneous electrical activity of a neuron in the snail (*Helix aspersa*); the normal activity in 20°C acclimated snails was blocked by cooling to 5°C. But after a 2 week at 5°C the levels of spontaneous activity in the neurone were comparable with those of warm acclimated snails at 20°C (Zecevic and Levitan, 1980), *Helix aspersa* shifted electrical activity of a neuron to the cold or warm acclimation temperature. Other workers have investigated compensatory changes in neuromuscular function. In the crayfish, *Astacus*, acclimated either 12 or 25°C, the break curve of resting membrane potential of the closer muscles of walking legs was 17 and 24°C in cold and warm acclimated crayfish

respectively. This indicates that thermal acclimation shift the RP toward the temperature of acclimation (Harri and Florey, 1979).

Resistance acclimation.

It is adaptive to increase the ability of animals to survive in extremes of temperature which may occur. It has been known that the normal range of temperatures varies from animals to animal, depending upon the evolutionary thermal experience of the species. Thus, polar species are cold tolerant and warm sensitive whereas tropical species are cold sensitive and warm tolerant. Measuring the incipient high and low lethal temperature for animals which acclimated to a range of constant temperatures is termed temperature tolerance polygon. This area between the incipient high and incipient low temperatures represents the combination of acclimation and lethal temperatures that 50% of the sample can withstand for an indefinite period (i.e. the zone of tolerance). Tolerance to high and low temperature is rarely fixed in a species is related to lifestyle of the species and to the seasonal range of temperatures that it usually encounters. So, the tolerance polygons also differences between stenothermic and eurythermic animals. Eurythermal bullhead (*Amieurus nebulosus*) for example, displayed resistance acclimation and a zone of tolerance that extended down to the freezing point of fresh water. Whereas the cold stenotherm chum salmon (*Onchorhynchus keta*) was more limited acclimation of heat resistance but more significant cold acclimation; and it is also important to recognize that in any sample of animals some are more resistant to lethal temperatures than others (Brettt, 1944; 1952). It was found that a high temperature may become lethal over a longer period, but it is clear from many studies that thermal death points (LD_{50}) can be altered by acclimation (Cossins and Bowler, 1987). In general, at thermal extremes, nervous systems often fail before other systems and the resulting behavioural failures occur before protein inactivation (Prosser and Nelson, 1981).

Membrane lipid structure.

In ectotherms, acclimation can be considered to occur at both metabolic and ultrastructural component. One aspect of this is the structural and function of cell

membrane. The model of the cell membrane structure proposed by Singer and Nicholson (1972), described the fluid mosaic model as cell membranes are composed of lipid bilayers with associated proteins (extrinsic proteins associated with the hydrophilic polar heads of the lipids, intrinsic proteins within the hydrophobic hydrocarbon domain of the lipid layers). It has been found that changes in temperature impact both the permeability of cell membranes, and the activity of integral membrane proteins, via changes in the physical characteristics of the lipid bilayer. These contain both the phase state of the hydrophobic portion and intermolecular packing of membrane constituents within a phase (Hazel, 1995). Changes in the specific lipid constituents of membranes are an important aspect of thermal acclimation by membranes; the chain length and extent of saturation of the lipids determine the melting point, or phase transition temperature of the membrane (Hazel, 1995). At physiological temperature the membrane is being relatively fluid, (liquid crystalline phase), (see Figure 1.2). However with decreasing temperature the membrane fluidity may be reduced, and at low temperatures the membrane may changed to another form (gel phase) this resulting in more ordered packing of the fatty acids. In contrast, increasing the temperature may be causes increased of diffusion; the observed effects could result from altered phospholipids in the membranes (Hazel, 1995). It has been shown for many kinds of organisms that fatty acids formed at low temperature are more unsaturated than those formed at high temperatures (Cossins and Bowler, 1987). Increased unsaturation maintains membrane fluidity at lower temperatures. In comparison, warm acclimated animal membrane lipid composition should be more saturated to reduce membrane fluidity in response to the increase in temperature. It was found that the maintenance of membrane function is more critical in excitable cells, Bowler *et al.*, (1973) for example reported that heat death in the crayfish *Astacus* is associated with a dramatic increase in the permeability of excitable membranes to cations. Its permeabilities increase dramatically at the gel/ liquid crystalline phase transition temperature (Singer, 1981). Ectothermic animals in general and crustaceans in particular are capable of regulation membrane fluidity in the face of changing environmental temperatures. Ectothermic animals change their membrane characteristics to maintain fluidity appropriate to the local temperature, to prevent temperature effecting animal; this phenomenon had been called homeoviscous adaptation

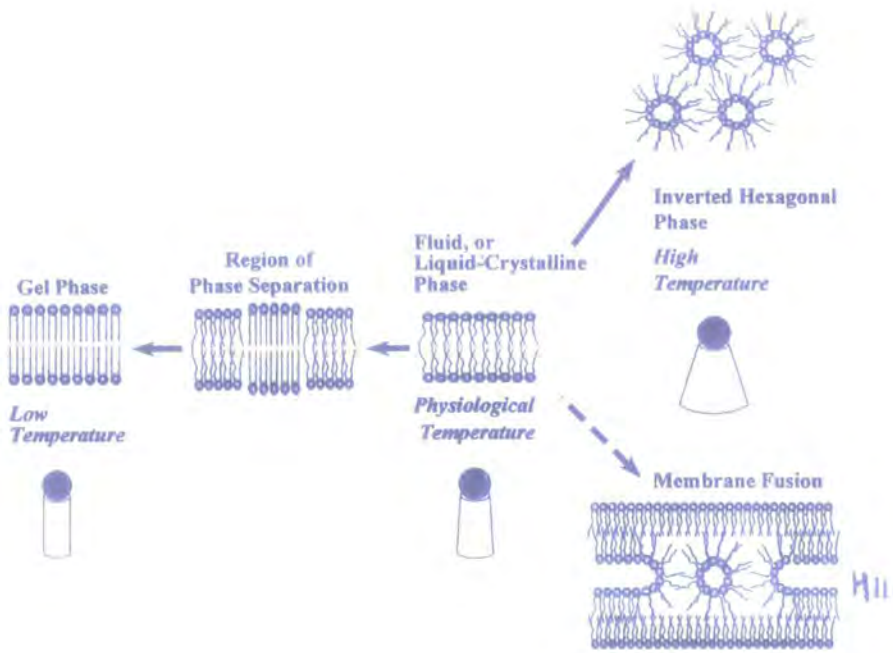


Figure 1.2: Indicating the phase states of membrane phospholipids. Solid arrows indicate the effects of either a rise or drop temperature. The dashed arrow illustrates the presumed involvement of the inverted hexagonal phase in membrane fusion (H_{II}). The physiological temperature refers to the temperature at which the organism is either adapted or acclimated (Taken from Hazel, 1995).

(Sinensky, 1974). This adaptation first described in the membranes of *E. coli* grown at low temperatures was more fluid than bacteria grown at higher temperatures. Homeoviscous adaptation was not always developed to the same extent. Thus, the degree of compensation (homeoviscous efficacy) varied from 75% in the basolateral membranes of carp intestinal mucosa to 25% in brain myelin, while in the sarcoplasmic reticulum no responses were observed (Gracey *et al.*, 1996). So, exposure to temperatures either above or below those required to maintain optimal fluidity initiates acclimatory within the lifetime or adaptational over evolutionary time, alterations in lipid composition that largely offset the direct effects of temperature on membrane lipid fluidity (Hazel, 1995). Smith and Ellory (1971) studied on goldfish intestinal mucosa, at cold acclimation, and it was suggested that the changes in membrane fluidity during thermal acclimation are responsible for changes in the activity of membrane bound enzymes by increases in the number for the Na^+ K^+ - ATPase. However, the specific activity was increased without any change in the number of active enzyme molecules. Other important properties of membranes are their permeability. It has been shown that the introduction of unsaturated hydrocarbon chains into phospholipids produces a substantial increase in the permeability of electrolytes and non electrolytes (Cossins and Bowler, 1987). It has reported that much greater permeability of artificial membranes and mitochondria prepared from cold acclimated animals than prepared from warm acclimated animals (Cossins, 1983).

Enzyme changes.

At the cellular level the process of temperature acclimation is the result of a complex reorganization of cell metabolism, and the changes depend upon the regulation of the activity of enzymes (Cossins and Bowler, 1987). The cellular concentration of an enzyme is a function of the rates of both synthesis and degradation. Sidell (1977) found that in the cold, synthesis was greater than degradation. This increase in the rate of protein synthesis in the cold may compensate for a temperature related reduction in enzyme activity, suggesting that this response may be by a direct effect of temperature at the cellular level. During cold acclimation metabolism of green sunfish brain *Lepomis cyanellus* is highly aerobic, and showed thermal acclimation of several enzymes. For

example pyruvate kinase activity of brain increased whereas it decreased in muscle (Shaklee *et al.*, 1977). In carp, cold acclimation leads to a rapid increase of 30-40 fold in the specific activity which results from a greater number of enzyme molecules (Cossins and Bowler, 1987). In general the rate of process increased when temperature increased by increased in the activity of enzymes. But enzymes exposure to a higher temperature may result in a decreased in activity; and the reaction rate may decrease due to proteins becoming unfold and loss of active site conformation (Somero, 1995). Thus enzymes become more ineffective with change the temperatures.

The Q_{10} is the factor by which reaction velocity of a process would increase for a 10°C rise in temperature (Van't Hoff rule). It has been used in biology as a measure of temperature effects on reaction rate, i.e. how much the metabolism increase or activity of enzymes increases over that temperatures range. Q_{10} of (2) for instance, something will be doubling its rate for every 10°C whereas Q_{10} of (3) its rate increasing 3 times; diffusion control processes have a low Q_{10} compare with enzyme control process which have a high Q_{10}. The Q_{10} of many biological processes is not constant over the normal temperature range, and with a temperature change all these different components of the system may have different temperature sensitivities. Because of that Q_{10} is very important measure of temperature sensitivity (Cossins and Bowler, 1987). Krogh (1914b) and Ege and Krogh (1914) indicated that the inconstancy of Q_{10} was not artifactual but systematic; that as temperature increases Q_{10} tended to decrease in a progressive manner. Comparisons of Q_{10} between different species are suspect because a given temperature interval may fall in quite different portions of their respective rate temperature curves. For example, 25°C would be quite favourable for many temperature fish species, but is injurious or even lethal for Antarctic fish or for mammals (Cossins and Bowler, 1987). There is another way to characterise the temperature sensitivity of a rate process as proposed by Arrhenius. The Arrhenius relationship was deduced from first principles by considering the activation energies of enzymes in a kinetic scheme. A non-linear, logarithmic relationship occurs between the rates of chemical reactions and temperature.

Resting membrane potential.

The central nervous system of multicellular animals plays a major role in controlling spontaneous activity and muscle recruitment as temperature changes, and the neuroendocrine system may be involved in controlling adaptive responses in other tissues. The nervous system is the primary organ of adaptation to the environment, important in several aspects of temperature adaptation (Lagerspetz, 1974). An adaptation of the nervous system to temperature is a major component of the thermal adaptation of animals (Prosser and Nelson, 1981). Each component function of the nervous system, such as resting membrane potential, synaptic transmission and axonal conduction is influenced by changes in temperature. The resting membrane potential (RP) reflects the conductance state of the membrane through which Na^+, K^+ and other ions passively diffuse and the activity and coupling of the Na^+, K^+ ATPase pump (MacDonald, 1990). The RP potential increases with increasing temperature; in shore crabs (*Pachygrapsus crassipes*) for example, resting membrane potential of stretcher muscle fibres was increased with temperature increasing in both cold (12°C) and warm (21°C) acclimated animals, in cold acclimated animals inflexion of the curves occurred at 12.7°C as compared with 18.7°C in warm acclimated. Thus RP shifted in an adaptive manner, can be modified by thermal acclimation (Stephens and Atwood, 1982). The hyperpolarization may be greater than that predicted by Nernst (Montgomery and MacDonald, 1990). However, in the presence of ouabain RP with temperature was reduced to that more closely predicted by Nernst (White, 1983; Montgomery and MacDonald, 1990). RP hyperpolarization of muscle fibre with increasing temperature due to thermal activation of membrane Na^+ /K^+ ATPase (Fatt and Katz, 1953). However, in acclimated animals, RP hyperpolarization may be due to changes in the activity or density of Na^+ /K^+ ATPase (Schwarzbaum *et al.*, 1991). The Na^+ /K^+ pump contribution to RP with temperature in crayfish was 1.5mV/°C (MacDonald, 1990). In crayfish leg muscles the RP became more negative on heating. However, in muscles treated with ouabin the gain in negativity was substantially reduced; hence the Na^+/K^+ pump is stimulated by warming, especially above 28°C (White, 1978). Some changes in the ion permeability mechanisms of the neuronal membrane occur at the inflection point,

may be linked to phase transition of membrane lipids occurring from the liquid crystalline state at high temperatures, to the gel state at low temperatures (Romey *et al.*, 1980). Poikilothermic animals die after much less cooling or heating than is required for metabolic failure; the nervous system fails before inactivation of metabolic enzymes. In general, the limits for normal function are narrower for integrated intact animals than for isolated tissues and cells; the limits for survival of enzymatic proteins or membrane phospholipids extend farther.

Electrical signalling.

The signals (electrical currents) used by nerve cells; generated across their surface membranes; the currents flow through the intracellular and external fluids and result from the movements of charges carried by sodium, calcium, and chloride ions. The electrical potential difference between the inside and the outside of the membrane of an excitable cell depends on the ionic concentration gradients and the selective permeability of the membrane; relatively high concentrations of potassium in the inside but low concentrations of sodium and chloride; and in the outside fluid the ratio is reversed. At rest muscle membranes relatively permeable to K^+ and Cl^- and relatively impermeable to Na^+; RP changes are mainly K^+ driven changes, whereas Cl^- stabilises the muscle fibre RP (Hill, 1992). Thus the RP is determined by the selective permeability of plasma membranes to different ions i.e. Na^+, K^+ and Cl^-. The relation between membrane potential and ions concentration can be calculated; RP calculated using the Nernst equation or the Goldman-Hodgkin-Katz equation if ion concentrations and selective permeabilities are known.

Nernst equation:

$$E_x = \frac{RT}{zF} \log_e \frac{[X^+]_o}{[X+]_i}$$

Where R= gas constant, T= temperature in Kelvin, z = valence of the ion, F Faraday constant, $[X^+]_o$ = extracellular ionic concentration and $[X^+]_i$= intracellular ionic concentration.

Goldman-Hodgkin-Katz equation:

$$V_m = \frac{RT}{zF} \log_e \frac{K_o + [P_{Na}/P_k]Na_o + [P_{Cl}/P_k]Cl_i}{K_i + [P_{Na}/P_k]Na_i + [P_{Cl}/P_k]Cl_o}$$

Where V_m = membrane voltage, K_o= external potassium concentration, K_i= internal potassium concentration, P_{Na}= permeability coefficient for sodium, P_k= permeability coefficient for potassium, P_{Cl}= permeability coefficient for chloride.
There is a temperature coefficient in both equations. Therefore it is possible to predict the extent of membrane potential changes as a function of temperature.

Action potential.

Action potentials are generated in terms of changes in sodium (Na^+) and potassium (K^+) conductance (Hodgkin and Huxley (1952a-b). During the action potential, the rapid depolarization of the membrane is produced by an increase in sodium conductance. Whereas repolarization is produced by an increase in K^+ conductance. Action potential conduction velocity is dependent on membrane resistance (R_m), the number of Na^+ channels and axon diameter (Stephens, 1985b); at higher temperatures membrane resistance decreases and ion shunts occur potentially blocking AP conduction (Fatt and Katz, 1953). Stephens and Church (1988) reported that in Pacific shore crabs (*Pachygrapsus crassipes*) action potentials are generated by calcium and sodium ions. Na^+ channels are responsible for the initial rise of an AP, the Ca^{2+} channel component appearing slightly slower and its conductance contributing to the AP width (Stephens and Church, 1988). Thus if either Na^+ or Ca^{2+} channels are then blocked AP initiation was still seen, but if both channels were blocked AP were prevented from being generated. Although, the AP propagation did not stop when either Na^+ or Ca^{2+} channels were blocked, it did lead to lack of a successful post synaptic change. The heat and cold tolerance limits of conduction were moved, Engelhardt (1951) for example acclimated frogs (*Rana temporaria*) to 15-20°C or to 5-10°C, the conduction velocity in the isolated sciatic nerve

was shifted by about 5°C to higher temperatures in the nerves of animals acclimated to 15-20°C.

Neuromuscular transmission.

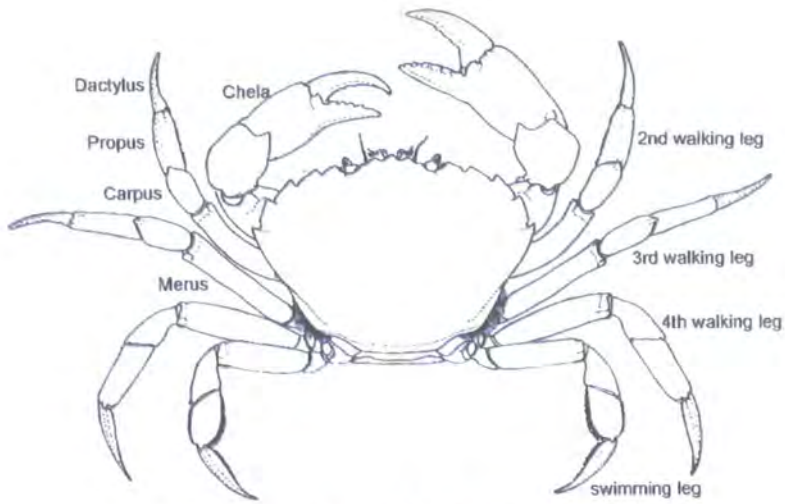
When the nerve impulse arrives at the terminals of an axon a chemical transmitter is released. The transmitter is synthesized in nerve terminals (presynaptic) and released from a presynaptic site, and acts on receptors in postsynaptic site. The small amount released of transmission is sufficient to initiate a muscle impulse; acetylcholine and glutamate are neuromuscular transmitters which are more sensitive to thermal stress either cold or heat than nerve or muscle conduction, in fish (Battle, 1926), frog sartorius (Li and Gouras, 1958; Jensen, 1972). Glutamate is the excitatory neurotransmitter in crustacea, whereas GABA is inhibitory transmitter (Atwood, 1976; White, 1983). Whilst postsynaptic potential is determined by the amount of neurotransmitter released, the exocytosis of neurotransmitter is considered to be the main temperature sensitive part of synaptic transmission (MacDonald, 1988). On the other hand, the liberation of acetylcholine at frog neuromuscular junction is greatly reduced and neuromuscular transmission blocked when one lowers the calcium or increase the magnesium concentration (Katz, 1966).

Neuromuscular junctions (synapses) are more thermolabile than contraction in muscle or nerve conduction (Montgomery and MacDonald, 1990). The effect of temperature acclimation on the neuromuscular function has been studied in invertebrates and vertebrates. In goldfish, during cooling or heating, behavioural disturbances starting with hyperactivity may result from removal of central inhibition and thus motor incoordination, leading to failure to swim, and finally shallow breathing coma and death (Friedlander *et al.*, 1976). These effects might relate to synaptic block. In crayfish, when temperatures for heat coma are approached, the potassium concentration in the hemolymph rises and central nervous function becomes impaired (Bowler, 1973). However, synaptic transmission in crayfish fails with less warming than is required to block post-synaptic depolarization by directly applied transmitter (glutamate). Synaptic block appears to result from decrease in transmitter liberation at temperatures above a critical level (White, 1983), transmission is more sensitive to cooling and heating than nerve conduction (Lagerspetz,

1974). The temperatures for cold and heat block can be altered by acclimation; for example in frog sartorius, cold block for 10°C and 25°C acclimated frogs was at 1 and 4°C respectively (Jensen, 1972). Synaptic block might also involve decreased Ca^{2+} entry presynaptically which would reduce neurotransmitter vesicle release leading to insufficient post synaptic channel activation (White, 1983). Inhibitory synapses are more thermosensitive than excitatory (White, 1983); in crayfish for example, leg muscle receive fast and slow excitatory plus inhibitory innervation. When animals were acclimated to 10°C, the inhibitory junction blocked at 29.9°C; 3°C lower than excitatory junctions. Whereas at 25°C acclimated, the heat block of inhibitory synapses was at 34°C; 4°C lower than excitatory synapses (White, 1978). Blocking temperatures can be shifted by acclimation (Prosser and Nagai, 1968).

Nervous system in crustacean.

The crustacean nervous system includes large diameter unmyelinated axons and ganglia (Nicol, 1964; Nguyen and Atwood, 1994; Liberman *et al*,. 1994). Most crustaceans have long segmented bodies e.g. crayfish and lobster, whereas crabs have one main body segment (Shepherd, 1988; Crothers, 1968). In crustacean, the central nervous system (CNS) is positioned posterior to the mouth. It consists of neurons and neuropil (mass of synaptic connections) connected to the ventral ganglionic mass by a circumoesophageal commissure. Ganglia maintain relatively independent action although being fused together, i.e. correct individual legs are auto mised and only one eye stalk is retracted when touched (Crothers, 1967). Figure 1.3 illustrates the crab walking legs of *Carcinus maenas* (shore crab) and inneravation pathways for motor and inhibitory nerves. The dactylopodite is controlled by the closer and opener muscles; the propodite is controlled by the stretcher and bender muscles; and the meropodite is controlled by the extensor and two flexor muscles. The opener muscle is innervated by a single motor axon and two inhibitory axons. Whereas the closer muscle is innervated by two motor axons and one common inhibitory nerve (Wiersma and Ripley, 1952). The two excitatory nerves innervating the closer muscle are termed slow (tonic) and fast (phasic) which are shown by the characteristic of the excitatory junction potential (EJP) when the nerve is stimulated



Carcinus maenas (shore crab): male, carapace 88 mm across.

Taken from Crothers and Crothers 1983

Innervation of the seven distal muscles. **O**-opener; **C**-closer; **S**-stretcher; **B**-bender; **F**-main flexor; **A**-accessory flexor; **E**-extensor. Solid lines indicate a motor axon, broken lines are inhibitory axons. Taken from Wiersma and Ripley (1952).

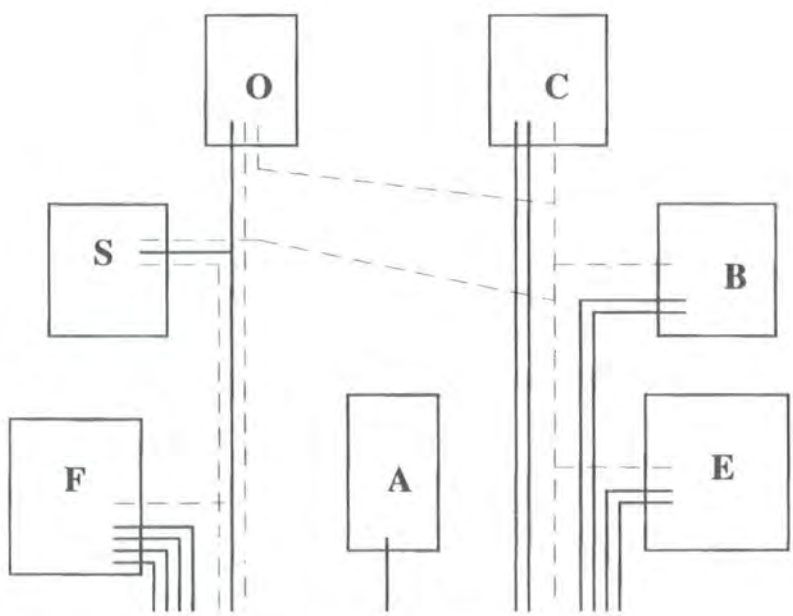


Figure 1.3: Innervation pathway of *Brachyuran* walking legs.

(Stephen, 1985). Tonic neurons are involved in general movement and behaviour, whereas phasic neurons are responsible for rapid defensive contractions (Atwood, 1976). The difference between both neurons is that the tonic axon fire at a low frequency to maintain posture, whereas phasic neurons are silent most of the time and then fire bursts. So, the slow fibers are always activated before fast fibers (Atwood, 1976). Also phasic axons innervate fast acting glycolytic muscles (fatigue easily) whereas tonic axons innervate slow acting oxidative muscle fibres (Atwood *et al.*, 1994; Atwood and Nguyen, 1995). In general, excitatory terminals have more synaptic endings than inhibitory terminals although inhibitory synapses are larger than excitatory. Inhibitory nerves, when stimulated, caused hyperpolarization of muscle fibre. But at low frequencies of stimulation presynaptic inhibition has more influence, whereas postsynaptic inhibition is more common at higher frequencies (Atwood, 1976). A single muscle fiber in crustacean has multiple neuromuscular synaptic terminals; the muscle is polyneuronally and polyterminally innervated (Atwood 1976; Atwood *et al.*, 1994). The closer muscle consists of four group of muscle fiber. Group I and group II show EJP when stimulated by both slow and fast axons. Whereas, group III and IV only show EJP when stimulated by the fast axon and no response from stimulation of the slow axon. They are not innervated by the common inhibitor (Rathmayer and Maier, 1987).

Optimal temperature.

An increase in temperature generally causes increased rates of animal activity or biological process in metabolism and other process. But there is an upper thermal limit to this rate effect of temperature (Cossins and Bowler, 1987). The ultimate thermal limit for living cells is 100°C, and no organisms are able to survive at or above this temperature. But some prokaryotic organisms can survive close to this temperature. Most animals live in environments with maximum temperatures lower than 50°C (Withers, 1992). The thermal limits tolerated by an organism are set genetically and may be adaptive in determining the geographical distribution of a species (Cossins and Bowler, 1987). Over the normal range of temperature an increased temperature causes an increased rate of processes, which is often variable but above the normal range an increased temperature may cause a decreased

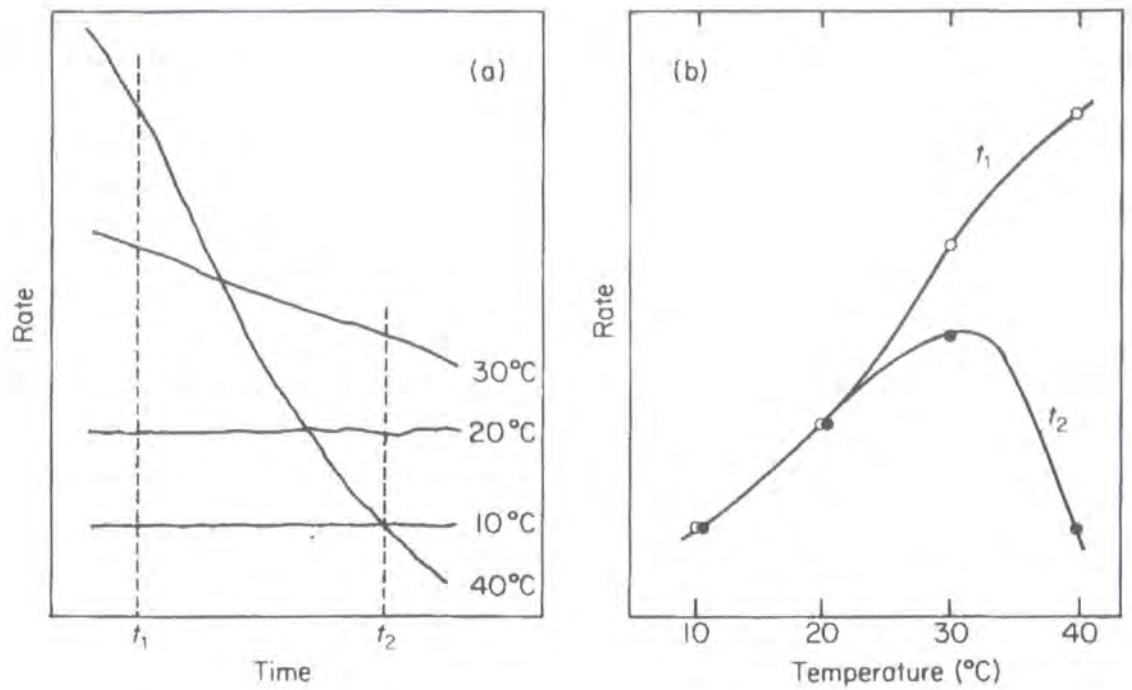


Figure 1.4: A hypothetical graph illustrating the effects of exposure at high temperatures upon the rate of a biological process. (a) At the lower temperatures the rate is quite constant whereas at the higher temperatures the rate declines. Rate measurements made at times t_1 and t_2 yield the rate temperature curves shown in (b), (Taken from Cossins and Bowler, 1987).

rate of biological processes, which become progressively more affected by the debilitating effect of temperature (Cossins and Bowler, 1987). The temperature at which the process rate is most effective is known as the optimal temperature. However, there may be large differences between the thermal optima of different organisms which may be correlated with their natural habitat. Figure 1.4 shows the hypothetical graph illustrating the effects of exposure at high temperatures upon the rate of a biological process. As shown at the lower temperature the rate is quite constant with temperatures up to 25°C. Whereas at 30°C although the rate is higher than at 25°C it slowly declines until it is less than at 20°C. The decline in rate is more dramatic at higher temperatures. Thus the optimal temperature would be about 40°C at time t_1 whilst at time t_2 the optimal temperature would be only 30°C. Therefore the optimal temperature decreases with increasing exposure to the various temperatures, and are not the same of individual animals (Cossins and Bowler, 1987). The rate and temperature dependence of many biological processes will depend to the level of activity of an animal. For example, the rate of oxygen consumption of active fish may be two to four times greater than that of quiescent fish (Fry, 1957). Animals which are exposed to increased temperatures for long periods tend to establish reduced rates of metabolism to compensate for the rate increasing effects of the temperature change. These physiological adjustments are part of an organism's normal response to changes in temperature, and generally take several days or perhaps weeks to become apparent (Cossins and Bowler, 1987).

Thermotolerance.

Thermal experience of a species includes various features of the thermal regime, such as maximum and minimum temperature experienced. Mean temperature and patterns and rates of temperature fluctuation on a daily, seasonal and annual basis and is an acclimatization feature (Cossins and Bowler, 1987). Depending upon the thermal experience of the species, the normal range of temperatures varies from animal to animal. Thus, winter animals are usually more cold tolerant and more heat sensitive than summer animals (Cossins and Bowler, 1987). The concept of critical thermal maximum was originally introduced by Cowles and Bogert (1944) and Lowe and Vance (1995), who

worked on species of reptiles, and defined the CTMax as the thermal point at which locomotory activity becomes disorganised and the animal loses its ability to escape from conditions that will promptly lead to its death (Lagerspetz and Bowler, 1993). The upper and lower limits that define thermotolerance are set by critical thermal maximum (CTMax) and critical thermal minimum (CTMin). Thus thermal acclimation and critical temperatures are important in the understanding of the biology, distribution and ability of the organisms to adapt to different thermal regimens. Most invertebrates and vertebrates have CTMax values over 30°C and some have values over 40°C. For example, in congeneric marine snails, species from the low inter-tidal to sub-tidal zone (*Tegula brunnea* and *Tegula montereyi*) die at a lower temperature (36°C) than the mid to low inter-tidal zone (*Tegula funebralis*) which die at 42.5°C (Tomanek and Somero, 1999). The criteria of survival are different for intact animals, tissues, isolated enzymes, and lipids. So, whole animals survive and may be active over narrower range of temperature than isolated tissues and cells; temperature limits for proteins (denaturation) and lipids (melting or solidification) are wider than for cell survival (Prosser and Heath, 1991). Furthermore, different tissues may have varying thermal sensitivity, for example, skeletal muscle of *R. pipiens* has about the same thermal tolerance as the intact adult, but sciatic nerve and heart have a higher thermal tolerance (Cossins and Bowler, 1987). Survival limits of organisms or parts of organisms can be modified (acclimated) by prior thermal experience (Prosser, 1991); as reviewed by Barclay and Robertson (2000); Stillman and Somero (2000); Lagerspetz and Bowler (1993); Pearson, 1998. In crustacean for example, the CTMax of adult *Asellus aquaticus* increased by 4-5°C by acclimation at 20°C as compared with the same animals at 10°C (Lagerspetz and Bowler, 1993).

Environments.

In most aquatic invertebrates body temperature (T_b) closely matches environmental ambient temperature (T_a), allowing T_b to be inferred from T_a. However, the high specific heat capacity of water and its high thermal conductivity make aquatic environments relatively thermostable (Cossins and Bowler, 1987). Aquatic environments are more thermostable compared to terrestrial environments, because water can absorb and lose

relatively large amounts of heat with only small changes in temperature. Whereas, the low specific heat of air, and its low thermal conductivity make terrestrial habitats the most thermally varied and complex (Cossins and Bowler 1987). Rocky shores are typified by steep environmental gradients between land and sea. The vertical gradient is essentially unidirectional with increasing stress from immersion to higher shore levels. There is also a horizontal gradient associated with exposure to wave action, as revised by Raffaelli and Hawkins (1996). Rocky shores are subjected to considerable natural environmental change, the most obvious is tidal amplitude that varies, on short term and long term time scales (Denny and Paine, 1998). There will also be diurnal and seasonal variation in stress experienced at low tides due to the prevailing weather and season (Thompson *et al.*, 2002). Intertidal zonation patterns have been shown to be due both to biotic factors such as competition and predation and to abiotic factors such as temperatures and air humidity (Connel, 1961). Intertidal organisms have a distribution pattern from high shore to low shore (Hofmann, 1999). Because of the difference in vertical distribution, these organisms may experience very different patterns of emersion during each tidal cycle, and the differences in frequency and duration of emersion, these are important in shaping the thermal characteristics of the vertical zones (Stilman and Somero, 1996). Also, changes from terrestrial to aquatic conditions occur in the intertidal zone; which aquatic condition more constant and terrestrial conditions more variable. Two species of intertidal porcelain crabs are common along rocky shores of the Northeastern Pacific. *Petrolesthes cinctipes* is found under rocks in the mid to high intertidal region, but *Petrolesthes eriomerus* is found under rocks in the low intertidal zone and subtidally to 80m. *Petrolesthes cinctipes* can be emersed during every low tide which is much grate of time, but *P. eriomerus* is only emersed during the lowest spring tides and on most days is not emersed at all. These emersion patterns indicate that individuals of *P. cinctripes* may spend as much as 50% of their time out of water. Because of their different vertical distributions, these two species experience very different levels of abiotic stress (Stilman and Somero, 1996). Temperatures measured underneath rocks in the mid intertidal zone were as high as 31°C, 15°C higher than maximal temperatures measured under rocks in the low intertidal zone. In air, at 25°C, large specimens of *P. ci*nctipes were able to maintain a higher respiration rate

than similarly sized *P. eriomerus*; and *P. c*inctipes were able to maintain aerobic metabolism during emersion, whereas *P. eriomerus* shifted to anaerobic metabolism. A suite of morphological, physiological and biochemical features allows *P cinctipes* to live higher in the intertidal region than *P. eriomerus* (Stilman and Somero, 1996).

Heat shock proteins as molecular chaperon.

In nature, organisms must be responsive to the changes in physical environment; these responses are often behavioural or metabolic. But one mechanism to prevent cellular damage an animal is the adjustment of gene expression in response to environmental change, namely the heat shock response (Pigliucci, 1996). During a heat shock response, heat shock proteins (HSPs), are induced in response to various stressors that are denaturing to the cellular protein pool (Lindquist, 1986; Parsell and Lindquist, 1993). These stresses are not limited to extreme temperatures and include a range of other environmental for example, ethanol, heavy metals, hypoxia, hyperoxia, changes in pH, toxins, ischaemia, osmotic shock, radiation (Feder, 1996). In stressed cells, denaturing proteins may enter one of two pathways: either degradation by a cellular protease or refolding, facilitated by molecular chaperones (Wickner *et al.*, 1999). Many organisms are able to synthesize proteins which offer some protection from cellular damage (Hightower *et al.*, 1985; Hightower, 1993). These proteins were first described in cells from *Drosophila melanogaster* during exposures to high temperature (Ritossa, 1962) and so the term "heat shock protein" (HSP) was applied. Subsequently a range of environmental stresses have been shown to induce heat shock proteins, and the term "stress proteins" has been used to describe these proteins (Lewis *et al.*, 1999). Many heat shock proteins are molecular chaperones, a class of proteins that function to minimize the problems that arise when other proteins are in non native conformation (Hartl, 1996; Gething, 1997; Feder *et al.*, 1995; Feder and Hofmann, 1998; 1999). These HSPs are universally found in wide variety of organisms, animal or plant, prokaryote or eukaryote. Two general categories of heat shock proteins occur, first those that are expressed constitutively under normal physiological conditions (HSCs) and secondly those that are expressed only in response to protein denaturing stress (HSPs).

Family members of heat shock proteins.

There are several families of heat shock proteins such as HSP110, HSP100, HSP90, HSP70, HSP60 and of smaller proteins such as HSP27, HSP10 and ubiquitin. Heat shock proteins can be divided into classes by mechanisms of transcriptional regulation, many HSPs as molecular chaperones, share a common mechanism in terms of how they interact with their substrate, non-native proteins (Feder and Hofmann, 1999). There are three major families of proteins: HSP90, with variants between 80 and 100 KiloDaltons (KDa); HSP70, with variants between 65 and 75 KDa; and HSP60 and the small HSPs ranging from 16 to 40 KDa. In addition there is a highly conserved ubiquitin around 8 KDa (Hightower *et al.*, 1985; Hightower, 1983); HSP90 is found in association with several cellular proteins; either enhances or inhibits the normal function of these proteins. The HSP70 family accounts for a majority of the translational activity in cells in response to environmental disorganization. HSP73 and HSP72 are two of at least 21 proteins in this group. HSP60 or chaperonin also referred to as Gro-EL, is found in *Escherichia coli* and in other related bacteria. It is as well found in the mitochondria and chloroplasts of eukaryotic cells. HSP60 is highly conserved, has low background levels and its synthesis is induced by stress (Lewis, 1999). HSP40 has been identified in mammalian cells; forms a cytoplasmic complex with HSP70, and functions co-operatively in refolding denatured proteins after heat shock (Yamane *et al.*, 1995). HSP20-30 (low molecular weight) these proteins are more species specific than the larger stress proteins and less conserved; are not synthesized under normal conditions. Ubiquitin involved in the nonlysosomal degradation of intracellular proteins in all eukaryotes, its synthesis is increased with increase in temperature (Lewis, 1999). Although originally recognised by their induction by heat or other stress, many of the HSPs are known to be expressed constitutively. The major advance of the function of stress proteins came about with the recognition of molecular chaperones (Parsell and Lindquist, 1993; Feder *et al.*, 1995). Although cellular proteins are typically folded in their native conformations while functioning in cells, proteins may be unfolded in several contexts i.e. during or after exposure to a protein-denaturing stress. At these times, unfolded proteins may be susceptible to inappropriate interactions with other cellular components. For example, interactions can result in aggregates of unfolded protein

that at best diminish the pool of functional protein and at worst are cytotoxic (Feder, 1999). Molecular chaperones in this state enable the cell to cope with this problem; can recognise and bind to the exposed side groups that characterise unfolded proteins; prevent the bound side groups from engaging in inappropriate interactions with other cellular components, as well as stabilising the bound proteins in an unfolded state. Typically in an ATP-dependent manner, molecular chaperones can then release bound proteins to allow them to refold properly. Alternatively, chaperones can target bound proteins for degradation or removal from the cell. According to this scheme, the heat shock cognates or constitutively expressed HSPs perform these roles for nascent polypeptides or proteins that unfold during normal cellular processes, while the inducible HSPs function primarily in response to the protein denaturation that occurs during or after stress. Thus molecular chaperones are not presently thought to assist a protein along its folding pathway; rather, chaperones reduce the probability of off pathway reaction such as the formation of aggregates (Feder, 1996).

Environmental regulation of HSP gene expression.

The regulation of the heat shock response is liked to the activity of a specific transcription factor, HSF (heat shock factor) (Morimnoto *et al.*, 1994). Craig and Gross (1991) and Morimoto (1993) proposed a model for the transcriptional activation of HSP genes in which inducible genes are controlled by single transcriptional factor, HSF, which activates the HSP genes in response to heat stress. In the absence of heat shock, HSF exists primarily in the cytoplasm and as a monomer. On heat shock, HSF trimerises and migrates to the nucleus, where the timer binds to HSEs (heat shock elements, consensus sequences in the promoters of genes encoding heat shock proteins) and initiates transcription (see Figure1.5). One heat shock protein, HSP70, plays a key role in the regulation of HSF trimerisation and hence transcription. HSP70, either by itself or as part of a protein may interact with HSF trimers to promote their dissociation from HSEs or otherwise inhibit their transcriptional activation. Unfolded proteins apparently compete with HSF monomer for interaction with HSP70. Thus, heat or other cellular stresses may denature proteins and thereby depress HSP70 mediated inhibition of transcription will decrease as will transcription of HSPs.

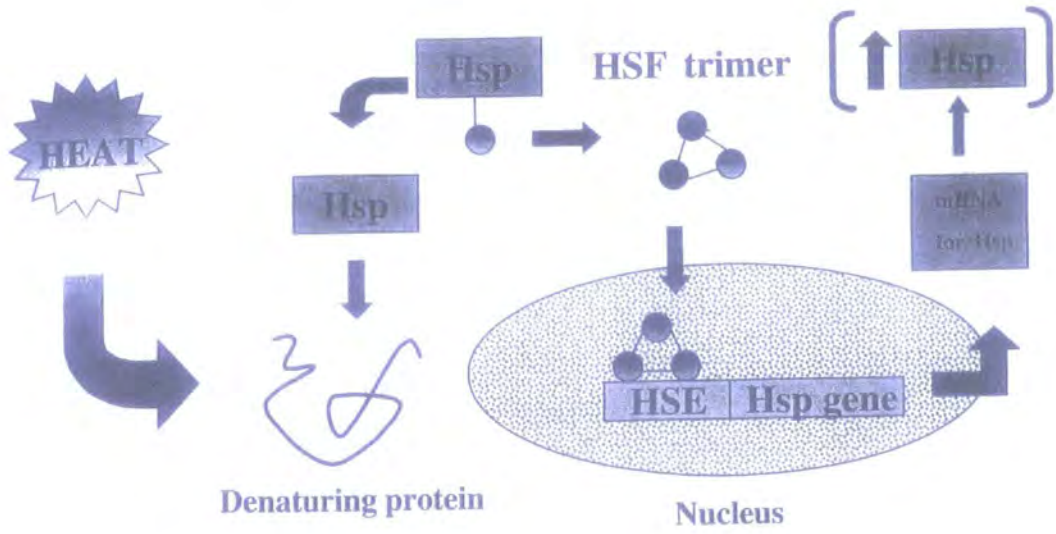


Figure 1.5: Proposal model for the transcriptional activation of HSP genes. Proposed by Craig and Gross (1991) and Morimoto (1993).

Tomanek (2002) hypothesized that the interspecific and acclimation induced variation in onset (T_{on}) of HSP70 synthesis is regulated through the interaction between HSF1, a transcription factor that binds to heat shock elements (HSE) which are located in the promoter of HSP genes, and several HSPs, among them the two known putative main repressors of the response, HSP70 and HSP90. This interaction is called the cellular thermometer which is the core of a model of transcriptional regulation of HSP expression (DiDomenico *et al.*, 1982; Craig and Gross, 1991). Under non stressful conditions several HSPs, e.g., HSP70, HSP40, and HSP90 keep HSF1inactive by binding to the monomer. But under stressful conditions HSPs preferentially bind to denaturing proteins and this leads to the release of HSF1, which subsequently can activate HSP synthesis (Tomanek and Somero, 2002; Tomanek, 2002), (see Figure 1.6). Many studies have shown that acclimatization and acclimation induce variation in the onset temperature of heat shock response, because of changes in endogenous levels of HSPs that repress HSF1 activation, (e.g. Tomanek and Somer, 1999). Thus, the functions of HSPs under stressful conditions are viewed as important in setting an organism's resistance to heat stress (Tomanek, 2002), and these patterns are may directly related to thermotolerance at the organismal level (e.g. Tomanek and Somero, 1999).

Expression patterns of HSPs in natural population.

The most significant ecological aspect of HSP synthesis is that HSPs confer thermotolerance to cells and organisms. But HSPs vary in response to environmental factor; total cellular levels of HSPs fluctuate with season (Hofmann and Somero, 1995). In seasonal variation for example, it has been found that HSP70 was greater in tissue from summer collected than in winter (e.g. Feder *et al.*, 1994; Hofmann and Somero, 1995; Roberts *et al.*, 1997). HSP70 levels were also greater in tissue collected from inter-tidal location than from a submerged population (e.g. Hofmann and Somero, 1995; Tomanek and Somero, 1999). For example, in the inter-tidal mussel, *Mytilus trossulus*, endogenous concentrations of HSP70 in gill tissue were significantly elevated in summer versus winter acclimatized mussels (Hofmann and Somero, 1995). In addition to changes in total amount of HSPs, induction set point, which is also called the threshold induction temperature, is

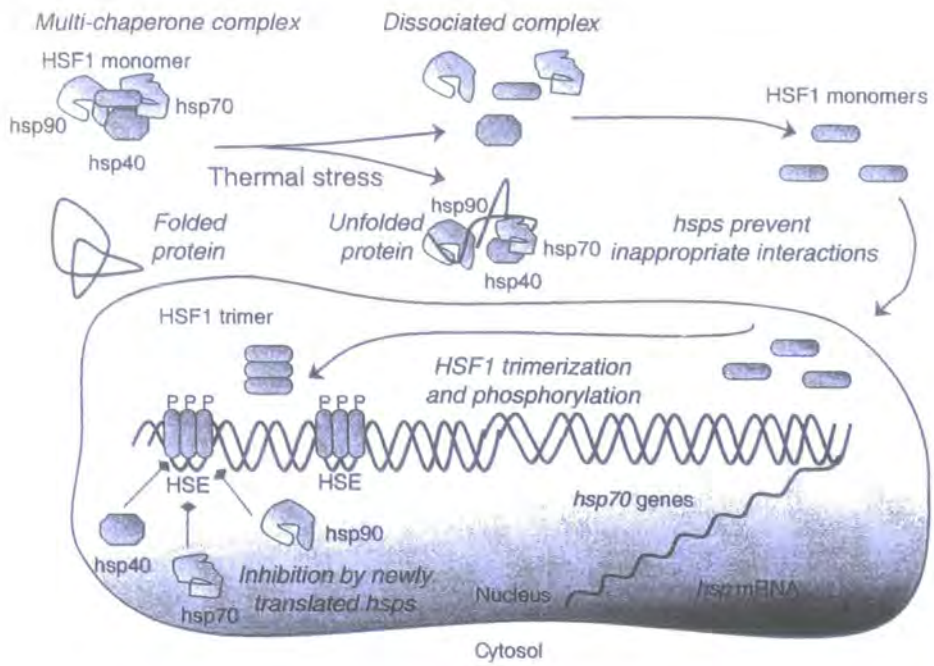


Figure 1.6: The regulatory model for the transcriptional activation of the *de novo* synthesis of heat shock proteins (hsps). Under non stressful conditions, heat shock factor 1 (HSF1) monomers are associated with at least hsp70, hsp40, and hsp90 (fro references see Tomanek and Somero, 2001). During stressful conditions hsps prevent proteins from denaturing and the complex between HSF1 and hsps dissociates. Free HSF1 monomers can form active trimers, which bind to the heat shock element (HSE) and are transcriptionally competent after phosophorylation. As hsp levels increase, they re associate with HSF1 and thereby lead to the repression of hsp gene expression (for references see modified after Tomanek and Somero, 2002).

altered by thermal history; with more warm acclimated organisms displaying a higher threshold for induction than cold acclimated organisms (Hofmann *et al.*, 2002). A similar pattern is also observed in the natural environment where the threshold induction temperatures show seasonal differences, rising to higher temperatures in the summer. For instance, in the summer mussels the induction temperature was 28°C, whereas it was 23°C in winter (Hofmann, 1999). Correlating to microhabitat and between species, Hofmann and Somero, (1996); Tomanek and Somero (1999) have found that different sets of congeners of rocky inter-tidal marine invertebrates have different stress responses despite being acclimated to the same temperature. For example, in marine snail genus *Tegula* the three temperate species that acclimated at 13°C differed in the onset temperature of HSP70 synthesis (T_{on}), of maximal HSP70 synthesis (T_{peak}) as well as the upper thermal limits of HSP synthesis (T_{off}) according to the species heat tolerance. Furthermore, specimens of the subtropical mid inter-tidal that acclimated at 23°C showed even higher temperatures for T_{on}, T_{peak} and T_{off} than its temperate mid inter-tidal congener (Tomandk and Somero, 1999). Thus, in natural population across environmental gradients and with thermal microhabitat, the stress response varies (for review see Helmuth and Hofmann, 2001).

Expression of heat shock proteins and thermotolerance.

In many studies, it has been found that patterns of stress protein expression can be correlated with the species' natural thermal environments; i.e. species from warm environments undergo a stress response at warmer temperatures than from cool environments (Lindquist, 1986; Huey and Bennett, 1990; Somero, 1995). The expression of HSPs during stress and in the recovery period after stress coincides with the induction of improved stress tolerance. In terms of high temperature stress, this induction of thermal tolerance requires minutes to hours and is thus more rapid than the acclimatory changes (Feder, 1996). Many studies have shown that subjecting cells or organisms to a sublethal temperature increase, causes a transitory increase in tolerance to a subsequent heat stress (e.g. Subject and Shy, 1986; Barclay and Robertson, 2000; Gray and Robertson, 1998), this expression may involve the acquisition of thermotolerance to normally lethal temperature, and the function of stress proteins in thermal tolerance seems to be the prevention of heat-

damaged protein accumulation in cell (Feder and Hofmann, 1999). In most organisms studied, HSP70 family of proteins (~70-80KDa) are among the most prominent proteins induced by heat, and these proteins do play a central role in the thermotolerance to high temperature, as they allow cell survival during and after thermal stress (Parsell and Lindquist, 1993). Expression of HSP70 is not detectable in non-heat shocked animals, but during heat shock it becomes the most prominent protein synthesized and could be an important component of the protective response. In *Drosophila*, most protein synthesis is down regulated following a heat shock, but there is a striking induction of heat shock protein 70 (Parsell and Lindquist, 1993). Thus, induction of thermal tolerance has been reported to correlate more closely with accumulation of HSP70 than other stress protein (Li and Werb, 1982).

Effect of thermal stress on nervous system.

Within nervous tissue, the question *is how the nervous system maintains adaptive function under different thermal regimes, and does heat shock protect neuronal function at high temperatures?* As heat shock proteins preserve cellular integrity by preventing protein misfolding, aggregation, and denaturation during exposure to abnormally high temperatures (Parsell and Lindquist, 1993), their increased production of HSPs could serve to stabilize and maintain synaptic transmission. Recent work has shown that prior heat shock not only enhances survival rate but in fact confers a functional neuroprotection at synapses (Dawson- Scully and Robertson, 1998; Karunanithi *et al.*, 1999; Barclay and Robertson, 2000; 2001). Karunanithi *et al.*, (1999) found that neurons and synapses can be altered and protected from heat stress by prior heat shock. In *Drosophila melanogaster* larva, brief exposure to 37°C induces HSP70 expression and preserves synaptic transmission during subsequent exposure to high temperature. Thus heat shock increases the upper temperature limit for synaptic transmission and stabilizes synaptic performance during a subsequent heat stress. It was also found that presynaptic and postsynaptic performance are improved at high temperatures; the percentage of failures of transmitter release is lower and the mean quantal output is higher in heat shocked individuals; and postsynaptic current retain a more stable amplitude at high temperatures >31°C compared

to non-heat shocked controls. In African migratory locust (*Locust migratoria*), a previous heat shock treatment can protect neuronal circuits responsible for generation of flight rhythms by increasing the temperature for heat induced failure and by decreasing their thermosensitivity (Robertson *et al.*, 1996b). Heat shock also protects synaptic transmission in motor circuits from a subsequent heat stress by increasing the upper temperature limit for synaptic transmission by 6°C, reducing the amplitude of postsynaptic potentials, and decreasing the time to recovery after heat induced failure (Dawson-Scully and Robertson, 1998; Barclay and Robertson, 2000).

Photoperiod.

Other environmental factors, e.g. photoperiod and nutrients may interact or counterbalance temperature in controlling locomotor activity (Prosser and Nelson, 1981). Temperature and day length are predominant cyclical factors in the natural environment. Seasonal changes in temperature and day length can lead to changes in primary productivity and seasonal variations in water characteristics such as oxygen, pressure, pH, total alkalinity and salinity. Roberts (1964) studied an effect of day length in sunfish, *Lepomis* by comparing the acclimated rate temperature curves for routine oxygen consumption of animals conditioned under long or short day photoperiods. He found higher rates of oxygen consumption under short day length compare with long day length, and suggested that this was probably due to increased routine activity and muscle tone during short day photoperiod. Many studies in which crabs were acclimated to different water temperatures have been carried out using a single, often 12:12 light-dark photoperiods (for example, Cuculescu, 1996; Pearson, 1998; Escamilla-Chimal *et al.*, 2001). This methodology may not always be appropriate, particularly when studying processes that occur at a whole animal level over the seasonal range. For example, in centrarchid fishes, variation in day length has been shown to influence whole animal metabolic rate, growth rate, and prespawning behavior. Thus the effect of temperature acclimation may be biased depending upon the photoperiod regime (Kolok, 1991).

Photoperiod may affect growth through a number of processes, including changes in behaviour, physiology, food consumption and or activity. Several studies have

investigated the effect of photoperiod on growth and survival. Brett (1989) and Berry (1997) studied the activity of lobsters under different photoperiods and determined that activity was significantly influenced by photoperiod. Most activity occurred during the dark period; because of this, it is suggested that a long day photoperiod may support better growth. Activity would be decreased at long day photoperiod and therefore use of energy is decreased and more energy can be directed towards growth (Berry, 1997). Crear *et al.*, (2003) studied the effect of different photoperiod on growth, survival, colour and activity of juvenile southern rock lobster (*Jasus edwardsii*). It was found that photoperiod had no effect on survival or colour, and the major peaks in activity occurred during dark periods. Furthermore, food consumption or activity does not appear to determine the rate of growth of lobsters in the different photoregimes but is affected by their energy expenditure. However, Gardner and Maguire (1998) studied the effect of different photoperiod and light intensity on survival, development and cannibalism of larvae of the Australian giant crab (*Pseudocarcinus gigas*) and found the similar result of survival that was not significantly affected by photoperiod or light intensity; larvae had shorter intermoult duration in treatments with longer photoperiods and brighter light with most rapid development. Size of stage 4 zoeas was affected by photoperiod with smallest zoeas in the continuously dark treatment, whereas all other treatments were similar. Other research studied the effects of photoperiod and temperature on growth, development and adult size in a pentatomid bug (*Dolycoris baccarum*) (Nakamura, 2002). It was found that the temperature size relationship was not stable but was highly affected by the photoperiod, and showed a geographical variation. Also there were variations in the developmental period and the growth rate.

Changes in survival and development of larvae in different lighting regimes are often simply attributed to effects on feeding. However, other aspects of behavior and physiology may be influenced by lighting including swimming activity (Gardner and Maguire, 1998), so light is one of the major factors influencing swimming. Kolok (1991) determined whether the critical swimming speed of juvenile largemouth bass (*Micropterus salmoides*) was influenced by prolonged exposure to seasonally inconsistent photoperiods. It was found that in early winter the critical swimming speed of largemouth bass

acclimated to 5°C and 12:12 L:D photoperiod was significantly reduced relative to that of fish field acclimatized to 5°C, but was not significantly different when compared to that of fish acclimated to 5°C and 9:15 L:D photoperiod. In early summer the critical swimming speed of largemouth bass acclimated to 10°C and 12:12 L:D photoperiod was significantly reduced relative to the critical swimming speed of fish either acclimated to 10°C and 15:9 L:D photoperiod or field acclimatized to 10°C. Thus the photoperiod alters the critical swimming speed at water temperatures of 5 and 10°C, and no significant differences were found in critical swimming speed of fish that were field acclimatized to 17°C or acclimated to 15 or 19C with photoperiods of 9:15, 12:12, or 15:9 L:D. Temperature and pairing of light are indicated as possible important exogenous determinants of the breeding activity in amphipods (*Hyalellaa azteca*). Kruschwitz (1978) studied the effects of temperature, photoperiod and mate stimulation on reproduction; generally, within the ranges studied higher temperature, longer photoperiods and pairing increased reproductive activity.

Previous studies have demonstrated seasonal effects on crab muscle membrane lipid composition and fluidity and also on thermal tolerance of *Carcinus maenas* (Cuculescu, 1996). Pearson (1998) establishes that the temperature experienced by the CNS and major endocrine organs during acclimation did not determine the responses to acclimation in the peripheral tissues, which remained a local phenomenon. However, they suggested factors other than temperature were involved in seasonal responses. They demonstrated that temperature causes direct local responses, but peripheral responses to photoperiod inputs must act through the central integration systems. Photoperiod is also more reliable indicator of the coming season than is temperature and it is not surprising that the literature reports that photoperiod can modify thermal acclimation.

In order to determine whether photoperiod might influence attainment of thermal acclimation as well as to have an effect on the extent of the acclimation response that may be evident a comparison of crabs subject to long and short photoperiod but having the same thermal acclimation experience would reveal such photoperiodic effects.

The objective of this research was to (1) determine whether the shore crab (*Carcinus maenas*), was influenced by prolonged exposure to different regimes of photoperiods either 8:16 L:D or 16:8 L:D with both cold acclimation (8°C) and hot

acclimation (25°C). Most research of *Carcinus maenas* acclimated to low or high temperature has been done either at a consistent photoperiod (12:12 L:D). We examined the effects of temperature and photoperiod on the neurophysiological parameters of synaptic transmission to see whether there were interactions between photoperiod and the acquisition of temperature acclimation. (2) Determine the upper thermal tolerance CTMax of the crab by following behaviour and survival and investigate the effect of heat shock on thermotolerance as measured by CTMax and the types and levels of heat shock proteins produced in the muscles of the shore crab *Carcinus maenas* (intertidal zone) and *Cancer pagurus* (subtidal zone). (3) Determine whether heat shock protein production has a thermal protective effect at synaptic transmission and protecting the EJP amplitude at high temperature.

Chapter Two

General materials and methods

2.1 Crabs.

Individuals of the species *Carcinus maenas* (L.) were obtained from either Dove Marine Laboratory, University of Newcastle or Millport Marine Station, Isle of Cumbrae. The crabs were held in the laboratory aquarium in tanks (5 L), which were supplied with filtered and aerated artificial sea water (Peacock Salt Ltd, Glasgow. Seamix). Animal were fed once a week on squid and the seawater was changed twice week at least. The aquarial rooms were temperature controlled (± 1/2°C) and had adjustable photoperiods using automatic timing clocks. On arrival from suppliers, crabs were held for at least three days at 8°C prior to acclimation.

2.2 Homothermal acclimation.

Environmental control rooms: crabs were maintained at either a short day photoperiod of 8hours Light: 16hours Dark (8L: 16D), or long day photoperiod of 16hours Light: 8hours Dark (16L: 8D) (Fluorescent illumination) at the acclimation temperature (either 8°C or 25°C) for 3 weeks before electrophysiological measurements. Previous work has established (Pearson, 1998) that this period was sufficient for temperature acclimation.

2.3 Dissection.

Walking legs were obtained from experimental animals by autotomy or severance at the basipodite. The main leg nerve bundle was exposed in the meropodite by using a small drill to make a window in the exoskeleton. The connective tissue membrane and overlying muscle were removed to expose the leg nerve. The nerve bundle was divided

into two parts longitudinally with a blunt microelectrode; this procedure invariably separated the tonic and phasic motor axons as subsequently measured by their different thresholds stimulation. A small window was opened in the exoskeleton towards the distal tip of the propodite to expose the closer muscle without damaging any of the fibres. During this operation, the leg was covered in saline solution of the following composition:

Solute	Concentration(mM)
HEPES(N-{2-Hydroxyethyl}piperazine-N'-{2-ethanesulfonic acid}) pH 7.4	10
KCl	8
NaCl	470
$CaCl_2$	20
$MgCl_2.6H_2O$	10

The pH of the saline solution was adjusted to pH 7.4 by addition of NaOH, and aerated prior to use.

2.4 Experimental procedure.

2.4.1 Electrophysiological Equipment.

Muscle membrane potentials were recorded with an intracellular microelectrode connected to a high input impedance amplifier. Signals were displayed on an oscilloscope and recorded on a PC using an analogue to digital circuit (see Figure 2.1); preparations were temperature controlled using a Peltier junction. The following experimental arrangement was used in all microelectrode recording experiment:

- **A microelectrode puller (Harvard):** was used to pull microelectrodes from borosilicate glass (2mm OD, 1mm ID). The type of microelectrode glass was GC200F-10 from Harvard with integral filament to facilitate filling. The tip of the microelectrode was

placed in saline solution to measure the tip resistance which was about 20MΩ to ensure the microelectrode tip was of suitable size when filled with microelectrode solution consisting of potassium acetate (3M), potassium chloride (200mM). Microelectrodes were connected to the input of the amplifier using chloridised silver wire. The bath reference electrode was also a chloridised silver wire.

- **Amplifier**: Electrical recording was made using a custom-built low-noise amplifier with input resistance of > 10^{12} ohms and amplification of 10^x or 100^x for digitization and display.
- **Faraday cage**: The experimental chamber was shielded from electrical interference using a Faraday cage and from mechanical disturbance by an air table (Technical Manufacturing Corporation).
- **Oscilloscope**: The time and voltages were measured by oscilloscope model PM 3206 PHILIPS.
- **Computer**: output from amplifier was displayed on an oscilloscope and recorded on a PC computer via an analogue to digital converter interface (see fig.). The SCAN synaptic current analysis program V3.0 was as provided by Dempster (Strathclyde University). It is an analogue to digital data acquisition and analysis program for signals from the variety of electrophysiological experiments. The output of the amplifier was connected to a Data Translation DT2812 Laboratory Interface card, and all electrophysiological experimental data was recorded on either Zip or floppy disks
- **Digitimer isolated stimulator**: the leg nerve was stimulated using a pair of platinum hook electrodes. Stimuli were applied through a stimulus isolation unit and timing was controlled using Digitimer. Stimulus timing and synchronization of data capture and oscilloscope display were controlled by a Digitimer (Devices model DS2A). Stimulus intensity was used to identify the low threshold axon type by splitting the nerve bundle longitudinally into two halves then stimulating one of the half bundles of the nerve on the hook electrodes with 2msec duration to identify the low threshold or tonic motor axon.

Figure 2.1: Diagram of experimental set-up for electrophysiological experiments. The dissected, immobilized leg was pinned with the ventral side facing outwards. The leg was pinned in a small plastic chamber containing an aerated extracellular saline solution; the floor was covered by Sylgard. A microelectrode (Me) was used for intracellular recordings, muscle membrane potentials were recorded with an intracellular microelectrode connected to a high input impedance amplifier (AMP) using chlorided silver wire. The recorded voltages were amplified using a custom-built low-noise amplifier with input resistance of $>10^{12}$ ohms and amplification of 10^{x} or 100^{x} for digitisation and display. The output from the amplifier (signals) was displayed on an oscilloscope and recorded on a PC computer via a digital to analogue converter interface. The SCAN software program (Dempster, University of Strathclyde) was used to record all electrophysiological experimental data on either Zip or floppy disks and for data analysis. A pair of platinum hook electrodes was used to stimulate the leg nerve through a stimulus isolation unit (SIU) and timing was controlled by Digitimer.

Figure 2.2: Diagram of the temperature of which was controlled using a Peltier junction. The hot junctions of the Pelteir were cooled by contact with a brass block. Cooled water was pumped through this block using a Geneline cooler/circulator. The temperature was changed by varying the currents through the Peltier element with a current control (Temp. control, Mectron). The saline temperature in a chamber was monitored with a thermocouple.

The experimental chamber was shielded from electrical interference using a Faraday cage and from mechanical disturbance by an air table (Technical Manufacturing Corporation).

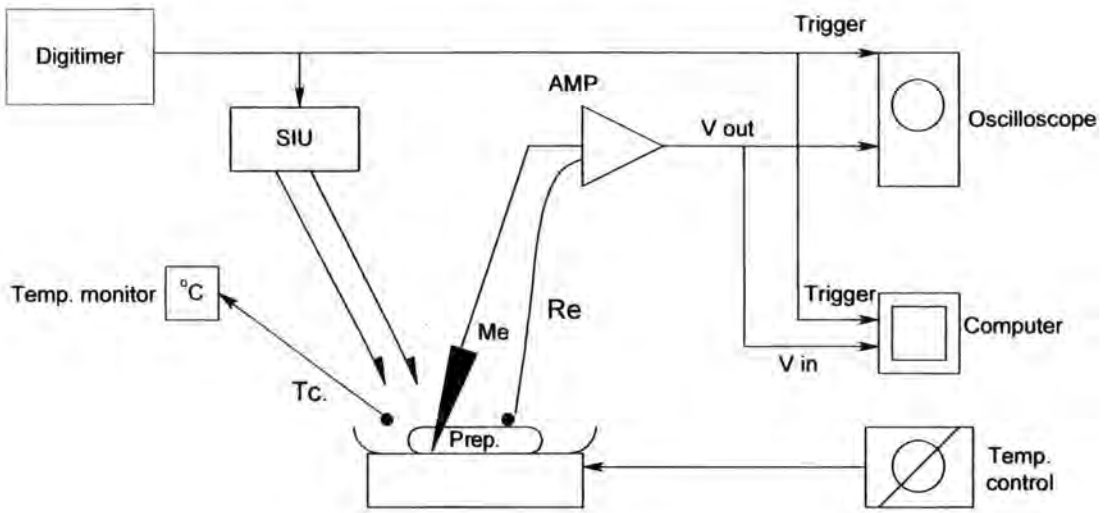


Figure 2.1: experimental set-up.

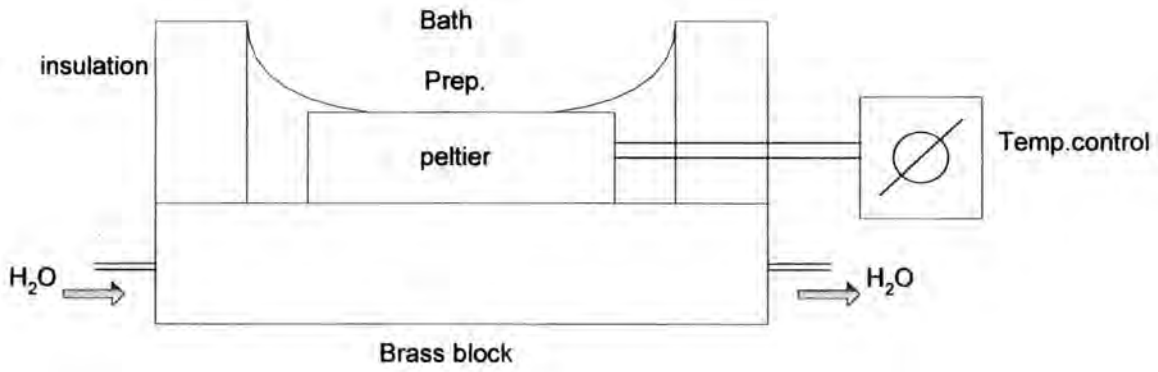


Figure 2.2: Peltier junction.

- **Digital thermometer**: experimental chamber was cooled with a Peltier element and the temperature was changed by varying the currents through the Peltier element with a current control (Mectron). The hot junctions of the Peltier were cooled by contact with a brass block. Cooled water was pumped through this block using a Geneline cooler/circulator. Measurement of the preparation temperature was carried out with a thermocouple placed in the saline solution close to the leg.
- **Microscope**: Dissections were carried out and placement of stimulation electrodes and insertion of microelectrode were carried out using a dissection microscope.

2.4.2 Recording Protocol.

- The immobilized leg was pinned in a small plastic chamber, the floor of which was covered by Sylgard (Dow Corning), and was covered with aerated saline solution.
- Successful penetration of muscle fibres was not observed visually but was indicated by the sudden appearance of a negative resting potential on advancement of the microelectrode tip into the superficial muscle fibre layer.
- Sixteen records of the excitatory junction potential (EJPs) were obtained at each temperature, then these sixteen records of EJPs were averaged. The range of measuring temperatures used was usually between 8 and 26°C.
- Stimuli applied to the nerve resulted in EJPs recorded from the muscle. EJPs were recorded in response to both single pulse and double pulse stimuli. The single pulse record of EJP was used to measure a variety of parameters displayed by the EJP. EJP's stimulated by a double pulse were used to measure facilitation. The interval between double stimuli was 40msec. The intervals were used between successive sets of records was 3sec to ensure that any possible facilitation from previous stimuli was no longer evident. Facilitation index was calculated as the difference in amplitude of the two evoked EJPs divided by the amplitude of the first EJP.
- An average of the mean EJP amplitudes, latent period, and decay constant values were determined and noted onto a computer using the SCAN synaptic current analysis program.

- All the data were then averaged and analysed using Excel spreadsheets and SPSS statistical program.

2.5 Heat shock treatments.

- *Carcinus maenas* acclimated at 8^0C were heat shocked at one of the following temperatures (30, 31.5, 33°C). These temperatures were chosen in relation to CTMax, around to below CTMax. Animals were transferred directly from 8°C to a heat shock temperature for 1h at 30, 31.5°C, and was 30min at 33°C. Following the period of heat shock the crabs were returned to their acclimation temperature for recovery during 1h.
- Following the recovery period, walking leg muscle tissue samples were collected from each group, mixed with 5ml of artificial seawater (saline solution), and then frozen immediately in liquid nitrogen, and stored at -80°C, chela and swimming legs were not used.
- For control animals, leg muscle tissue samples were collected from non-heat shocked animals acclimated at the same conditions, according to the same protocol.

2.6 Critical thermal maximum, (CTMax), before and after heat shock.

Animals were exposed to high temperatures to determine CTMax using a separate temperature controlled tank. The volume of water in the tank was 11L, and was controlled using a Techne heater model TU-20C Tempunit.

CTMax was defined as the temperature at which crabs did not show a righting reflex. Adult *Carcinus maenas* were used to determine the CTMax, size was chosen 65-85 mm carpus width. Crabs were acclimated at 8°C before their CTMax was determined. The protocol was used as follows:

- Three groups of crabs were used. Each group was contained 4 crabs.
- The first group was placed in seawater at 20°C and was then slowly heated at a rate of 0.2°C per min using a programmed heater (Techne).

- The righting reflex was tested every 1°C rise in temperature by turning the crab onto its back by hand, and 5mins was allowed to right itself.
- The CTMax was recorded for each crab at the temperature at which a crab was not able to right itself within 5mins.
- Then the crabs were transferred back into seawater at 8°C to recover.
- The protocol was repeated with other groups.
- The average CTMax was calculated using the pooled data for all 12 crabs.
- The effect of heat shock on subsequent CTMax was measured as follows:
- Three groups of animals not previously heat shocked of *Carcinus maenas* were chosen, each group contained 9 animals and the carpus width was between 65-95 mm. They were heat shocked at temperature 1°C lower than the CTMax value (32°C) for 20mins, followed by either 1h (9 animals), 2h (9 animals), or 4h (9 animals) recovery at 8°C.
- CTMax after this heat shock was determined in each group as described above.
- Additional group of animals was subjected to osmotic stress using 75% seawater + 25% distilled water.

2.7 Measurement of EJP after heat shock.

Electrophysiological microelectrode recordings of excitatory junction potentials (EJP) were measured in control and heat shocked animals as follows:

- Animals were placed directly from seawater at 8°C into seawater at 32°C for 30min, followed by recovery at 8°C.
- The EJP's after heat shock were measured in the recovery period using the protocol described in electrophysiological procedures.
- During the recovery period legs were rapidly dissected and prepared for microelectrode recording. All measurements were taken up to 90mins from the end of heat shock.

2.8 Muscle force measurements.

Muscle force was measured on homothermally acclimated *Carcinus maenas* either at 8°C or 25°C, and in each case with either short day or long day photoperiod. Muscle force was also measured in pre and post heat shocked crabs at 8°C. The crabs were all adults of comparable size. An isometric transducer unit was used for the measurement of the closer muscle force. A small window was drilled in the propodite above the dactylopodite opener muscle, and then it was damaged (by tweezers), and then the tip of the dactylopodite connected to the transducer. Muscle force measurements were then made at different temperatures increasing from 8°C until failure of contraction. The nerve was stimulated at different frequencies of 10, 20, and 50Hz as increased force was generated with increasing frequency of stimulation. The interval between bursts was 3secs.

2.9 Heat shock protein (HSP).

2.9.1 Sample preparation for heat shock protein analysis.

- Muscle tissue was thawed on ice and placed into 1.5ml pre-weighed Eppendorf tubes. Tissue weight in each sample was measured and 1volume of homogenizing buffer (0.01M Tris-HCl, pH6.8) with protease inhibitors added for each 1volume of sample.
- Samples were homogenized using hand-held homogenizer on ice.
- A volume of Phenylmethyl-sulphonyl fluoride (PMSF) stock (10mM in isopropanol) was added to give a final concentration of 100µg/ml PMSF (60µl stock PMSF for 1ml of homogenizing buffer).
- Homogenates were centrifuged at 12000rpm and 4°C for 30mins.
- The supernatant was transferred into new tubes and the amount of protein measured for each sample using the Bradford method (Bradford, 1976).
- The remaining aliquots were frozen at -80°C.
- Supernatants were mixed 1:2 with sample loading buffer (0.5M Tris-HCl, pH6.8, glycerol, 10%SDS, 0.5%bromophenol blue, B-Mercaptoethanol), boiled for 5mins, and centrifuged for 2mins in a bench Eppendorf centrifuge.

- Supernatants were collected and applied to the gel for SDS-PAGE.
- Samples were loaded onto the gel and separated at 200V for 45-60 minutes. An aliquot of prestained molecular weight standards solution (30,000-120,000Da) on gels to be stained with Coomassie blue.

2.9.2 Equipment.

Gel electrophoresis was carried out using Mini-PROTEAN II Electrophoresis Cell (Bio Rad).

2.9.3 Reagent.

a. Bradford.

The protein content of different samples was measured using the Coomassie blue method of Bradford (1976) with Bovine Serum Albumin as a standard.

b. Homogenizing buffer.

Homogenizing buffer consisted of 0.01MTris-HCl, (pH6.8) with protease inhibitors (aprotinin 2µg/ml, antipain 2µg/ml, leupeptin 2µg/ml, pepstain 1µg/ml), and 1volume of homogenizing buffer was added to the sample weight (1/1w/v).

c. PMSF (Phenylmethyl-sulphonyl fluoride).

A volume of PMSF stock (10mM) was also added to the sample to give a final concentration of 100µg/ml (60µl) stock PMSF for 1ml of homogenizing buffer).

d. Gel electrophoresis.

Proteins were separated using polyacrylamide gel electrophoresis SDS-PAGE (Laemmli, 1970).

The gels used were: resolving gel 8% and stacking gel 5%, with the monomer solutions (5%, 8%) prepared as follows:

Percentage of gel	DDI H_2O (ml)	30% Degassed acrylamide Bis-AA solution (ml)	Gel buffer	10%SDS (ml)
5%	5.7	1.7	2.5ml of 0.5M Tris-HCl,pH6.8	0.1
8%	2.3	1.3	1.3ml of 1.5M Tris-HCl, pH8.8	0.05

All reagents were mixed well. Immediately prior to pouring each gel the following were also added:

Percentage of gel	10%APS (ml)	TEMED (ml)
5%	0.05	0.01
8%	0.05	0.003

The mixtures were swirled gently to initiate polymerization The stacking gel was poured first and allowed to set for 45-60 min (with a layer of distilled water on top), then the stacking gel, which also needed 45-60 min to set. The gel plates were assembled to form the upper buffer chamber, etc. as described in the Biorad SDS-PAGE manual.

2.10. Data analysis.

Data were averaged using the SPSS statistical software and analysed for significance using the two sample F-test, two sample t-test, regression of slopes, ANCOVA and ANOVA. Results were graphed using the Excel software.

Chapter Three

Effect of temperature and photoperiod on neurophysiological properties of neuromuscular transmission in *Carcinus maenas*.

Introduction.

Temperature is one of the most important abiotic stresses because it affects many biological processes at all levels of biological organization, from the whole organism to molecular processes (Hochachka and Somero, 1984; Somero, 1997). In endotherms, animals which maintain relatively constant body temperature, the nervous system operation is to a large extent protected from variations in the physical characteristics of the environment such as temperature. However, in ectotherms species their internal temperatures vary as ambient temperature fluctuates (Robertson *et al.*, 1996). They live in environments with dramatic thermal fluctuations at both short (daily) and long term (seasonal). They may survive in wide ranges of temperatures by behavioural or compensatory mechanisms that allow them to function within a wide range of temperatures (Prosser and Nelson, 1981; Cossins and Bowler, 1987). The compensatory effects are adaptive to maintain the relative constancy of the vital functions in spite of changes in the environmental temperature (Lagerspetz, 1974). Acclimation and acclimatization are compensatory, reversible phenomena that result in adaptive changes that are considered to contribute to increased fitness to the new conditions (Cossins and Bowler, 1987). In ectotherms, acclimation and acclimatization contribute to the maintenance of the constancy of vital processes, but not to the maintenance of the constancy of the internal environment (Prosser, 1969). In many cases animals are able to make physiological and biochemical adjustments when the seasonal temperature changes to maintain fitness over the range of temperatures through the processes of acclimatization (Cossins and Bowler 1987). Within limits, animals are capable of compensating for high body temperature by acclimation (in the laboratory), acclimatization (in field) and adaptation (over evolutionary time). Adaptive

change in a function is related to an adaptive change in the system controlling that function. In multicellular animals, the control systems are the nervous system and endocrine system which show a degree of mutual interaction (Lagerspetz, 1974).

Temperature acclimation affects many life functions of animals. The direct effects of temperature on the various functions of organisms result in compensatory effects, which are adaptive to maintain the relative constancy of the functions in spite of the changes in the environmental temperature (Lagerspetz, 1974). Changes in environmental temperature may result in response of organism's behaviour and metabolism/ physiology (Prosser and Heath, 1991; Cossins and Bowler, 1987). It is possible that many of the effects of temperature acclimation are mediated by the nervous system. It seems to be the most sensitive site to temperature changes, and the primary organ of adaptation to the environment. In addition, there is the possibility of temperature compensation through the recruitment at different temperatures of neuronal populations with different, either fixed or variable temperature characteristics (Lagerspetz, 1974). The nervous system often fails at lower temperatures than other systems and the resulting behavioural failures occur at lower temperature than protein inactivation. (Prosser and Nelson, 1981). Conduction, transmission and neurosecretion are three functions of the nervous system at the cellular level. They undergo changes during temperature acclimation, which may compensate for the direct effects of temperature on the nerve cells (Lagerspetz, 1974). Temperature change has a major impact on the function of the nervous system and its components, including altering synaptic gain and changing synaptic and conduction delays. Harri and Florey (1977) studied the effects of temperature on neuromuscular transmission in the closer muscle of walking legs of the crayfish *Astacus leptodactylus*. They found that rapid alteration in temperature over a range of 6-30°C provoked changes in membrane potential, and changes in amplitude, time course, and facilitation of the post-synaptic muscle junction potential.

There have been numerous studies of temperature acclimation in the nervous system of different animals (for review see Lagerspetz, 1974). These studies have been done at different acclimation temperature and different acclimation periods, either in intact animals and isolated preparations; this makes a comparison difficult because of the

different conditions under which the experiments carried out. Roots (1968) and Roots and Johnston (1968) have further studied the effects of temperature acclimation on the phospholipids of the goldfish brain (*Carassius auratus*) acclimated to 5, 15, 25 or 30°C. They found that acclimation involves a change in the degree of unsaturation of the fatty acids of phospholipids, which increased when the acclimation temperature decreased. In general, ectothermic animals change their membrane characteristics to maintain fluidity appropriate to the local temperature, to prevent temperature effecting animal; this phenomenon had been called homeoviscous adaptation (Sinensky, 1974). The ionic conductance determines the resting potential (RP) and the cell activity. For example, The RP is determined by the selective permeability of plasma membranes to ions (i.e. Na^+, K^+ and Cl^-). At rest, muscle membranes are relatively permeable to K^+ and Cl but relatively impermeable to Na^+ (Hill, 1992). Thus the RP changes are mainly K^+ driven changes whereas Cl^- generally stabilizes the muscle fibre RP (Hill, 1992). At rest the ionic concentration gradients are kept relatively steady by active transport utilizing Na^+/K^+ ATPase. Lagerspetz *et al.*, (1973) studied the effect of temperature on the nerve cord from earthworms (*Lumbricus terrestris*) acclimated to 14 or 25°C. It was found that the Na^+/K^+ ATPase activity showed a higher activity at 14°C (acclimated) than 25°C (acclimated) which was interpreted as compensatory acclimation at low temperatures. This compensates for the fact that the Na^+/K^+ ATPase activity would be reduced at low temperatures due to the direct effect of temperature. Thus the subsequence change in activity of the enzyme is an acclimatory response. Tanaka and Teruya (1974); Kimelberg and Papahadjopoulos (1974) suggested that increased unsaturation of the fatty acids in the reacting phospholipids also makes the activity of Na^+-K^+ ATPase higher at low temperatures. Such changes in membranes may also affect the time relations of the changes in the ionic conductance and thus the time course of membrane potential changes such as the propagation of the action potential and the time course of synaptic potentials (Lagerspetz, 1974). Many studies have been carried out on crustaceans (for example, Harri and Florey, (1979); Orkand, (1962); Atwood *et al.*, (1994); Fischer and Florey, (1981); Cooper *et al.*, (1995); Kivivuore and Lagerspetz, (1982); White, (1983); Stephens and Atwood, (1982); Stephens, (1985); Kivivuori *et al.*, (1990)). Including crabs, (Cuculescu (1996); Stillman

and Somero, (1996); Stephens and Church, (1988). Pearson (1998) carried out both homothermal (the whole animal acclimated at one temperature, either 8°C or 22°C) and heterothermal (the animal with one part of its body 22°C whilst the other part was kept 8°C) acclimation in *C. maenas* and *C. pagurus*. In these experiments the crabs were heterothemally acclimated with the periphery (legs) acclimated to a different temperature than the central CNS. The purpose of these experiments was to establish whether acclimation in peripheral structures were influence by the independent acclimation temperature of the CNS. The electrophysiological parameters of dactylopodite closer muscles of walking legs on nerve stimulation were measured between 6 and 26°C. He found that the acclimation was dependent on local tissue temperature and was relatively independent on CNS or hormonal influence, except possibly at high CNS temperatures.

Photoperiod is another important abiotic factor of the environment and will depend on the latitude. In most latitudes there are seasonal changes in the length of the photoperiod but at the equator the day lengths are approximately 12Light:12Dark through the year. Whereas towards the Polar regions the variation in the photoperiod is much more extreme. There is a relationship between photoperiod and the onset of breeding behaviour, growth, reproduction, feeding or other activity may be response to the photoperiod (Crear *et al.*, 2003). Brett (1989) and Berry (1997) studied the activity of lobsters under different photoperiods and determined that activity was significantly influenced by photoperiod. Most activity occurred during the dark period, this suggested that a long photoperiod may support better growth because activity and use of energy is decreased and more energy can be directed towards growth (Berry, 1997). Regulation of activities according to the different seasons may be difficult for many organisms in temperate or higher latitudes, where conditions for feeding, survival and reproduction vary through the year. Little relevant work has been published on the interacting or combined effect of photoperiod and temperature on neurophysiological properties. However, there are several studies of photoperiod regulation of growth; survival and reproduction in other aquatic species. Crear *et al.*, (2003) have studied the effect of photoperiod on growth, survival, colour and activity of juvenile southern rock lobster (*Jasus edwardsii*). Gardner and Maguire (1998) have studied the effect of photoperiod and light intensity on survival, development and

cannibalism of larvae of the Australian giant crab (*Pseudocarcinus gigas*). They found that survival of *Pseudocarcinus gigas* larvae was not significantly affected by photoperiod or light intensity. But development was affected by photoperiod with smallest zoeas in the continuously dark treatment, whereas all other treatments were similar. Circadian and photoperiod are two slightly different things. For day length, all days are of the same duration (24hours), but animals may start to breed as a result of increasing the light phase compare with the dark phase during the day length. So within these 24hours period the time which remains light can trigger of a variety different responses.

Temperature and light are often considered major exogenous determinants of the breeding cycles of crustaceans and other animals (Barnes, 1963). In insects, the effect of photoperiod was studied on the size-temperature relationship in a pentatomid bug (*Dolysoris baccarum*) it was found that the size-temperature relationship was not stable but was highly affected by the photoperiod, and there were variations also in the developmental period and the growth rate (Nakamura, 2002). The question is how photoperiod may affect the activity of animals independent of temperature. In many previous studies of temperature acclimation the photoperiod either was not controlled or stated. Some studies have not actually recognized that photoperiod may be a contributing factor, and was not controlled. Very few studies have been carried out on the two factors together. This is a significant omission as both average temperature and photoperiod change together with latitude. Photoperiod is likely to be additional to temperature change as a factor driving the attainment of seasonal acclimation. It is possible that photoperiod perhaps alters the attainment of acclimation to temperature as well as the extent. It is therefore planned to study whether the attainment of acclimation to temperature is modified by photoperiod. The work in this chapter was to identify the photoperiod effects on the extent of thermal acclimation by comparison of crabs subject to long and short photoperiod in combination with thermal acclimation to either low or high temperatures.

The shore crab *Carcinus maenas* is one of the most extensively studied of all intertidal animals. They live in environments with daily and seasonal fluctuations of temperature and photoperiod. Because average temperature and photoperiod (day length) are related it might be expected that both have an influence on the acclimatory responses of

tissues. In the present study, the experimental design was to compare the acclimation responses under different photoperiod, either short day photoperiod (8:16 L:D) or long day photoperiod (16:8 L:D) from animals acclimated either low (8°C) or high temperature (25°C). Within the nervous system in the adult animal, the most important functional processes are synaptic transmission. In this chapter we examined in the laboratory the effects of temperature and photoperiod in the leg neuromuscular synaptic transmission to see whether there interaction between photoperiod and the acquisition of temperature acclimation.

Methods.

The effect of different acclimation and photoperiod were studied in *Carcinus maenas*. For dissection and electrophysiological procedure see Chapter 2 (sections 2.3 and 2.4.2). Briefly, animals acclimated to either 8°C and 25°C at long day photoperiod (16:8 L:D) and short day photoperiod (8:16 L:D) for 3 weeks. Crabs acclimated at 25°C were initially started at 8°C for 3 days and then increased to 12°C for a further 3 days. At subsequent three day intervals the temperature was increased to 16°C then 20°C and finally 25°C. Animals were acclimated at this final acclimation temperature for at least three weeks. After this acclimatory period the leg nerve bundle was exposed and the part containing the tonic axon supplying the closer muscle was isolated by splitting the leg nerve. The each nerve bundle was stimulated at the lowest voltage and the voltage increased until the dactylopodite movement was observed. This method of stimulation was used to identify the tonic motor axon (low threshold). After muscle fibre impalement, axon action potentials were stimulated and resting potential (RP), excitatory junction potential amplitude (EJP), latent period, decay time constant and facilitation were recorded from the muscle using intracellular microelectrodes. For all experiments, bath or leg temperature was controlled using Peltier unit, driven by a standard temperature control circuit (see Fig 2.2). Temperatures were measured directly adjacent to the axon with a thermocouple and the range of temperatures used to measure the electrophysiological characteristics were between 8- 26°C.

Results.

Resting membrane potential (RP).

The effect of temperature on resting membrane potential (RP) was measured in two ways. First with increasing temperature from 8-26°C and secondly with decreasing temperatures from 26-8°C in order to see if is there any difference. The result shows that there is no difference between them and all the experiments were usually using increasing temperature. Figure 3.1 shows RP change in short day photoperiod between 8°Cand 25°C acclimation. It can be seen that the RP hyperpolarized with increasing temperature in both cold and warm acclimation, analysis (Regression) of the data showed no significant difference between them ($p>0.05$). But analysis (ANCOVA) of the data showed a significant difference between them ($p<0.01$). The RP changes with temperature for both cold and warm acclimated animals were 1.6mV/°C and 1.2mV/°C respectively, significantly greater than that predicted by the Nernst equation (0.3163 mV/°C). These slopes were not obtained from the regression line but calculated from the data points at 10°C and 20°C because the relationship flattens out above 20°C. These two values of the slopes of the RP relationship of acclimation short day crabs were similar but the relationship from warm acclimated crabs was displaced to the right. The RP of cold acclimated crabs was more hyper-polarized than warm acclimated group, RP at warm acclimated crabs was hyperpolarized through range up to 26°C whereas cold acclimated crabs were relatively an affected by acute temperature changes above about 20°C. The differences between RP values indicated an acclimatory shift (Cossins and Bowler 1987) but it is not very large. The acclimatory shift was calculated from the horizontal displacement of the relationships of RP versus temperature. This value was expressed as a percentage of the displacement that would give complete compensation. It was estimated that cold acclimated crabs RP acclimatory shift was 13%. Figure 3.2 shows the RP-temperature function in animals acclimated with long day photoperiod at both 8°C and 25°C temperatures. RP hyperpolarized with increasing temperature in both low and high temperature acclimation. Analysis (Regression) showed a significant differences of RP

Figure 3.1: Resting membrane potential (RP) of leg muscles from *Carcinus maenas* acclimated at 8°C (solid diamonds) and at 25°C (light square) with a short day (8:16 L:D) photoperiods. Data is presented as mean ± S.E. mean; numbers of experiments were, 25°C (n= 20), 8°C (n= 25). For compare using comparison of the slopes were found to be statistically not significant (Regression, $P>0.05$). The differences in two data sets were found to be statistically significant (ANCOVA, $p<0.01$).

Figure 3.2: Resting membrane potential (RP) of leg muscles from *Carcinus maenas* acclimated at 8°C (solid diamond) and at 25°C (light square) with long day (16:8 L:D) photoperiods Data is presented as mean ± S.E. mean; numbers of experiments were, 25°C (n= 27), 8°C (n= 22). For compare using comparison of the slopes were found to be statistically significant (Regression, $P<0.01$).

Figure 3.1: Resting membrane potential (RP), changes in *Carcinus* leg muscles from animals acclimated at 8°C and 25°C with a short-day photoperiod (8hr light: 16hr dark).

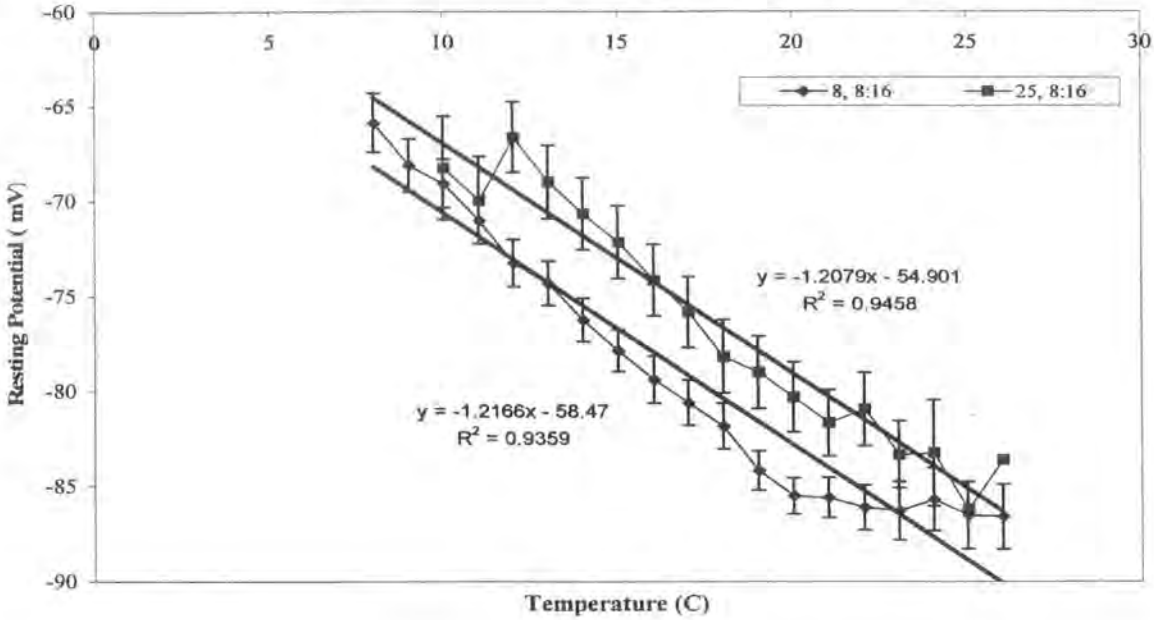


Figure 3.2: Resting membrane potential (RP), changes in *Carcinus* leg muscles from animals acclimated at 8°C and 25°C with a long-day photoperiod (16hr light: 8hr dark).

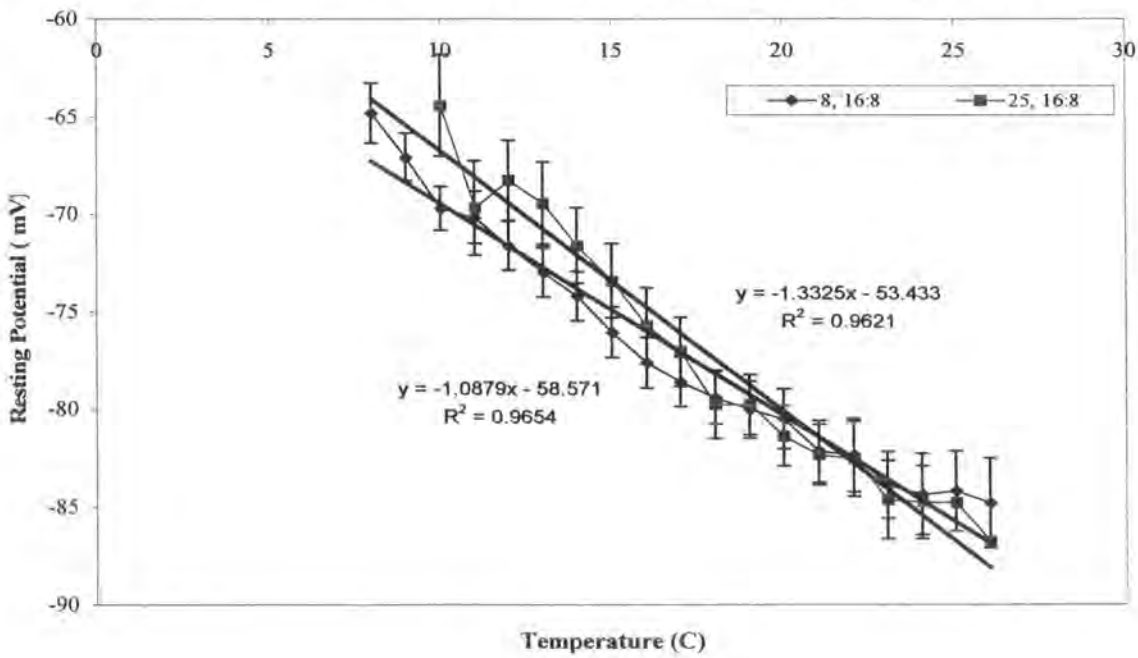


Figure 3.3: Resting membrane potential (RP) of leg muscles from *Carcinus maenas* acclimated to short day (8:16 L:D, solid diamonds) and long day photoperiods (16:8 L:D, light square) at 8°C. Data is presented as mean ± S.E. mean; numbers of experiments were, short day (n= 25), long day (n= 22). For compare using comparison of the slopes were found to be statistically significant (Regression, $P<0.05$).

Figure 3.4: Resting membrane potential (RP) of leg muscles from *Carcinus maenas* acclimated at short day (8:16 L:D, solid diamonds) and long day photoperiods (16:8 L:D, light square) at 25°C Data is presented as mean ± S.E. mean; numbers of experiments were, short day (n= 20), long day (n= 27). For compare using comparison of the slopes were found to be statistically not significant (Regression, $P>0.05$).

Figure 3.3: Resting membrane potential (RP), changes in *Carcinus* leg muscles from animals acclimated at 8°C with either short or long day photoperiod.

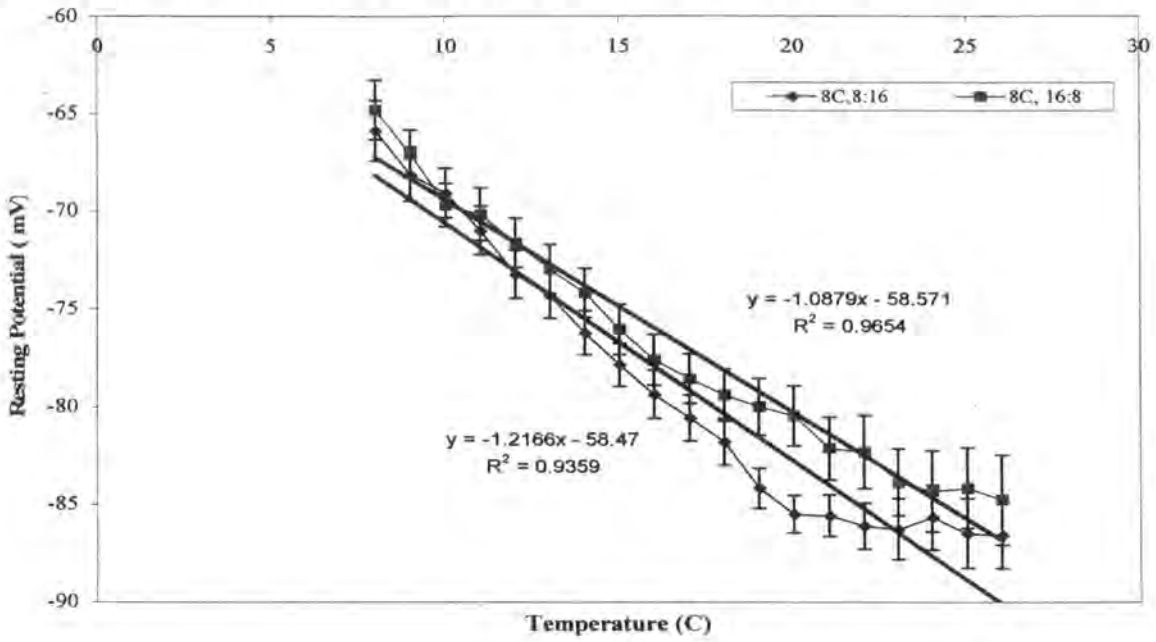
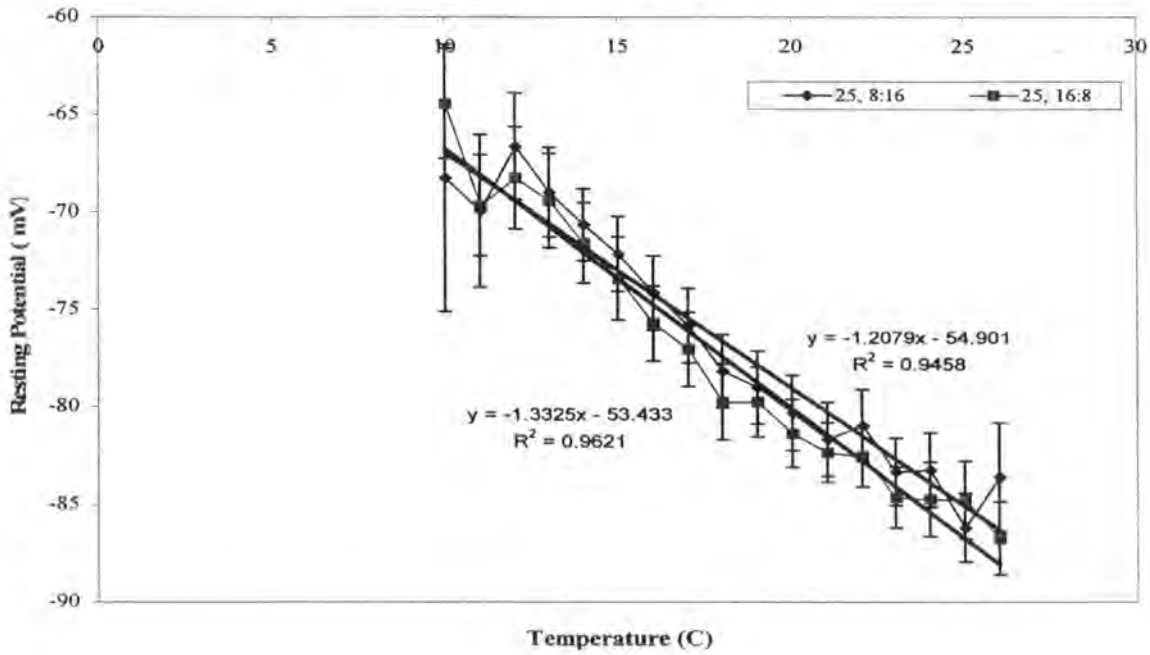


Figure 3.4: Resting membrane potential (RP), changes in *Carcinus* leg muscles from animals acclimated at 25°C with either short or long day photoperiod.



($p<0.001$) between cold and warm acclimation. In cold acclimated animals RP was more hyper-polarized at 8-17°C than warm acclimated animals. However, RP's from warm acclimated crabs between 16°C and 26°C were not different. RP change with temperature was 1.4mV/°C for 8°C, 16:8 L:D, whereas 1.7mV/°C for 25°C, 16:8 L:D. The RP changes with temperature for both cold and warm acclimated animals were significantly greater than that predicted by the Nernst equation (0.3163 mV/°C). It is clear that photoperiod appears to influence the extent of acclimation to low versus high temperatures. In order to reveal the effects of photoperiod on acclimation to either low or high temperatures the data were plotted in Figures 3.3 and 3.4.

Figure 3.3 presents resting membrane potential (RP) change with experimental temperature (8-26°C) from 8°C acclimation at short and long day photoperiod. It can be seen that RP hyperpolarized in both short and long day photoperiod with increasing temperatures, and the RP was more hyper polarized in the short day photoperiod especially noticeable at higher experimental temperatures. Analysis (Regression) showed a significant differences of RP ($p<0.05$) between short and long day photoperiod. As seen with long day photoperiod acclimated crab the RP curve shifted to a right compared with animals acclimated to the same temperature but with short day photoperiod. The differences between RP values indicated an acclimatory shift (Cossin and Bowler 1987). The acclimatory shift was estimated to be 13.6%. Thus, in cold acclimated animals a change in the photoperiod from short day to long day resulted in a shift of the RP versus temperature relationship, with the data for long day photoperiod being displaced to the right along the temperature axis. In Figure 3.4 both short and long day photoperiod from 25°C acclimated crabs exhibited hyperpolarizing RPs with increasing experimental temperatures. Analysis (Regression) showed no significant differences of RP ($p>0.05$) between short and long day photoperiod.

Excitatory Junction Potential (EJP) amplitude.

Figure 3.5 shows EJP amplitude versus acute temperature relationships of animals acclimated at short day photoperiod with either 8°C or 25°C. It was generally found that EJP amplitudes decreased with increasing temperatures. EJP amplitudes of cold acclimated

Figure 3.5: Effect of temperature on EJP amplitude. All animals were acclimated using a short day photoperiod (8:16 L:D) and either at 8°C (solid diamonds) or at 25°C (light square). Data is presented as mean ± S.E. mean; numbers of experiments were, 25°C (n= 16), 8°C (n= 22). Statistical analysis (ANOVA) determined no significant differences between pairs of data points at each temperature ($p>0.05$), except at 14°C ($p<0.05$).

Figure 3.6: Effect of temperature on EJP amplitude. All animals were acclimated using a long day photoperiod (16:8 L:D) and either at 8°C (solid diamonds) or at 25°C (light square). Data is presented as mean ± S.E. mean; numbers of experiments were, hot (n= 16), cold (n= 22). Statistical analysis (ANOVA) determined no significant differences between pairs of data points at each temperature, ($p>0.05$).

Figure 3.5: Effect of temperature on EJP amplitude. *C. maenas* were acclimated using a short-day photoperiod (8hr light: 16hr dark) and either at 8°C or 25°C.

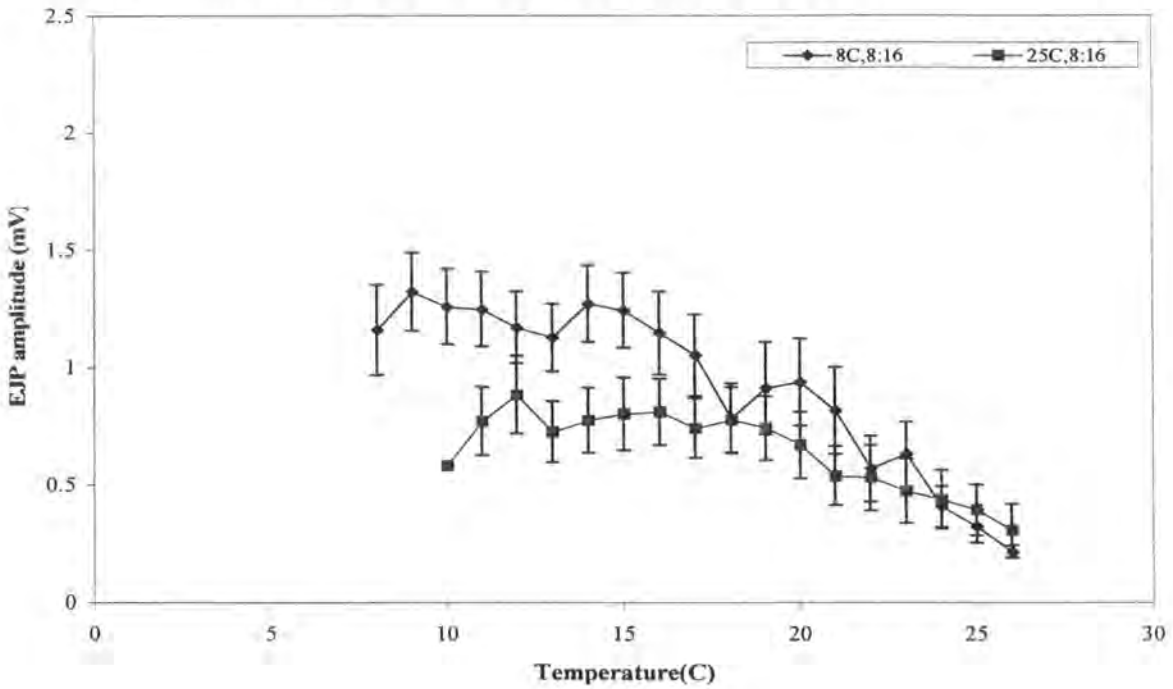


Figure 3.6: Effect of temperature on EJP amplitude. *C. maenas* were acclimated using a long-day photoperiod (16hr light: 8hr dark) and either at 8°C or 25°C.

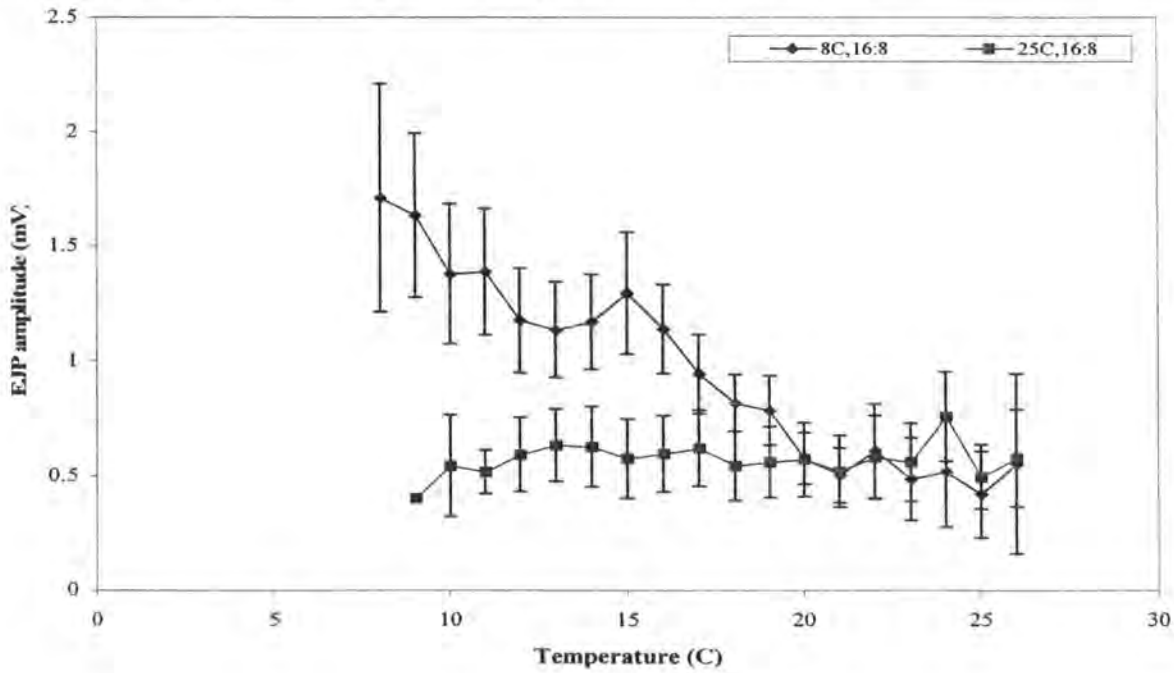


Figure 3.7: Effect of temperature on EJP amplitude. All animals were acclimated using a cold acclimation (8°C) and either a short day photoperiod (8:16 L:D, solid diamonds) or a long day photoperiod (16:8 L:D, light square). Data is presented as mean ± S.E. mean; numbers of experiments were, short day (n= 22), long day (n= 17). Statistical analysis (ANOVA) determined no significant differences between pairs of data points at each temperature, ($p>0.05$).

Figure 3.8: Effect of temperature on EJP amplitude. All animals were acclimated using a warm acclimation (25°C) and either a short day photoperiod (8:16 L:D, solid diamonds) or a long day photoperiod (16:8 L:D, light square). Data is presented as mean ± S.E. mean; numbers of experiments were, short day (n= 16), long day (n= 11). Statistical analysis (ANOVA) determined no significant differences between pairs of data points at each temperature, ($p>0.05$).

Figure 3.7: Effect of temperature on EJP amplitude. *C. maenas* acclimated at 8°C with short-day (8hr light: 16hr dark) and long-day (16hr light: 8hr dark).

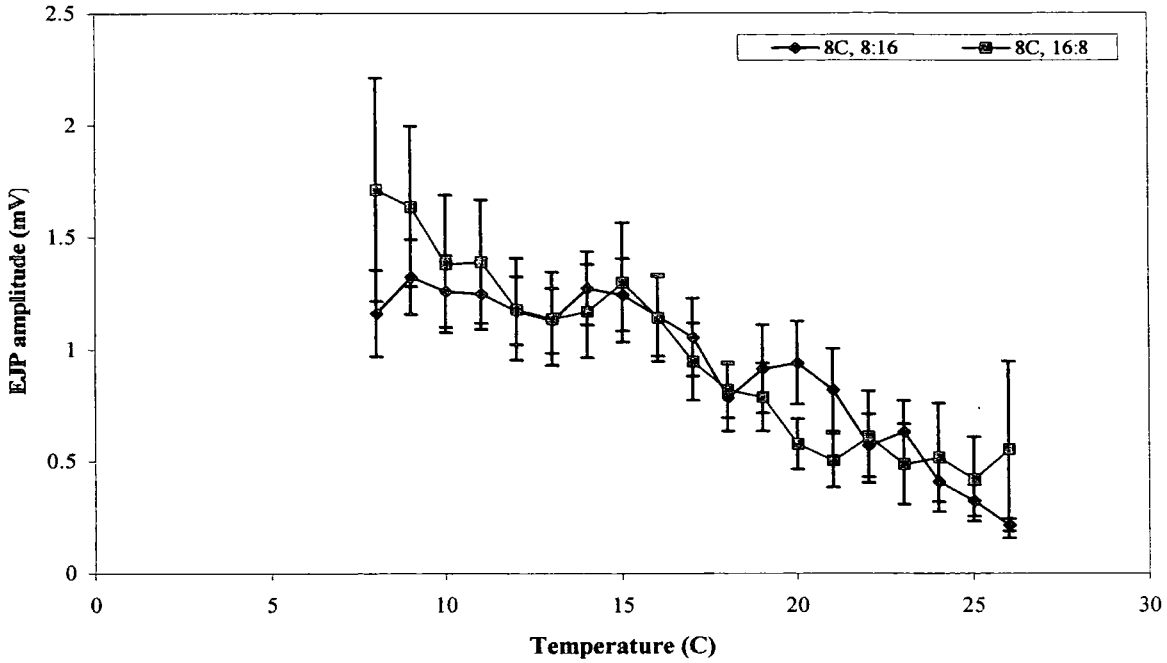
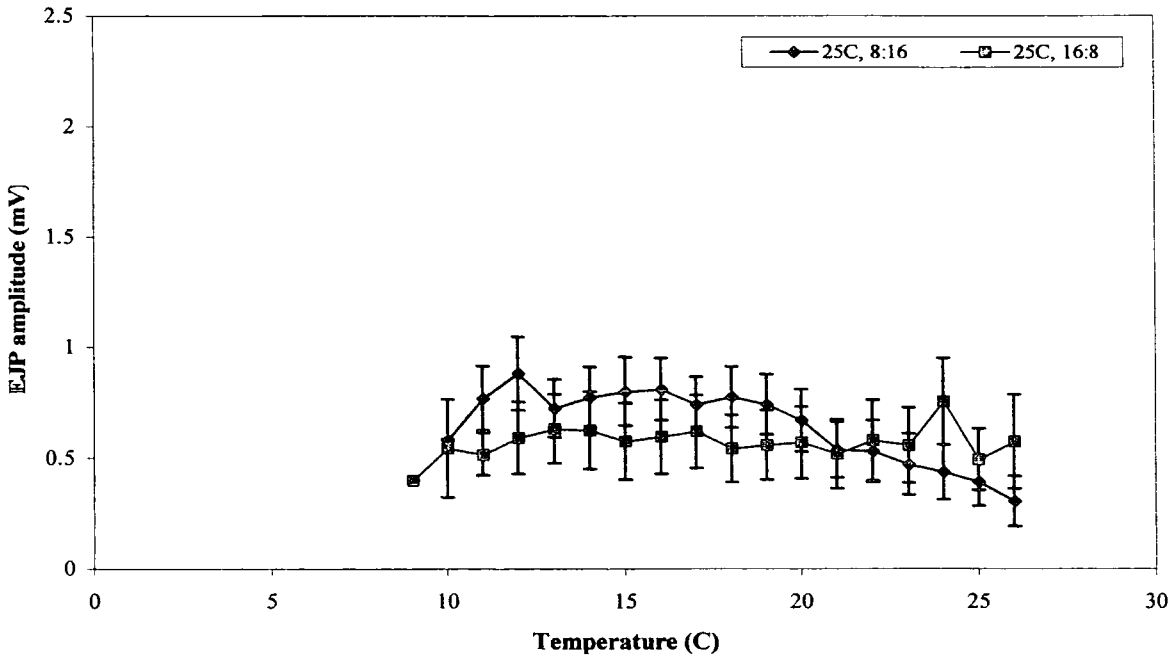


Figure 3.8: Effect of temperature on EJP amplitude. *C. maenas* acclimated at 25°C with short-day (8hr light: 16hr dark) and long-day (16hr light: 8hr dark).



animals at short day photoperiod decreased rapidly with increasing temperature over 9-13°C, 15-18°C, 21-22°C and 24-26°C. In comparison EJP amplitudes of warm acclimated animals at short day photoperiod were generally little affected by acute temperature change over 13-18°C but then decreased at higher temperature. EJP amplitudes were consistently larger in animals acclimated to 8°C compared with 25°C. With these differences being greater at lower acute temperature. Statistical analysis (ANOVA) revealed no significant differences between cold and warm acclimated crabs at short day photoperiod ($p>0.05$), except at temperatures 14°C ($p<0.05$). In animals acclimated to cold and warm with long day photoperiod, EJP amplitudes were shown in Figure 3.6. In cold acclimated crabs, EJP amplitudes decreased rapidly with increasing temperature over 8-22°C. In contrast EJP amplitudes from warm acclimated animals at long day photoperiod were generally constant over the whole experimental temperatures 8-26°C. Statistical analysis (ANOVA) showed no significant differences between cold and warm acclimation crabs at long day photoperiod ($p>0.05$).

The data presented in Figures 3.5 and 3.6 are replotted in Figures 3.7 and 3.8 in order to compare the effects of photoperiod at each acclimation temperature. Figures 3.7 shows EJP amplitude over the experimental temperature range (8-26°C) for short and long day photoperiod in 8°C *Carcinus maenas*. Statistical analysis (ANOVA) revealed no significant differences between short and long day photoperiod walking leg EJP amplitudes ($p>0.05$). EJP amplitudes from short and long day photoperiod with warm acclimated crabs are shown in Figure 3.8. Short day photoperiod and warm acclimated animals EJP amplitudes were changed little over 13-20°C but then decreased with increasing temperature. In comparison long day photoperiod and warm acclimated crabs EJP amplitude were generally constant over the whole experimental temperature (8-26°C). Statistical analysis (ANOVA) showed no significant differences between short and long day photoperiod at warm acclimation animals ($p>0.05$).

Latent period.

Figure 3.9 shows latent period of EJP of animals acclimated at short day photoperiod with either 8°C or25°C, all data was fitted to a log/linear plot. Figure 3.10

Figure 3.9: Effect of temperature on latency of EJP. All animals were acclimated using a short day photoperiod (8:16 L:D) and either at 8°C (solid diamonds) or at 25°C (light square). Data is presented as mean ± S.E. mean; numbers of experiments were, 25°C (n= 16), 8°C (n= 23). For compare using comparison of the slopes were found to be statistical significant (Regression, $P<0.01$).

Figure 3.10: Effect of temperature on latency of EJP. All animals were acclimated using a long day photoperiod (16:8 L:D) and either at 8°C (solid diamonds) or at 25°C(light square). Data is presented as mean ± S.E. mean; numbers of experiments were, 25°C (n= 11), 8°C (n= 17). For compare using comparison of the slopes were found to be statistical not significant (Regression, $P>0.05$).

Figure 3.9: Effect of temperature on latent period of EJP. *C. maenas* were acclimated using a short-day photoperiod (8hr light: 16hr dark) and either at 8°C or 25°C.

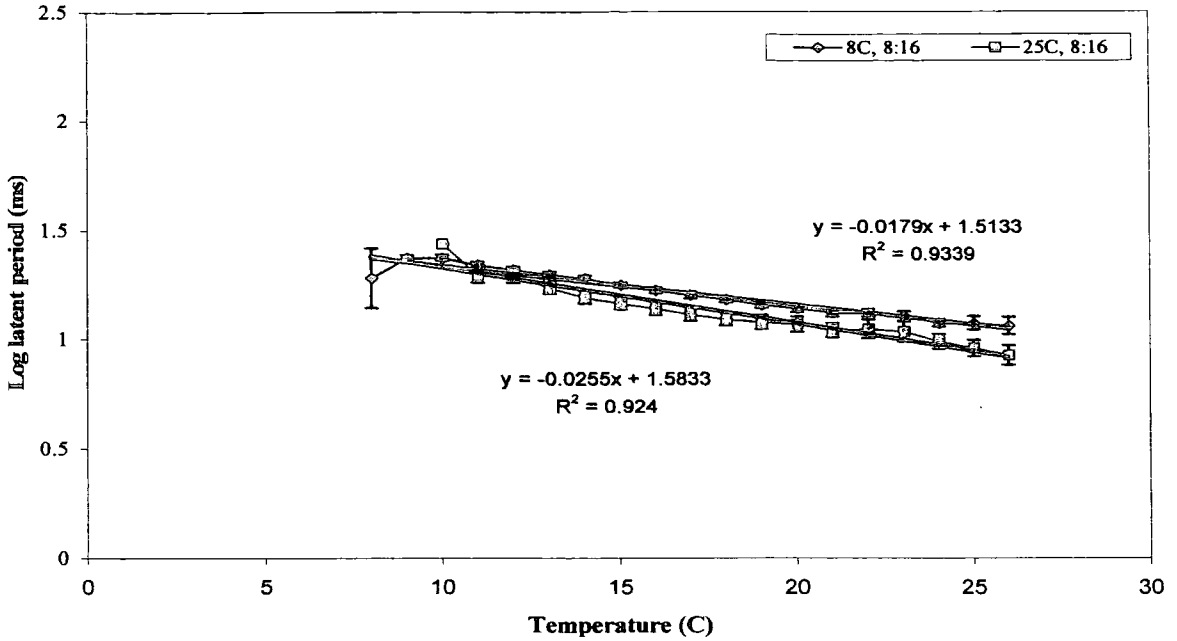


Figure 3.10: Effect of temperature on latent period of EJP. *C. maenas* were acclimated using a long-day photoperiod (16hr light: 8hr dark) and either at 8°C or 25°C.

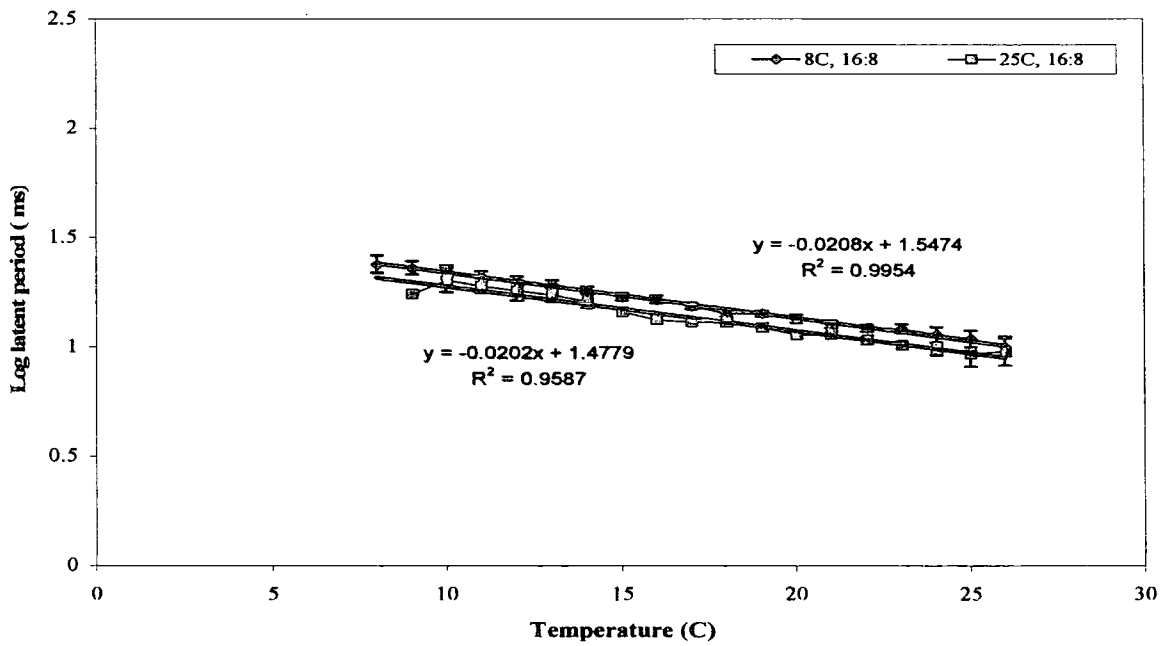


Figure 3.11: Effect of temperature on latency of EJP. All animals were acclimated using a cold acclimation (8°C) and either a short day photoperiod (8:16 L:D, solid diamonds) or a long day photoperiod (16:8 L:D, light square). Data is presented as mean ± S.E. mean; numbers of experiments were, short day (n= 23), long day (n= 17). For compare using comparison of the slopes were found to be statistical significant (Regression, $P<0.05$).

Figure 3.12: Effect of temperature on latency of EJP. All animals were acclimated using a warm acclimation (25°C) and either a short day photoperiod (8:16 L:D, solid diamonds) or long day photoperiod (16:8 L:D, light square). Data is presented as mean ± S.E. mean; numbers of experiments were, short day (n= 16), long day (n= 11). For compare using comparison of the slopes were found to be statistical significant (Regression, $P<0.05$).

Figure 3.11: Effect of temperature on latent period of EJP. *C. maenas* acclimated at 8°C with short-day (8hr light: 16hr dark) and long-day (16hr light: 8hr dark).

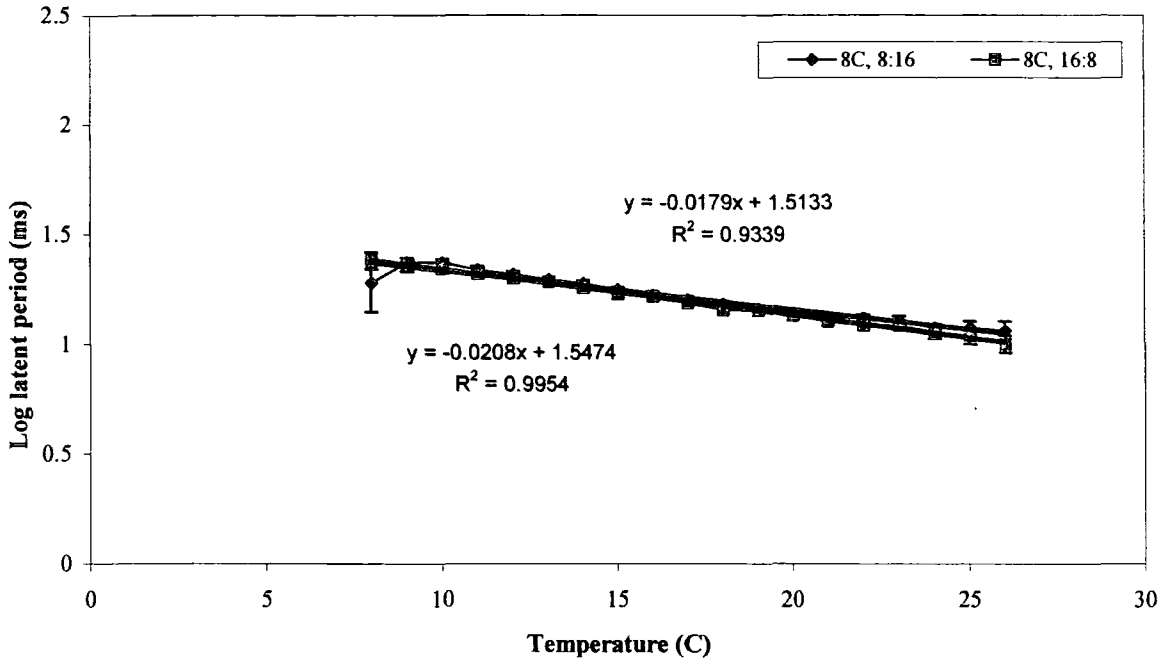
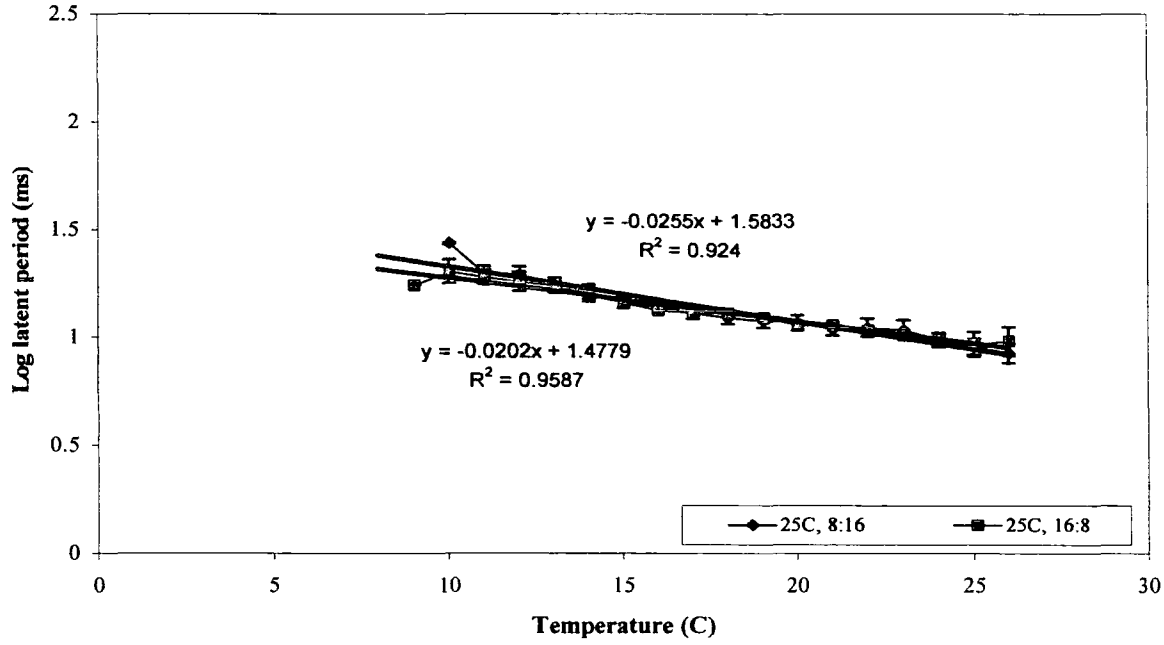


Figure 3.12: Effect of temperature on latent period of EJP. *C. maenas* acclimated at 25°C with short-day (8hr light: 16hr dark) and long-day (16hr light: 8hr dark).



shows the latent period of EJPs of animals acclimated at long day photoperiod with either cold or warm temperature. In both figures presented, the latent periods of EJP decreased with increasing temperatures. Statistical analysis (Regression) shows a significant difference between cold and warm acclimation on short day photoperiods ($p<0.01$) but not significant difference between cold and warm on long day photoperiods ($p>0.05$).

In order to compare the effects of photoperiod at either cold or warm acclimation temperature. Figure 3.11 shows latent period of EJP change with experimental temperature (8-26°C) of animals acclimated to cold with either short or long day photoperiod. Figure 3.12 shows latent periods of EJP of animals acclimated at warm with either short or long day photoperiod. It can be seen that in both Figures, the latent periods of EJP decreased with increasing temperatures. Statistical analysis (Regression) showed significant differences between short and long day photoperiods on both cold ($p<0.05$) and warm acclimations.

EJP decay time constant (Tau).

EJP decay constants were fitted with a least-squares exponential decay function using the SCAN program. Figure 3.13 shows the decay constant of EJP from animals acclimated at short day photoperiod with either cold or warm temperatures, all data were fitted to a log/linear plot. EJP decay time constant decreased with increasing experimental temperature. Tau from short day photoperiod at cold acclimated crabs was generally larger than that from crabs acclimated at short day photoperiod and warm. Statistical analysis (Regression) shows no significant differences between short warm and short cold acclimation ($p>0.05$). Figure 3.14 shows EJP decay time constant from animals acclimated at long day photoperiod with either 8°C or 25°C. Tau decreased with increasing experimental temperature and statistical analysis (Regression) showed significant differences between two groups ($p<0.05$).

In order to compare the effects of photoperiod at either cold or warm acclimation temperature. Figure 3.15 shows EJP decay time constants from animals acclimated to cold with either short or long day photoperiods. EJP decay time constants decreased with increasing experimental temperature. The decrease in Tau for both short and long day

Figure 3.13: Effect of temperature on decay constant of EJP. All animals were acclimated using a short day photoperiod (8:16 L:D) and either at 8°C (solid diamonds) or at 25°C (light square). Data is presented as mean ± S.E. mean; numbers of experiments were, 25°C (n= 16), 8°C (n= 23). For compare using comparison of the slopes were found to be statistical not significant (Regression, $P>0.05$).

Figure 3.14: Effect of temperature on decay constant of EJP. All animals were acclimated using a long day photoperiod (16:8 L:D) and either at 8°C (solid diamonds) or at 25°C (light square). Data is presented as mean ± S.E. mean; numbers of experiments were, 25°C (n= 11), 8°C (n= 16). For compare using comparison of the slopes were found to be statistical significant (Regression, $P<0.05$).

Figure 3.13: Effect of temperature on decay constant. *C. maenas* were acclimated using a short-day photoperiod (8hr light: 16hr dark) and either at 8°C or 25°C.

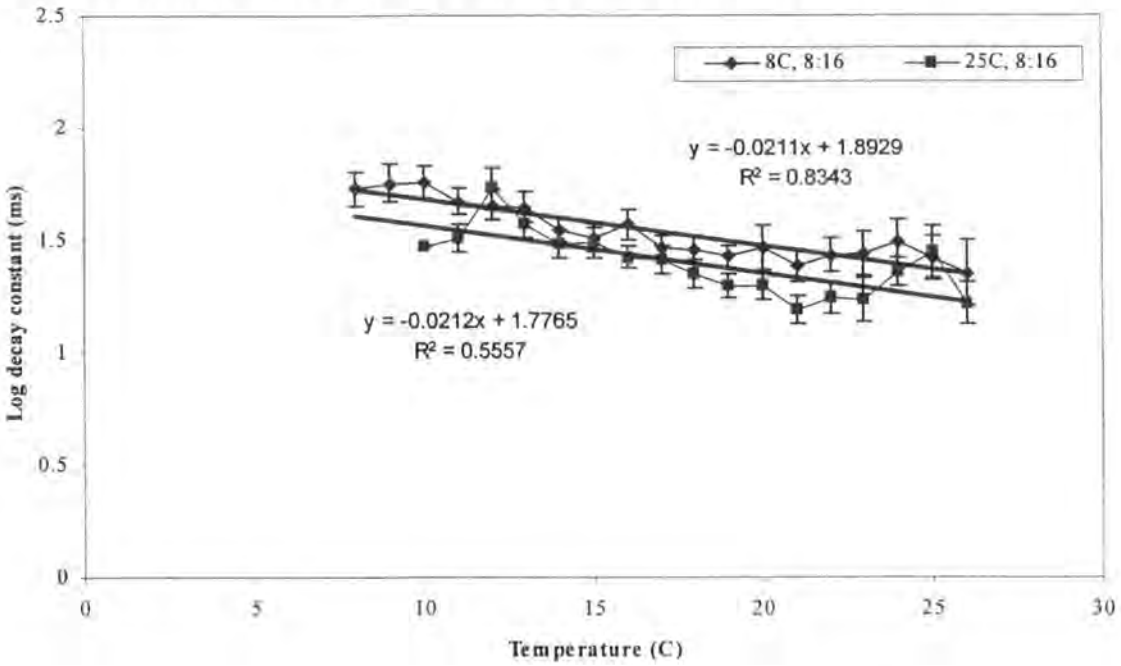


Figure 3.14: Effect of temperature on decay constant. *C. maenas* were acclimated using a long-day photoperiod (16hr light: 8hr dark) and either at 8°C or 25°C.

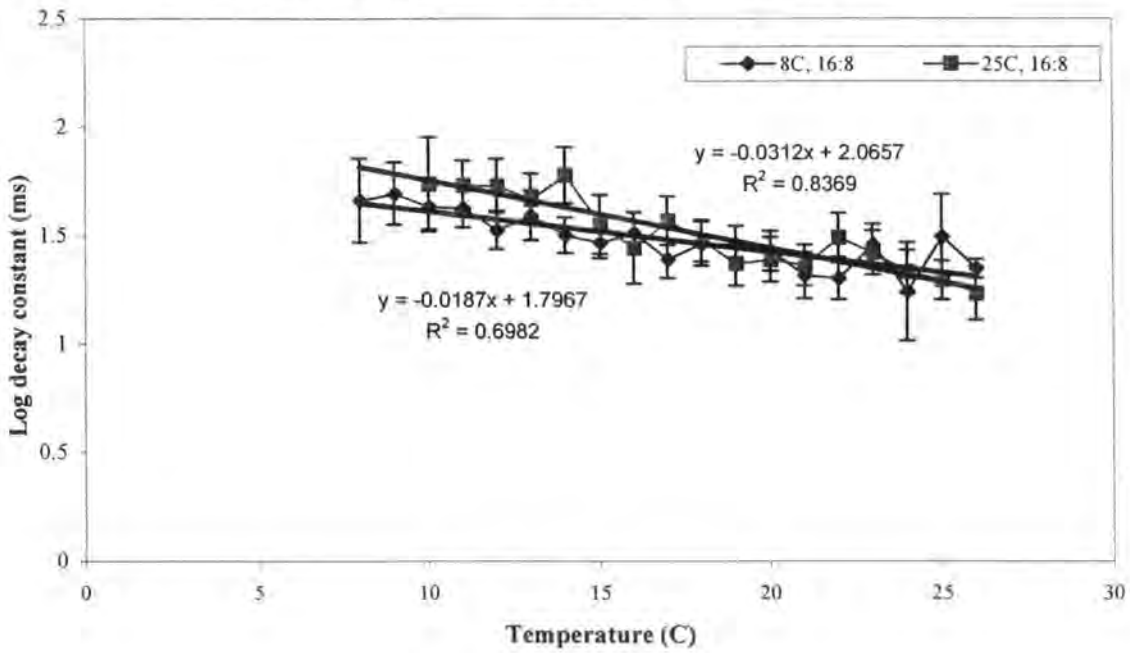


Figure 3.15: Effect of temperature on decay constant of EJP. All animals were acclimated using a cold acclimation (8°C) and either a short day photoperiod (8:16 L:D, solid diamonds) or a long day photoperiod (16:8 L:D, light square). Data is presented as mean ± S.E. mean; numbers of experiments were, short day (n= 23), long day (n= 16). For compare using comparison of the slopes were found to be statistical not significant (Regression, P>0.05).

Figure 3.16: Effect of temperature on decay constant of EJP. All animals were acclimated using a warm acclimation (25°C) and either a short day photoperiod (8:16 L:D, solid diamonds) or a long day photoperiod (16:8 L:D, light square). Data is presented as mean ± S.E. mean; numbers of experiments were, short day (n= 16), long day (n= 11). For compare using comparison of the slopes were found to be statistical not significant (Regression, P>0.05).

Figure 3.15: Effect of temperature on decay constant. *C. maenas* acclimated at 8°C with short-day (8hr light: 16hr dark) and long-day (16hr light: 8hr dark).

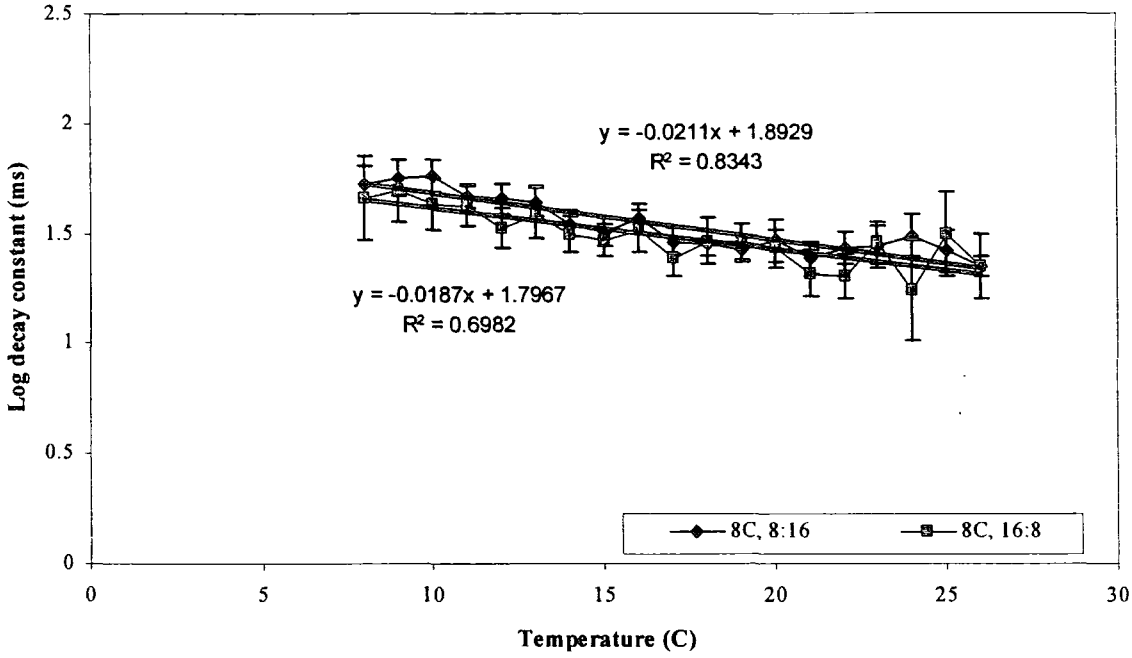
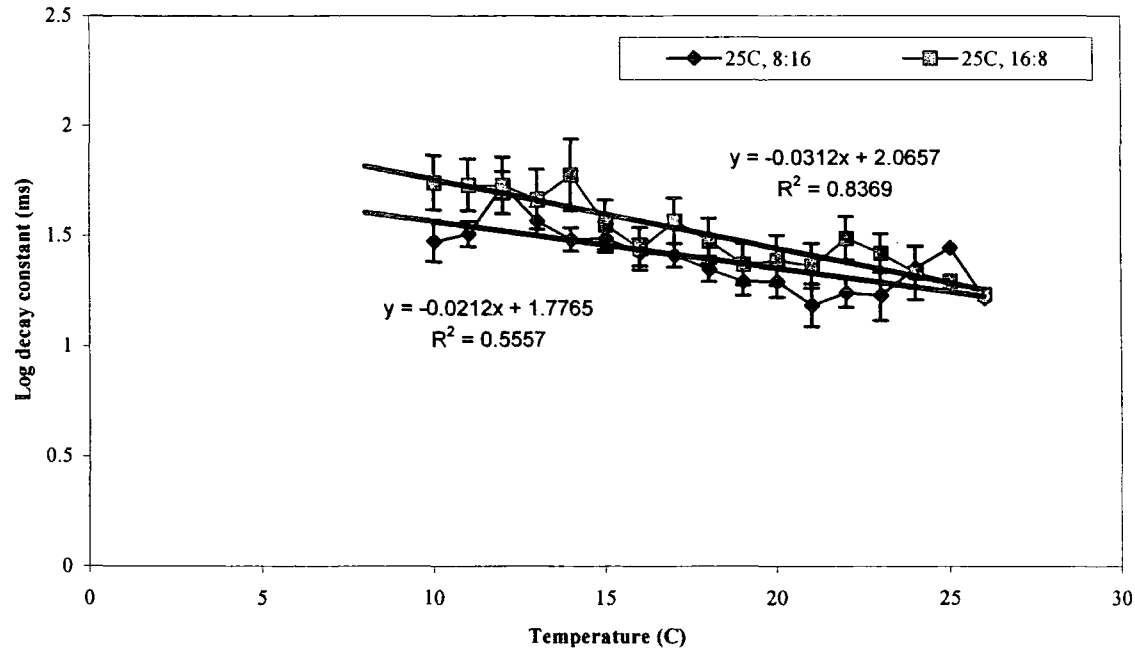


Figure 3.16: Effect of temperature on decay constant. *C. maenas* acclimated at 25°C with short-day (8hr light: 16hr dark) and long-day (16hr light: 8hr dark).



photoperiods was qualitatively and quantitatively similar. Statistical analysis (Regression) shows no significant differences between short and long day photoperiods ($p>0.05$) with cold acclimation. Although EJP decay time constant decreased by an experimental temperature increase, there was no statistical difference between short and long day photoperiod (Regression, $p>0.05$). This is shown in Figure 3.16 for warm acclimated crabs with either short or long day photoperiods. Tau was generally larger in long day photoperiod than short day photoperiod.

Facilitation.

Figure 3.17 shows EJP facilitation over experimental temperature in animals acclimated to 8°C and 25°C with short day photoperiod. In both cold and warm acclimated crabs it was found that there was little or no effect of temperature on EJP facilitation except possibly at high temperature over 20- 25°C. In comparison between two groups there was no significant differences (Regression) over the experimental temperature range ($p>0.05$). Figure 3.18 shows EJP facilitation in both 8°C and 25°C acclimated animals at long day photoperiod. It was found that in warm acclimated crabs there was no effect of temperature on EJP facilitation. The same result was found in cold group over 8- 21°C but over 21- 26°C EJP facilitation increased. There was a significant difference between two groups (Regression, $P<0.001$). EJP facilitation in warm acclimated animals was larger than cold acclimated animals.

In order to compare the effects of photoperiod at either cold or warm acclimation temperature. Figure 3.19 shows EJP facilitation changes over experimental temperature in animals acclimated to cold with either short or long day photoperiod. In both short and long day photoperiod it can be seen that there was no effect of temperature on EJP facilitation except possibly at higher temperature. There was an increase in EJP facilitation at high temperatures, over 21-26°C range. Short photoperiod crabs acclimated at 8°C had EJP facilitation values that were not significantly (Regression, $P>0.05$) larger than EJP facilitation in long photoperiod crabs acclimated at 8°C. Figure 3.20 shows EJP facilitation changes over experimental temperature in animals acclimated to warm with either short or

Figure 3.17: Effect of temperature on facilitation of EJP amplitude. . All animals were acclimated using a short day photoperiod (8:16 L:D) and either at 8°C or at 25°C. Data is presented as mean ± S.E. mean; numbers of experiments were ,25°C (n= 13), 8°C (n= 18). For compare using comparison of the slopes were found to be statistical not significant (Regression, P>0.05).

Figure 3.18: Effect of temperature on facilitation of EJP amplitude. All animals were acclimated using a long day photoperiod (16:8 L:D) and either at 8°C (solid diamonds) or at 25°C (light square). Data is presented as mean ± S.E. mean; numbers of experiments were, 25C (n= 10), 8°C (n= 11). For compare using comparison of the slopes were found to be statistical significant (Regression, P<0.001).

Figure 3.17: Effect of temperature on facilitation. *C. maenas* were acclimated using a short-day photoperiod (8hr light: 16hr dark) and either at 8°C or 25°C.

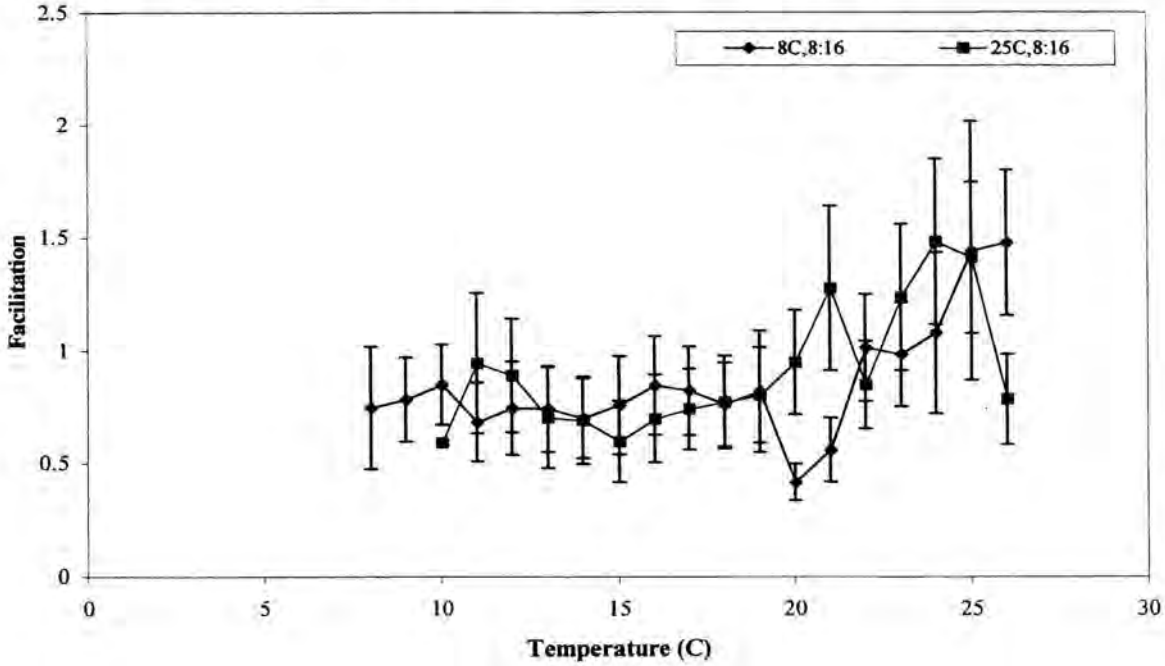


Figure 3.18: Effect of temperature on facilitation. *C. maenas* were acclimated using a long-day photoperiod (16hr light: 8hr dark) and either at 8°C or 25°C.

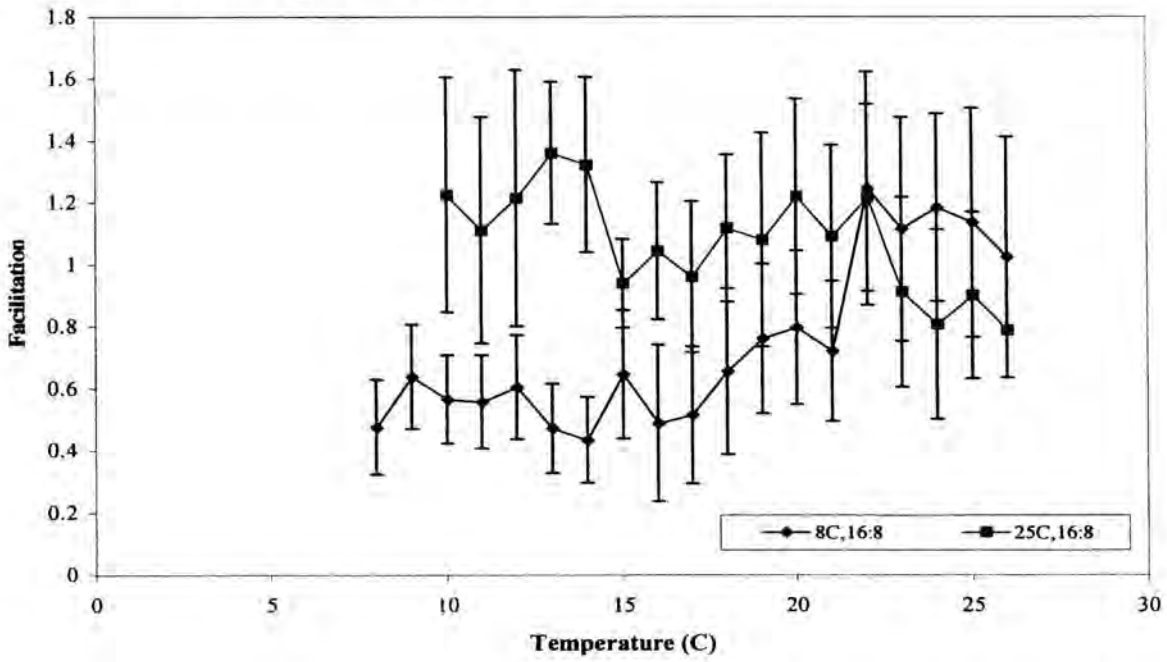


Figure 3.19: Effect of temperature on facilitation of EJP amplitude. All animals were acclimated using a cold acclimation (8°C) and either a short day photoperiod (8:16 L:D, solid diamonds) or a long day photoperiod (16:8 L:D, light square). Data is presented as mean ± S.E. mean; numbers of experiments were, short day (n= 18), long day (n= 11). For compare using comparison of the slopes were found to be statistical not significant (Regression, $P>0.05$).

Figure 3.20: Effect of temperature on facilitation of EJP amplitude. All animals were acclimated using a warm acclimation (25°C) and either a short day photoperiod (8:16 L:D, solid diamonds) or a long day photoperiod (16:8 L:D, light square). Data is presented as mean ± S.E. mean; numbers of experiments were, short day (n= 13), long day (n= 10). For compare using comparison of the slopes were found to be statistical significant (Regression, $P<0.05$).

Figure 3.19: Effect of temperature on facilitation. *C. maenas* acclimated at 8°C with short-day (8hr light: 16hr dark) and long-day (16hr light: 8hr dark).

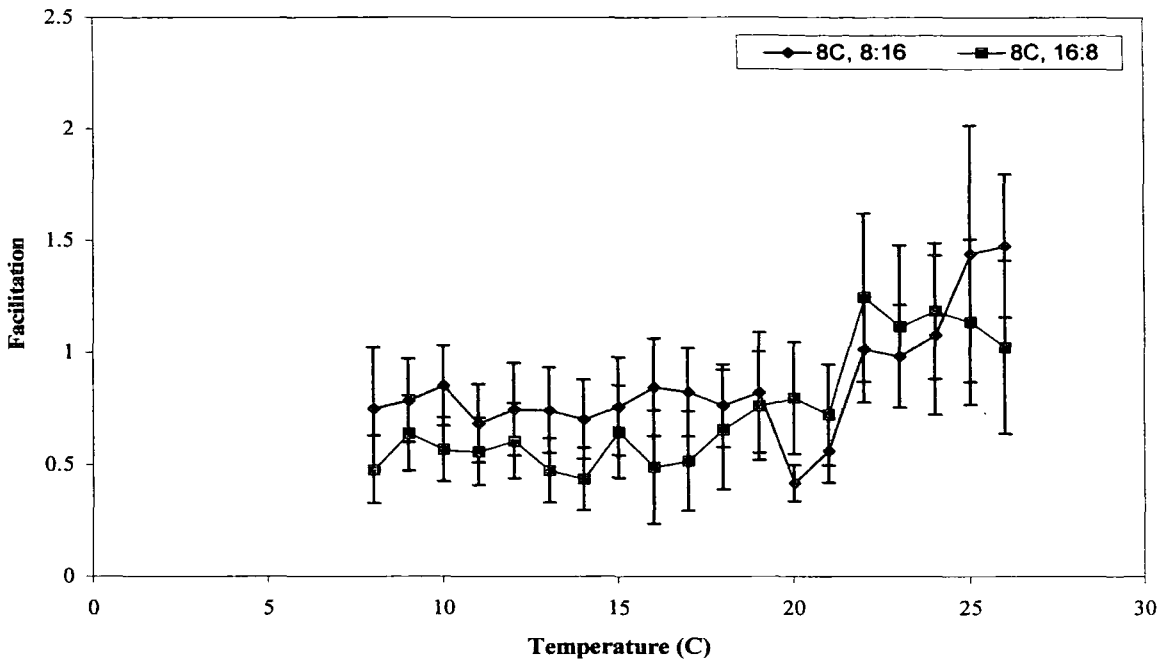
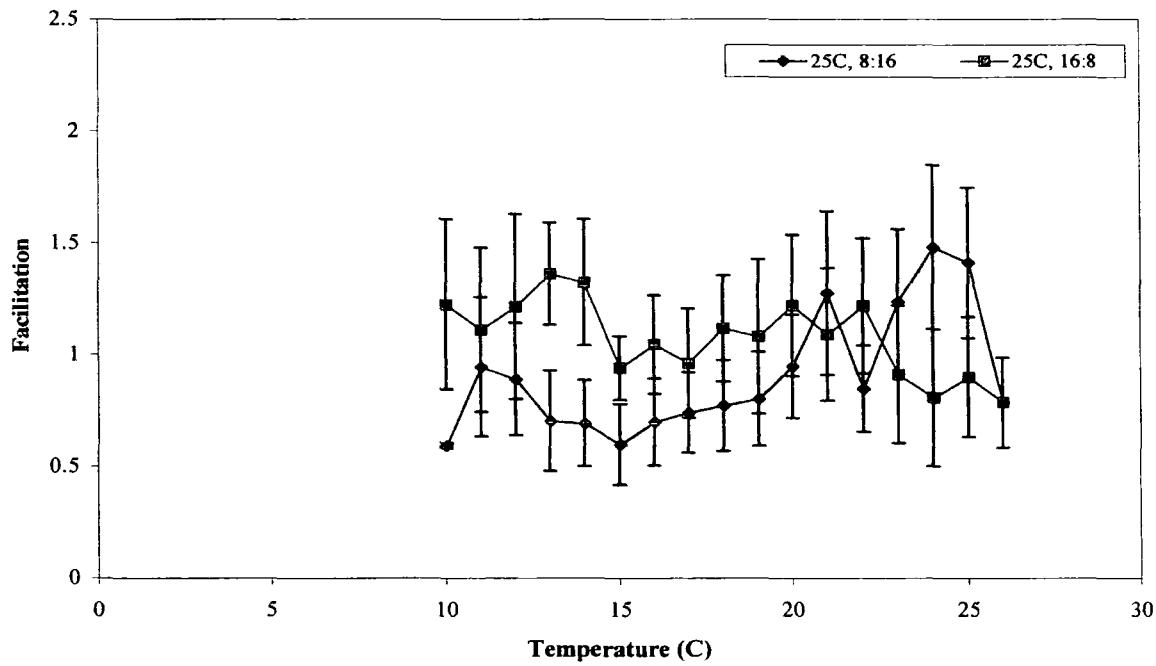


Figure 3.20: Effect of temperature on facilitation. *C. maenas* acclimated at 25°C with short-day (8hr light: 16hr dark) and long-day (16hr light: 8hr dark).



long day photoperiod. Long day photoperiod and warm acclimated animals showed generally irregular shape over experimental temperature. Short day photoperiod and warm acclimated crabs showed a decline of EJP facilitation with increasing temperature over 10-15°C and then showed an increase over 16- 21°C. Long day photoperiod crabs acclimated at 25°C had EJP facilitation values were significantly (Regression, $p<0.05$) larger than short day photoperiod animals acclimated at 25°C.

Discussion.

The results presented here can the analyzed in two ways. Firstly, the effects of temperature at fixed photoperiod showed reveal the extent to which acclimation to temperature alone occurs. Examination of the data for short day photoperiods for the neurophysiological parameters measured reveal that for the RP there was a compensatory shift in the temperature-RP relationship, namely a displacement to higher temperatures for the higher acclimation temperature relationship (Figure 3.1). However, for long day photoperiod the temperature-RP relationships were not significantly different at the two acclimation temperatures. In both cold and warm acclimated animals the RP became more hyperpolarized with increasing experimental temperature for both photoperiods either short or long day. The RP change with temperature was 1.6mV/°C for cold and short day acclimated crabs, and was significantly different from the RP change with temperature of warm and short day acclimated crabs at 1.2mV/°C (Figure 3.1). The RP changes with temperature were similar to the 1.1-1.3mV/°C found in 26°C acclimated closer muscle of *Ocypode ceratophthalma* (Florey and Hoyle, 1976). Hyperpolarization of RP with increasing temperature has also been seen in the closer muscle of crayfish *Astacus leptodactylus* (Harri and Florey, 1979), stretcher muscle of the Pacific shore crab *Pachygrapsus crassipes* (Stephens and Atwood, 1982), E2 axon of *Pachygrapsus crassipes* (Stephens, 1985b) and in the closer muscle of the crabs either *Cancer pagurus* or *Carcinus maenas* (Pearson, 1998). The RP change with temperature in cold and long day acclimated crabs was 1.4mV/°C, which was not significantly different compared to warm and long day acclimated crabs (1.7mV/°C) (Figures 3.2). The hyperpolarization of the RP

with temperature were greater than that predicted by the Nernst equation (0.32mV/°C). However, the Nernst equation only takes into account the prediction from passive and fixed permeability to a single ionic species. Prosser and Nelson (1981); White (1983); MacDonald (1990) explained that as temperature changes the ratio of ion permeabilities or the activity of the electrogenic Na^+/ K^+ ATPase pump or both may change. In general, membrane potentials reflect the conductance of the membrane through which ions diffuse, and the activity, and coupling of the Na^+/ K^+ ATPase pump (MacDonald, 1990). This was clear in the lateral giant neuron in the buccal ganglion of the freshwater snail *Helisoma trivolis*. The RP of snails acclimated at 25°C increased with increasing experimental temperature to a greater extent than snails which were acclimated at 5°C. In the presence of ouabain the increase in RP with temperature was similar in cells from cold and warm acclimated snails. They concluded that the pump component is greater at high than at low temperatures, and pump acclimation tends to keep the RP of cold acclimated cells more constant than that of warm cells (Merickel and Kater, 1974). Carpenter and Alving (1968) found that in *Aplysia* neurons the RP hyperpolarized on warming by amounts exceeding the K^+ equilibrium potential and which were depolarized by ouabain. The change in RP which exceeded Nernst prediction was attributed to alterations in the K^+/Na^+ permeability ratio rather than to a pump component (Klee *et al.*, 1974). But White (1983) concluded that increases in the K^+/Na^+ permeability ratio cannot be responsible for the extreme hyperpolarization. Prosser and Nelson (1981) noticed that membrane resistance decrease with warming and little effect on the diffusion component but a marked effect on the Na/K ATPase of RP. Changes in K^+/Na^+ permeability are more important at lower temperatures and probably the contribution of an electrogenic Na^+/K^+ pumps is more evident at high temperatures. Hyperpolarisation beyond $E_K{}^+$ may arise through activation of the electrogenic Na^+ pumps. So, the initial RP hyperpolarization which occurs with increasing temperatures from 8°C may be more a consequence of membrane permeability changes to Na^+/K^+ ratio (MacDonald, 1990). Whereas, at warmer temperatures the continued RP hyperpolarization beyond $E_K{}^+$ may be due to increased active pump a activity (White, 1983; MacDonald, 1990). Pearson (1998) investigated the effect on the Na^+/K^+ATPase activity by incubated legs of crabs in the 1mM ouabain for 25minutes. He found that the

effect of ouabain was to reduce the RP change with temperature (from 1.34 to 0.41mV/C°) closer to the value predicted by Nernst (0.32mV/C°).

A further feature evident in the results reported here was relative insensitivity of the RP in cold acclimated crabs at higher experimental temperature (Figure 3.1). The RP in this case became more hypolarized with increasing temperature up to about 20°C but at higher temperatures not much further change in RP was recorded. In cold acclimated crabs at short day photoperiod, the RP change with temperature was the same as that of warm acclimated crabs (~22.5mV for 8 to 26°C). However, throughout this range the RP of cold acclimated crabs was consistently more hyperpolarize than that from warm acclimated crabs by about 6mV. Figure (3.1) establishes that partial temperature acclimation occurs during constant short day photoperiods. The slopes of the RP were similar (-1.2079 in warm acclimated crabs and -1.2166 in cold acclimated crabs). The main conclusion is that a warm acclimation shifts the RP relationship to the right along the experimental temperature and exhibits partial acclimation about 13% (Type III after Precht, 1958). Different acclimatory shifts in RP have been reported by Pearson (1998) for *Carcinus* acclimated at 8°C and 22°C, 12:12 L:D photoperiod. The percentage of acclimation in that study was 53.6% and the acclimation shift was more complete than was found in this report. With long day photoperiod (Figure 3.2) the two curves of temperature-RP relationship from animals acclimated at the two temperatures were much closer together and their S.E. bars overlap, with no significant difference between them. Thus a comparison between Figures 3.1 and 3.2 shows that there was an effect of photoperiod on temperature acclimation. At short day photoperiod there was a partial compensatory acclimation to temperature but at long day photoperiod this did not occur.

Figures 3.3 and 3.4 replot these data shown in Figures 3.1 and 3.2 such that the effects of different photoperiods at a single temperature can be compared. It can be clearly seen that the RP-temperature relationship for the cold acclimated group at different photoperiods were significantly different (Figure 3.3) with a small displacement of the relationship to the higher temperatures in the long day acclimated group. In contrast crabs acclimated at the higher temperature showed no significant shift of the RP-temperature relationship for the two different photoperiodic regimes.

EJP amplitudes are dependent on the amount of transmitter released (Fatt and Katz, 1953; Matthews, 1996) and the number and sensitivity of post synaptic receptors (White, 1983). In addition muscle membrane resistance affects the EJP amplitudes (MacDonald, 1990). EJP amplitude was relatively insensitive to experimental temperatures in crabs that were warm acclimated irrespective of photoperiod. However, in cold acclimated cabs the EJP was larger at lower experimental temperature than warm acclimated crabs and furthermore the amplitude decreased with increased experimental temperature such that at high experimental temperatures the EJP amplitudes for both photoperiodic groups converged to the same values. Stephens and Atwood (1982) found in stretcher muscle fibers of Pacific shore crab *Pachygrapsus crassipes* acclimated either to 12°C or 21°C, that the maximum EJP amplitudes occurred at the acclimation temperature. This is qualitatively similar in cold acclimated crabs that have the maximum EJP amplitudes at 8°C in both short and long day photoperiod. A possible explanation for the reduction of EJP amplitude with experimental temperature change may be because of conduction block in nerve branches (Hatt and Smith, 1975; Lang and Govind, 1977), or to temperature induced changes in quantal content (White, 1983), perhaps produced by alteration of the amount of calcium entering the terminal of nerves (Charlton and Atwood, 1979). For high acclimation temperatures either short or long day photoperiod, the EJP varies very little with experimental temperature and is relatively constant in both cases over most of the experimental temperature range. Thus, acclimation to low and high temperatures has a significant effect possibly on the mechanisms of transmitter release or action. In contrast to the acclimatory effects of temperature on EJP amplitude there was no significant difference between EJP amplitudes from short and long day photoperiod in either temperature acclimated groups. The stable EJP amplitudes recorded from warm acclimated in either short or long day photoperiod indicates maintained muscle function over a wide range of temperatures.

The latent period of EJP is a timing interval between the stimulus and the initial response. It includes axonal conduction to the synaptic cleft the release of neural transmitter substances, diffusion of transmitter and post synaptic response through muscle fibre depolarization. It has been shown previously that latency in crustacean neuromuscular

junctions was affected by temperature (Florey and Hoyle, 1976). It has been shown previously that change in experimental temperature if large enough can block conduction at a synapse (Montgomery and MacDonald, 1990) and such a block may occur at neuronal branch points (White, 1983; Atwood and Nguyen, 1995). Conduction velocity determines the time taken for an action potential (AP) to travel from stimulation point to the axon terminal; it is dependent on axon diameter and the number of Na^+ channels. The latent periods of EJPs in all experimental groups decreased with increasing experimental temperature. Florey and Hoyle (1976) found that the latent periods in warm and cold acclimated ghost crabs were reduced by increased experimental temperature to the same extent; by Cuculescu (1996) and Pearson (1998) who worked on warm and cold acclimated *C. maenas* and *C. pagurus*. The decrease in latent period was attributed to the thermal effect on conduction, and thermal activation of Na^+ conductance (Prosser and Nelson, 1981), and decreased electrical resistance (Montgomery and MacDonald, 1990.) There appears to be no effect of photoperiod on latency in either cold or warm acclimated crabs (Figures 3.11 and 3.12). However, there is a small effect of temperature acclimation on latency (Figures 3.9 and 3.10) with slightly shorter latencies for warm acclimated crabs. Cuculescu (1996) has been shown shorter latent periods in warm acclimated *C. maneas* and *C. pagurus* compared with cold acclimated crabs. Pearson (1998) reported no differences in latent period between the warm and cold acclimated *C. maenas*. This indicated latency was not changed with acclimation temperature.

Decay constant (Tau) is a function of membrane capacitance and membrane resistance or the number of open channels (Fatt and Katz, 1953). Membrane capacitance is considered to be relatively constant as it depends on the thickness of the membrane and is not normally affected by temperature (MacDonald, 1990). For all acclimatory groups it can be seen that tau (R_m) decreased with increasing experimental temperature, which indicated decreasing membrane resistance or an increase in the number of open channels (Fatt and Katz, 1953). A similar reduction in time constant with experimental temperature was found by Pearson, (1998) in *C. maenas* and *C. pagurus* acclimated either at cold or warm temperatures. Photoperiod had no significant effect on the reduction in time constant in cold acclimated animals. However, there was a significant difference found between short

and long day photoperiod in warm acclimated animals. In cold and warm acclimation at short day, showed input resistance changes. Only in short day photoperiod was there a small but significant difference in cold and warm acclimation, with a decay constant of the colder acclimated group being larger than that of the warm acclimated group. No significant difference was found between cold and warm acclimated groups in long day conditions.

Facilitation was determined by recording two EJPs evoked by a double pulse stimulus (40ms apart) over the experimental temperature range 8-26°C. The amplitude of the second EJP was corrected for the overlap with the first EJP and then the amplitude of the corrected second EJP was by the amplitude of the first EJP. Hence a value of zero indicates no facilitation whereas a value greater than zero indicates increased facilitation. A positive facilitation value denotes facilitation, whereas a negative facilitation value is obtained when the second EJP is smaller than the first, and reflects de-facilitation or depression (Stephens and Atwood, 1982). Synaptic transmission is generally recognized by five stages: the release of the neurotransmitter, diffusion across the synaptic cleft, binding to post synaptic receptor-channel, the opening/closing response of the channel and finally the removal, recycling and synthesis of the neurotransmitter (MacDonald, 1990). Neurotransmitter exocytosis is triggered by Ca^{2+} influx into the presynaptic terminal where it initiates exocytosis (Katz, 1965). An increase in experimental temperature may result in a general decrease in Ca^{2+} sensitivity and hence a reduction in transmitter exocytosis (Stevens and Godt, 1990). Facilitation quite clearly was affected by temperature acclimation in the long day photoperiod group with greater facilitation occurring for the warm acclimated group compared with the cold acclimated group. No such significant difference was found in short day condition. The warm acclimated groups in long day photoperiods have a greater facilitation than the cold groups over almost all the experimental temperature range, between10-22°C. Cuculescu (1996) reported small shift facilitation with warm acclimation compared with cold acclimated *C. maenas* and *C. pagurus*. It is interesting note that EJP amplitudes from both short and long day photoperiod with cold acclimated crabs were maximal around 22-26°C, this may be related to changes in neurotransmitter release, changes in membrane resistance or changes in

calcium sensitivity which are all known to change with temperature (see White, 1983; Montgomery and MacDonald, 1990). It is also interesting to note that in cold acclimated crabs (Figure 3.19) the facilitation index was below unity for experimental temperatures below about 22°C, whereas it became greater than unity above 22°C. This effect in low temperature acclimated crabs is independent of photoperiod (Figure 3.19). Similarly the facilitation index in warm acclimated crabs is below unity in short day photoperiods for temperatures below about 20°C, rising to value above unity at higher temperatures. Whereas by contrast, the facilitation index of warm acclimated crabs in long day photoperiods are generally greater than unity for the lower range of experimental temperatures falling to below unity in the higher temperature range

Chapter Four

Heat shock: changes Critical Thermal Maximum (CTMax) and the response of the neuromuscular system in shore crab *Carcinus maenas*.

Introduction.

Heat death of an animal has been proposed to be the end point of a cascade of events; firstly acute heat disturbs the cellular membrane functions which in turn leads to other disturbances and finally to the death of the animal (Bowler *et al.*, 1973). Marine intertidal animals are subjected to a variety of abiotic stresses, including aerial exposure and wide ranges of temperature depending on the tidal cycle, the time of day and season. The rocky intertidal zone is the area of the shore that is regularly covered and uncovered by the movement of the tides. Therefore, the rocky intertidal animals have been an important study organism for physiological ecology because of their unique tolerances to physical factors of the marine and terrestrial worlds (e.g, Newell, 1979) and has become an area of particular interest of heat shock proteins (HSPs) (Hofmann *et al.*, 2002). A major problem these animals encounter is how to maintain activity and preserve adequate function in the face of the physical variability of their habitat. The question arises whether changes in HSP production occur naturally during period experience to thermal stress, such as might occur in intertidal habitat.

Although aquatic ectothermic organisms have body temperatures equal or close to that of their surroundings, a variety of strategies have been exploited to compensate for the environmental temperature variation experienced. Organisms may avoid the stress by behavioural responses allowing them to exploit microhabitats that could otherwise be harmful. In some cases behavioural selection of favorable thermal niches may help to stabilize body temperature, and makes behavioural avoidance of thermal extremes important. Zimmerman *et al.*, (1989) found that largemouth bass *Micropterus salmoides*,

displayed behavioural thermoregulation in a reservoir subject to large temporal and spatial thermal gradient. Naïve bass exhibited a range of reactive thermoregulatory behaviours which led half of them to seek cool water refuges in the reservoir. This behaviour allowed 30% of the naïve fish to survive extreme thermal exposure. Prosser and Nelson, (1981) noted that nervous systems often fail before other systems, and synaptic transmission is a highly temperature sensitive and has a greater sensitivity to temperature than does impulse conduction. Therefore, behavioural failures occur before protein inactivation (Prosser and Nelson, 1981). Most researchers recognize that temperatures in many environments can exceed the tolerance limits of ectotherms (e.g., Gunter, 1941). Most animals have behavioural responses to avoid thermal extremes, however, animals in conditions that do not allow escape will often die from heat. For example lizards trapped in rocks, and aquatic organisms in or near hot springs (e.g., Bailey 1955; Heatwole and Harrington, 1989). On the other hand, many intertidal animals simply endure daily thermal challenges; this is particularly the case for sedentary species, barnacles and some mollusces. In addition to daily temperature change intertidal animals experience a seasonal variation in environmental temperature. A large literature shows that these animals are able to make changes to their physiology and biochemistry that compensate for the direct effect of seasonal temperature changes, Helmuth and Hofmann (2001); Whiteley *et al.*, (1998); Hofmann and Somero (1995).

In the laboratory these responses to a single environmental factor are called acclimations (International Union of Physiological Sciences Commission for Thermal Physiology, 2001). In response to a temperature change they occur over a relatively long term course and persist as long as the new temperature regime continues. These laboratory acclimations are thought to be an expression of the situation in nature where animals experience changes in a variety of other environment factors well as temperature, where they are termed acclimatizations (IUPS commission for thermal physiology, 2001), e.g. day length, oxygen, tension, osmotic pressure, and maybe produced in response to a variety of influences. Many studies have concerned thermal acclimation e.g. Stillman and Somero (2000); Lagerspetz and Bowler (1993); McGaw (2003); Pearson, 1998).

On a longer time scale genetic changes in population may alter fitness in response to long term changes in climate; these are genetic responses and become fixed in the population and are called adaptations (IUPS commission for thermal physiology, 2001). The different modes of adaptation have been recognized. These are short term phenotypic acclimations or acclimatizations and long term evolutionary adaptation that require genetical change.

Thermal acclimation is a strategy, crabs employ to compensate for seasonal changes in temperature but the daily changes experienced during the tidal cycle would occur too quickly for acclimation to occur. It is possible that the stress response mechanism is involved in responding to such environmental variability in the short term. This response is thought to be a consequence the production of HSPs. Lagerspetz *et al.*, (1995) noted that *Anodonta cygnea* mussels or their isolated gills after transferred from 4°Cto 20-24°C, the heat resistance of gills was increased after 1 day and remained at this level for 3 further days. There was therefore an increase of the heat resistance time of ciliary activity of gills, at least in isolated gills. It was found that a synthesis of 90KDa protein was induced in isolated gills by 24 hrs, but this did not affect cell membrane fluidity. A further increase of the heat resistance time (thermal acclimation) occurs only in whole animals, over a period of 2 or more days in time and is proposed to be associated with homeoviscous acclimation of cell membrane fluidity. The short term heat shock responses and longer term acclimation responses are additive in increasing resistance to lethal conditions (Korhonen and Lagerspetz, 1996). Thus, temperature compensatory acclimation of the properties of synaptic membranes would be predicted to correlate with recovery of behavioural capacities, such as membrane fluidity and behavioural traits changed more quickly during warm than cold acclimation (Somero *et al.*, 1996). For instance, Lagerspetz and Bowler (1993) found that the rate of attainment of acclimation was faster from 10 to 20°C than from 20 to 10°C.

The ability of a cell or organism to survive a normally lethal heat stress called thermotolerance, this is induced by exposure to elevated, but sublethal temperatures. Critical thermal maximum (CTMax) defined as the thermal point at which locomotory activity becomes disorganized and the animal loses its ability to escape from conditions

that will promptly lead to its death (Cowles and Bogert 1944). CTMax is an indication and measurement for evaluating the thermal death and physiology of an organism (Lutterschmidt and Hutchison, 1997), determined by acute exposure to lethal temperatures often employed to measure the thermal resistance among species. There are two major methods for determining thermal tolerance (1) the static method, which measures the time to death at constant test temperatures, and (2) the dynamic method, which involves increasing the test temperatures until an end point is reached. The first method determined the ultimate and lower lethal temperatures (UILT and LILT) and mark the boundary between the zone of tolerance and zone of resistance. The UILT and LILT are derived from temperatures at which 50% of the population survives an indefinitely long exposure. This method was originally introduced by (Brett, 1944; Fry *et al.*, 1942; Fry, 1957). Cowles and Bogert (1944) and Lowe and Vance (1955) introduced the concept of critical thermal maximum (CTMax) and critical thermal minimum (CTMin). In both the static and dynamic methods, tolerance of high temperatures increases with acclimation temperature only to a certain point. At temperatures above this point, thermal tolerance (CTMax or thermal resistance) does not increase (Lutterschmidt and Hutchison, 1997). Lagerspetz and Bowler (1993) found in *Asellus* that the CTMax increased by 4-5°C by acclimation at 20°C compared with the same animals at 10°C. Many organisms exhibit increased thermal tolerance following brief exposure to high temperatures. This induced thermal tolerance allows survival at temperatures that would otherwise prove lethal (Parsell and Lindquist 1994). The response to the exposure of a non-lethal heat shock is to induce a temporary increase in tolerance to the applied stressor, and increases resistance to potentially lethal temperatures (Loeschcke *et al.*, 1994; 1997; Feder *et al.*, 1996). Feder and Hofmann (1999) suggested that this increase in fitness is related to the biosynthesis of heat shock proteins (HSPs). Korhonen and Lagerspetz (1996) found in *Asellus aquaticus* that heat shock at 28°C for 60min increased heat tolerance only for a period of 6-7h after heat shock, during this time the CTMax of heat shocked animals was higher than in non HS controls, the effect of heat shock on CTMax is significant 1h after the shock not 24h after it. But the effect of thermal acclimation on CTMax is significant at 24h after the transferred from

10°C to 23°C and later. This suggested that the effects of heat shock on CTMax are rapid and transient, but the effects of thermal acclimation slow and cumulative.

The heat shock response was first identified by Ritossa (1962) who described a puffing pattern in *Drosophila* chromosomes in response to heat-shock. This discovery led to the identification of a large family of heat shock proteins (HSPs) (Tissiéres *et al.*, 1974; Storti *et al.*, 1980). Heat shock proteins are produced in many cells, prokaryotic and eukaryotic, in *vivo* and in *vitro* (Kiang and Tsokos 1998). The genes encoding HSPs are highly conserved and occur in every species, are found from bacteria to humans. In eukaryotes, many families comprise multiple members that differ in inducibility, intracellular localization, and function (Feder and Hofmann, 1999). Accordingly, HSPs are equally well termed stress proteins, their expression is termed the stress responses, HSPs are useful markers because their induction is sensitive to stress. HSPs are known to play diverse roles, even in unstressed cells, in the successful folding, assembly, intracellular localization, secretion, regulation, and degradation of other proteins (Gething, 1997). Many HSPs function as molecular chaperones, which are a class of proteins that can recognize and bind to non-native proteins in order to prevent or ameliorate nonproductive folding and aggregation. Feder and Hofmann (1999) noted that HSPs recognize and bind to other proteins when these other proteins are in non-native conformation, whether due to protein denaturing stress or because the peptides they comprise have not yet been fully synthesized, folded, assembled, or localized to an appropriate cellular compartment. In the unstressed cell, HSC70 which is a constitutively expressed chaperone plays a vital role in the folding of newly synthesized proteins. Whereas exposure to stress leads to the induction of additional HSP synthesis. HSP70s for example are inducible and are thought to prevent irreversible protein aggregation during stress.

There are two types of HSPs: constitutive and stress-inducible. First, the constitutive proteins are present all the time, frequently very abundant (Parsell and Lindquist, 1993), and those that are expressed constitutively under normal physiological conditions. Secondly, the inducible ones are produced in response to a variety of stresses, including extremes of temperature, cellular energy depletion, and extreme concentrations of ions, other osmolytes, gases, and various toxic substances to cope with stress-induced

denaturation of protein (i.e., HSPs). The degree of induction of HSP depends on the level and duration of exposure to stress, the increase is transient and the time it lasts differs in various cell types (Kiang and Tsokos, 1998). The roles of HSPs have been listed in the review of Feder and Hofmann (1999), there are many members of HSPs family and several families of stress inducible proteins. The 85-90 KDa and 68-72 KDa families are predominant and lower molecular weight proteins of 20-30 KDa are also commonly found (McLennan and Miller, 1990). But HSP 70 family is the most highly conserved of the heat shock proteins and has been identified in several species of many marine invertebrates (Hightower *et al.*, 1985; Hightower, 1993; Williams *et al.*, 1996), and there is a positive correlation between the abundance of HSP70 in the presence of heat and the ability of cells to withstand increasing temperatures or stressed conditions (Craig, 1985). Parsell and Lindquist (1993) reported that the heat inducible forms of HSP70 do play a central role in stress tolerance, either by promoting growth at moderately high temperatures and/or by protecting organisms from death at extreme temperatures. Kiang and Tsokos (1998) found that under normal conditions, levels of constitutive HSPs increase during the cell cycle and during development, and two kinds of increase in constitutive HSPs are observed, one transient and the other sustained. Thus HSPs are constitutively present in large quantities (Yu *et al.*, 1994). These have been referred to as heat shock cognates (HSC), These HSCs are 75% identical in DNA sequence in the protein coding region to HSP70 (Craig, 1985). The amount of HSCs produced under normal conditions is unknown. Therefore, it is important to determine this level in order to determine how much HSP70 is produced under stressed conditions whilst, in unstressed cells heat shock-related proteins direct the folding and assembly of other cellular proteins, under stress, the heat induced proteins are produced in response to increased protein disruption and they play different roles (Pelham 1984). Denatured proteins, therefore, serve as a stimulus for the induction of the expression of HSP70, and its in turn can restore protein structure and function (Sharp *et al.*, 1999). This suggests that it is vitally important for cells to accumulate rapidly HSP70s during times of stress; their induction has the character of an emergency response, being extremely rapid and very strong. (Parsell and Lindquist, 1993). As long as cells are maintained at high temperatures, HSPs continue to be the primary product of protein

synthesis, but when cells are returned to normal temperatures, normal protein synthesis gradually resumes (DiDomenico *et al.*, 1982). For instance, Karunanithi *et al.*, (1999) found that control samples of *Drosophila* larvae showed no detectable HSP70, whereas *Drosophila* larvae were exposed to 36°C heat shock for 1h showed a robust induction of HSP70. When placed at a recovery temperature (25°C), a further increase in HSP70 was noted in the succeeding 1/2hr recovery, after which HSP70 rapidly declined and was barely detectable by 6h recovery time at 25°C. In addition, both constitutive HSC70 and inducible HSP70 contribute to acquire thermotolerance in *Poeciliopsis* hybrids (dilorio *et al.*, 1996). Feder and Hofmann (1999) have remarked that there is little physiological insight into exactly how the activity of HSPs culminates in the enhanced stress tolerance of multicellular eukaryotes and the cells and tissues that they comprise.

In nature, it has been shown that synthesis of heat shock proteins is activated near the upper lethal temperatures in different species (Hofmann and Somero, 1995; Roberts *et al.*, 1997; Tomanek and Somero, 1999). When cells or whole organisms are exposed to high but sublethal temperatures, as well as to other stressors, synthesis of a set of special proteins in induced that are thought to protect normal cellular protein during heat or other stress and to facilitate cellular recovery after the stress is removed (Craig, 1985; Morimoto *et al.*, 1990). The HSP response may be a mechanism for surviving what would otherwise be a lethal temperature (Spotila *et al.*, 1989). Ulmasov *et al.*, (1992) noted that the amount of HSP in ectotherms may be correlated with the mean temperatures of their habitats as well as whole animal thermoresistance. However, Feder *et al.*, (1994) studied seasonal variation in heat shock proteins (HSP70) in different kinds of stream fish in their environment. In all stream fish it was found that there was the highest level of HSC/HSP70 in spring and the lowest level in winter, coincident with increasing environmental temperatures and a rise in water temperature. The increase in environmental temperature was 10.5-16.7°C from winter to spring but 4.7-7.7°C from spring to summer. Therefore, it was suggested that these fish acclimatized to warmer conditions in the stream and reduced HSP70 production. Thus the induction of HSP70 may be dependent more upon the relative increase in environmental temperature than upon the absolute temperature experienced by these fish. The minimum heat shock temperature required to induce the heat shock

response (T_{on}), the temperature of maximal response (T_{max}), and the shut off temperature (T_{off}) appear to be species specific and reflect the evolutionary history of organisms (Dietz and Somero, 1993; Tomanek and Somero, 1999). The threshold temperature for HSP induction is correlated with the typical temperatures at which species live. In the extreme an Antarctic fish may suffer heat injury death at 6 to 10°C and HSPs may not be induced whereas some hot spring bacteria tolerate very high temperatures and HSPs may only be produced a temperatures close to 100°C (for review see Feder and Hofmann, 1999). In general, threshold induction temperatures for HSPs are positively correlated with ambient environmental temperatures (Dietz and Somero, 1992; Buckley *et al.*, 2001). Koban *et al.*, (1991) suggested that organisms native to cold habitats will synthesize HSPs at lower temperature compared with organisms adapted to warm or hot habitats. But Feder and Hofmann, (1999) showed that seasonal variation in water temperature can alter the heat shock response in summer and winter acclimatized fish. For example, Roberts *et al.*, (1997) found that summer acclimatized intertidal mussels (*Mytilus californianus*) induce HSPs at a threshold that is 6°C higher than the threshold in winter acclimatized mussels. Barua and Heckathorn, (2004) have been documented that there was no difference in acclimation in the heat shock response set points between eurytherms and stenotherms, whereas, sessile organisms exhibited lower acclimation compared to mobile organisms. The extent of acclimation differed among different heat shock proteins and the heat shock response temperature set points (induction, maximal response and shutoff). In response to growth temperature heat shock response induction temperatures change fashion. However, these authors based their conclusions on a review of the existing literature encompassing a very wide range of organisms from different kingdoms (plants, fungi, protists, monera and animals). They acknowledge the limited data set and were unable to comprehensively compare patterns within specific taxa or control for non-independence of data.

A short- time exposure to elevated temperature caused in many ectothermic animals and in cultured cells a rapid but transient synthesis of stress proteins (Lindquist, 1986; Lindquest and Craig, 1988). This has been observed also in marine bivalve molluscs (Sanders, 1993). There may be two states of thermaltolerance, a temporary response that does not require synthesis of HSPs and longer term delayed response that does require

synthesis of new HSPs (Boon-Niermeijer *et al.*, 1986). Heat shock causes a production of additional proteins (HSPs) that correlates with the development of thermotolerance. However, it is reported that some degree of thermotolerance occurred in the absence of protein synthesis and other mechanisms must contribute to the survival in the absence of heat shock protein synthesis (Carretero *et al.*, 1991). For example, changes in the milieu (internal cellular environment) can influence the thermal responses of a large number of proteins (Somero, 1992); can contribute to the stability of protein structure in two ways. Firstly, by temperature-dependent pH changes, this can stabilise the function and structure of enzymes for activity or maintenance of structural integrity (Reeves, 1977; Somero, 1986). Secondly, by low molecular weight organic solutes that is capable of stabilizing proteins (Yancey *et al.*, 1982; Somero, 1992). For example, Hottiger *et al.*, (1987) noted that when yeast cells were heat shocked it was found that the accumulation of high concentrations of trehalose, and it is a powerful stabilizer of protein and membrane structure. These changes in the milieu may arise as a consequence of a change in cell temperature and include pH, low molecular weight solutes (Somero, 1992).

The heat shock proteins impart protection not only from temperature but also from many other physiological stresses (Parsell and Lindquist, 1993; Feder and Hofmann, 1999) such as low oxygen tension, salinity, and heavy metals and xenobiotics. Hightower (1991) recorded that high levels of abnormal and unfolded proteins are cytotoxic, and that the heat shock response has evolved to protect cells and organisms from such "proteotoxic" insults. Thus the response to heat shock is an indicator of how a species can increase its CTMax to survive stressful temperature (Feder and Hofmann, 1999).

Species living higher in the inter-tidal zone generally have a greater resistance (tolerance) to abiotic factors than do species living in the lower inter-tidal zone (Connell, 1961). Cuculescu (1996) studied the thermal tolerance of marine crabs with different temperature acclimation. It was found that the average of the CTMax of warm acclimated crabs (22°C) was increased of both species *Carcinus maenas* and *Cancer pagurus* than cold acclimated crabs (8°C) by 3.2°C and 6.39°C respectively. And the CTMax of *Carcinus maenas* which live in intertidal zone, acclimated at cold was 32.32°C, whereas *Cancer pagurus* which live in subtidal zone, at the same condition, the CTMax was

24.79°C. Stillman and Somero, (2000) studied the upper thermal tolerance limits of eastern Pacific porcelain crabs, and it was found that whilst inter-tidal species have high thermal tolerance limits, the sub-tidal species have lower thermal tolerance than inter-tidal species. Furthermore, the change in heat tolerance during thermal acclimation is greater for sub-tidal than for inter-tidal in 20 congeneric species of porcelain crabs, genus (*Petrolisthes*) throughout the eastern Pacific. This suggests that in some inter-tidal species, the upper thermal tolerance limits might near current habitat temperature maxima and may affect the distribution limits of inter-tidal species to a greater extent than for sub-tidal species. Expression patterns of HSPs in intertidal animals are also affected by distribution such as microhabitat and different heights within the intertidal zone. For example, mussels (*Mytilus californianus)* which live in location higher on the shore showed elevated levels of HSP70 isoform as compared with mussels from the lower shore (Roberts *et al.*, 1997). In the tropical reef coral *Goniopora djeboutiensis*, transplantation from the subtidal habitat to the intertidal habitat for 16 and 32 days resulted in elevated constitutive levels of HSP70 compared with control, low constitutive levels of HSP70 were found in all control samples compared with levels of HSP70 following heat shock temperatures of 33°C and above (Sharp *at al.*, 1997). The ability of *Collisella* limpets to tolerate elevated temperatures was much greater for *Collisella scabra* (which lives in the high intertidal region) than *Collisella pelta* (which lives in the upper midtidal region). Animals were heat shocked by transfer from their acclimated temperature of 20°C to elevated temperatures that ranged from 33 to 41°C for 15min, then were allowed to recover at 20°C for 24h. It was noticed that there was no mortality at 33°C for *Collisella pelta* but all animals died upon exposure to 36°C, whereas *Collisella scabra* were not killed until exposure to 41°C. In heat shocked *C. scabra* it was found that an increase in HSP70 levels in comparison with controls (Sanders *et al.*, 1991).

The question arises whether animals that inhabit environments with little stress have a different or reduced stress response compared with organisms from environments that are more stressful. In this chapter the levels and effects of heat shock proteins is investigated. The shore crabs, *C. maenas* lives in an intertidal zone with widely fluctuation temperature whereas *C. pagurus* lives mainly sub-tidally with a narrower range of

temperature fluctuation. The question arises therefore whether heat shock may play a role in these crabs. Therefore for *C. maenas* we compared CTMax before and after heat shock to test the hypothesis that heat shock proteins synthesis may be induced and that such a production would lead to increased thermotolerance was measured by elevation of CTMax. In addition also investigated is the effect heat shock may have or the neurophysiological properties of neuromuscular transmission.

Methods

a. Critical thermal maximum and heat shock

The effect of a heat shock on the CTMax, was measured in adult crabs *Carcinus maenas* acclimated to cold temperature (8°C). The CTMax procedure was exactly the same as those stated in (Chapter 2 section 2.6). Crabs were heat shocked at a temperature 1°C below the CTMax as previously determined. Experimental groups were subsequently heat shocked at 32°C for 30min, and allowed to recover at 8°C for different times (1h, 2h, and 4h), the controls were not heat shocked.

The effect of heat shock on the electrophysiological parameters was also measured. The heat shock protocol described in (chapter 2 section 2.7). Briefly, animals acclimated at 8°C, were placed in a bath at 32°C for 30min. The crabs were then returned to a bath at their acclimation temperature for recovery period. During the recovery period legs were rapidly dissected and prepared for microelectrode recording. This procedure took between 20-30min. Briefly, RP's and EJPs were measured using intracellular microelectrodes from closer muscles of the propodite in response to stimulation of the tonic excitatory leg nerve (see chapter 2 section 2.4). Data was displayed on an oscilloscope and recorded on a PC using an analogue to digital circuit. Then measurements of electrophysiological parameters of neuromuscular transmission were taken over a subsequent period up to 90min from the end of heat shock. During this time the temperature of the preparation was increased gradually from 8°C to a temperature at which no further recording of resting potential was possible. This period of temperature increase usually covered between 2-5mins. In the case failure of the RP there was a sudden loss of recording of any potential.

When this occurred attempts were made to repenetrate the muscle to establish whether or not any potential could be recorded. In the case of EJP the point of failure was the inability to measure any EJP following stimulation of the nerve. EJP failure preceded the loss of the RP. In order to cover a wide range of temperatures, from 8°C to 32°C two series of experiments were carried out; one in which the temperature was changed from 8-16°C within that period and another in which the temperature was changed from 16-32°C.

b. Tissue preparation and gel electrophoresis

The heat shock procedures were the same as stated in (Chapter 2, sections 2.5 and 2.9). In brief, adult *Carcinus maenas* acclimated to 8°C and heat shocked at either 30, 31.5, or 33°C. These were chosen below and above the CTMax to ensure a regime that resulted in heat shock. Heat shock period was 1hour for 30, 31.5°C, whereas in order to prevent mortality it was reduced to 30min at 33°C. For comparison with the heat shock treatment, animals were also subject to osmotic stress using 75%seawater + 25% distilled water. Following heat shock, animals were allowed to recover for 1h at 8°C. At least 3 animals were used in each experiment. Upper lethal temperature limits were examined in *C. maenas* which were acclimated to 8°C following the different temperature of heat shock. Heat shock at 30 and 31.5°C caused no mortality whereas at 33°C one of the three crabs used died. The mortality percentage of 75% seawater + 25% distilled water was zero as shown in Table below:

Heat shock temperature (°C)	% Mortality (n = 3 for each temperature)
30	0
31.5	0
33	33.3

After the end of the recovery time autotomised legs were collected from each animal of each group and muscle tissue was rapidly dissected and transferred into tubes containing 5ml of cold saline solution consisted of (HEPES 10mM, KCl 8mM, NaCl 470mM, $CaCl_2$ 20mM, $MgCl_2.6H_2O$ 10mM) then each tube was frozen immediately in

liquid nitrogen and stored at -80°C. The same protocol was used for control animals acclimated at the same temperature.

To prepare homogenates, the frozen samples were thawed on ice and then homogenized with a hand-held homogenizer. One volume of homogenizing buffer that consisted of 0.01MTris-HCl, (pH6.8) and protease inhibitors (aprotinin 2μg/ml, antipain 2μg/ml, leupeptin 2μg/ml, pepstain 1μg/ml)} was added to the sample weight (1/1w/v) then centrifuged at 12000rpm and 4°C for 30min. The supernatant was removed and stored at -80°C. Protein concentration was determined using the Bradford assay. Proteins were then separated by 5% and 8% polyacrylamide gel electrophoresis (Biorad SDS-PAGE) for 45-60 min at 200V (3.3μg μl^{-1} of protein per well) following staining with Coomassie blue, (refer to Chapter 2 section 2.9).

Summer acclimatized adult crabs were also heat shocked; specimens of the two species (*Carcinus maenas* and *Cancer pagurus*) were collected near Millport, Isle of Cumbrae in Scotland in August 2002. These two species collected are found in different habitats. After the CTMax determination in each species, *C. maenas* was heat shocked at 32°C whereas *C. pagurus* was heat shocked at 23°C (in both cases, near their CTMax) for 30 min, followed by 1hour recovery at 11°C. Tissue samples were collected at the end of recovery period. The autotomised legs were collected from different animals in each species before and after the heat shock, frozen immediately in liquid nitrogen, transported in liquid N_2 to the University of Durham and stored at -80°C. For tissue preparation and gel electrophoresis the same protocol was used as described above.

Results.

Heat tolerance after heat shock.

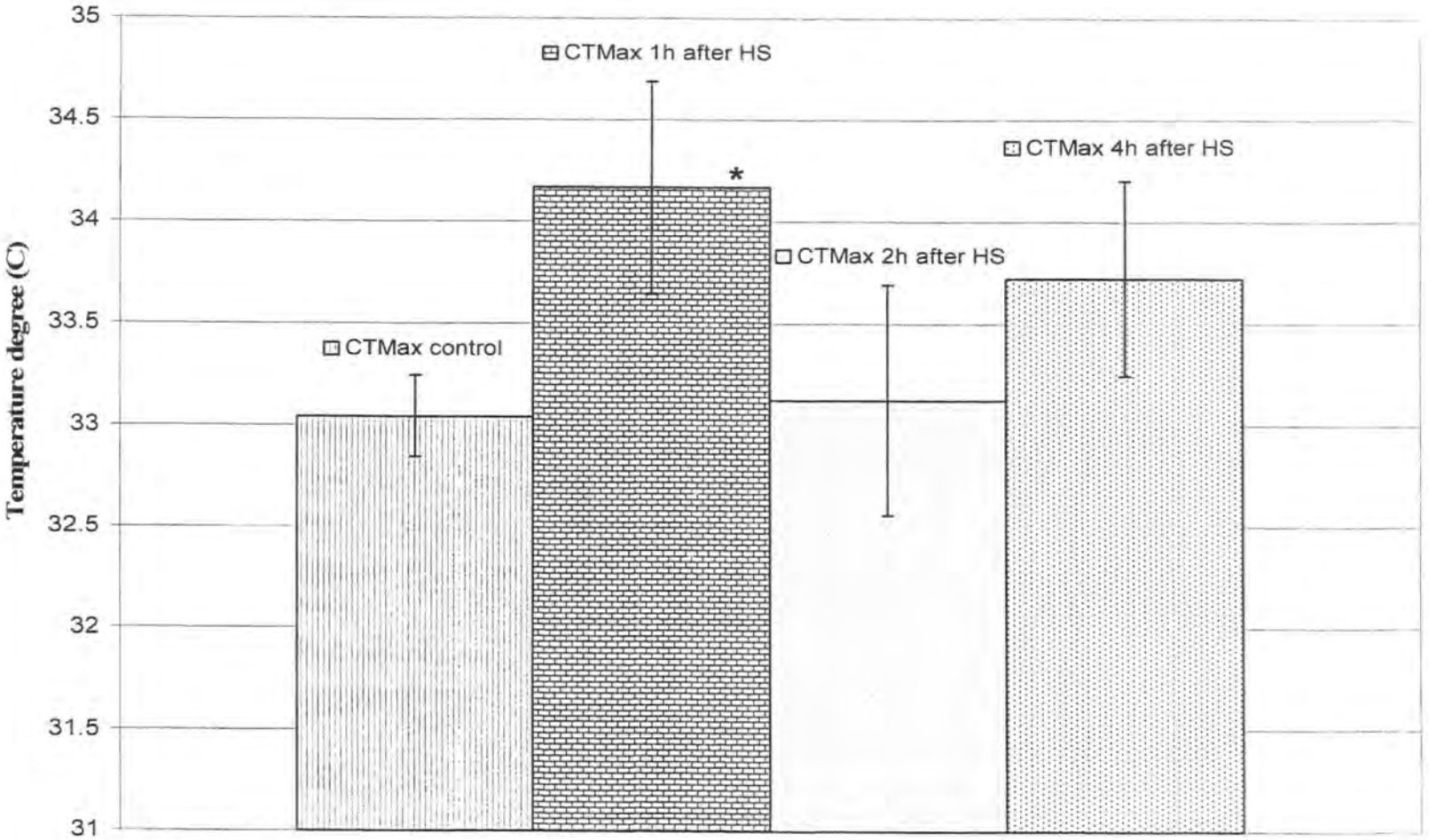
Figure 4.1 shows the CTMax values for control and heat shocked *C. maenas* as a function of time after the heat shock period. As can be seen the control value for CTMax (33.04 ±0.20°C) and only 1h after heat shock was CTMax significantly higher (34.17

Figure 4.1: Critical thermal maximum (CTMax) was measured in control and post heat shock (32°C for 30min) animals returned to their acclimation temperature (8°C) for periods of 1h, 2h or 3h. Numbers of animals were; control (n= 12), 1h after HS (n= 9), 2h after HS (n= 8), and 4h after HS (n= 9).

Mean values and Error bars represent SEM. were plotted and analyzed for significant differences (Student's (t-test).

Significantly different in CTMax between control and heat- shocked group denoted on the figure by a symbol (*), ($p < 0.05$).

Figure 4.1: Critical thermal maximum (CTMax) in control and in heat- shocked *C. maenas* acclimated at 8°C, during recovery at 8°C for 1, 2, and 4h.



±0.52°C) than the controls. No significant differences between control and heat shock animals were found after 2h or 4h recovery.

Neurophysiological parameters following heat shock.

The study shown in Figures 4.2 and 4.3 was undertaken to determine whether following heat shock the RP measured was dependent the length of time of the experiment. RP was recorded with a time at a relatively constant temperature (between 15.7- 16.8°C), and records were taken every 5mins, after heat shock. The initial period (not shown) corresponds to the time taken to make the preparation and establish microelectrode recording. Figure (4.2) shows that RP was not affected by the length of the time after heat shock up to a recording period of 90min. We also considered that EJP might be dependent on the length of time during which measurement took place. Figure 4.3 showed the EJP amplitude was also constant at 1.3 to 1.6 mV. Ninety minutes was chosen because this was the time taken to achieve the recordings shown in Figures 4.4 to 4.10.

Resting Potential (RP).

Figure 4.4 shows the RP change over 8-15°C of experimental temperature for control and heat shocked *Carcinus maenas* acclimated at 8°C. It can be seen that the RP hyperpolarized with increasing measuring temperature in both control and heat shock preparations, analysis (ANCOVA) of the data showed no significant differences between control and heat shocked *C. maenas* over the temperature range 8-15°C, ($p>0.05$).

In both control and heat shock groups the change in RP at temperatures above 16°C is shown in Figure 4.5. The control and heat shock results were significantly different (ANCOVA, $p<0.001$). In controls the RP was more hyperpolarized than in heat shocked crabs over the range 16-26°C. Failure of RP occurred at a lower temperature in control animals as compared with heat shocked animals. In both sets of experiments the RP at the starting temperatures (8°C in Figure 4.4 and 16°C in Figure 4.5) was more depolarized for the heat shock crabs than in controls. This difference was greater at the higher starting temperature.

Figure 4.2: Resting potential (RP) of leg closer muscles from *C. maenas* acclimated to 8°C and heat shocked (32°C for 30min). Following HS, animals were maintained at a relatively constant temperature (15.7- 16.8°C) in order to measure changes in RP. Data were recorded every 5min following the end of the heat shock. Numbers of animals were; HS (n= 7). Mean values and Error bars represent SEM.

Figure 4.3: Excitatory junction potential (EJP) of leg closer muscles from *C. maenas* acclimated to 8°C and heat shocked (32°C for 30min). Following HS, animals were maintained at a relatively constant temperature (15.7- 16.8°C) in order to measure changes in EJP. Data were recorded every 5min following the end of the heat shock.. Numbers of animals were; HS (n= 7). Mean values and Error bars represent SEM.

Figure 4.2: Resting potential (RP) recorded for up to 90mins following HS in cold acclimated *C. maenas* measured at a relatively constant temperature of 15.7-16.8°C.

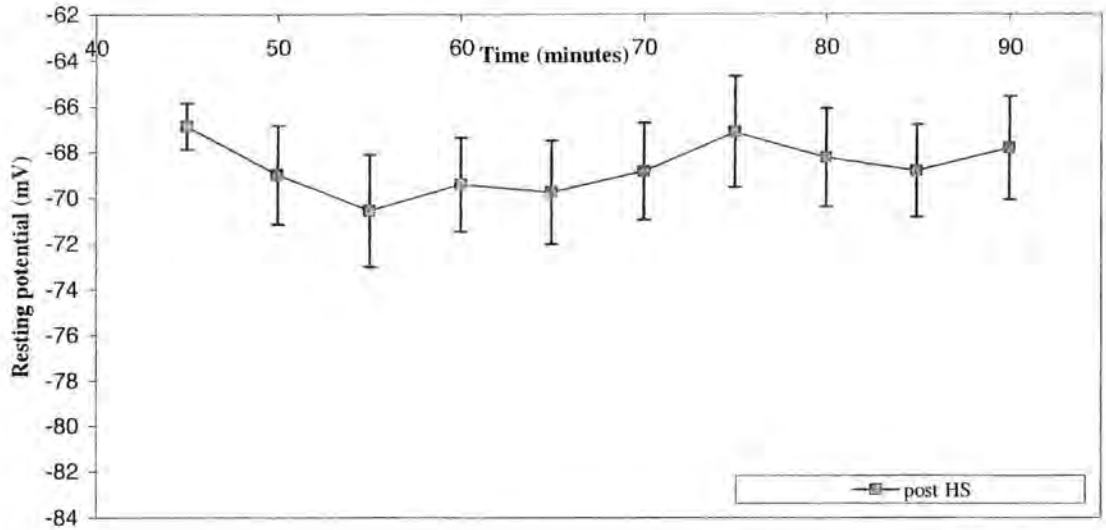


Figure 4.3: Excitatory junction potential (EJP) recorded for up to 90mins following HS in cold acclimated *C. maenas* measured at a relatively constant temperature of 15.7-16.8°C.

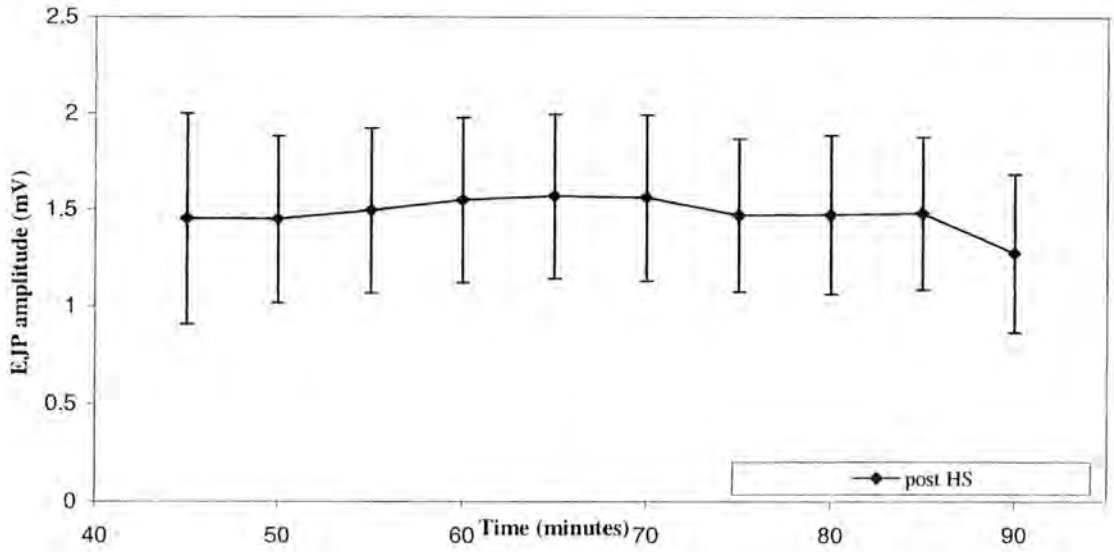


Figure 4.4: Effect of heat shock (30min exposure to 32°C) on the temperature-RP relationship (8-15°C) in leg closer muscles of *C. maenas* acclimated to 8°C. Control groups received no heat shock treatments. Data is presented as mean ± S.E. mean; numbers of experiments were, control (n= 22), post HS (n= 8). Statistical analysis (ANCOVA) determined no significant differences between two groups, ($p>0.05$)

Figure 4.5: Effect of heat shock (30min exposure to 32°C) on the temperature-RP relationship (above 16°C) in leg closer muscles of *C. maenas* acclimated to 8°C. Control groups received no heat shock treatments. Changes in RP with temperature in control and HS are shown. Numbers of experiments were, control (n= 22), post HS (n= 16). Data is presented as mean ± S.E. mean. Statistical analysis (ANCOVA) determined significant differences between control and HS ($p<0.001$)

N.B. In control experiments RP could not be recorded above 26°C donated by a symbol (*).

Figure 4.4: Effect of heat shock on the temperature-resting membrane potential (RP) relationship (8-15°C) in *C. maenas* acclimated at 8°C in control and post HS.

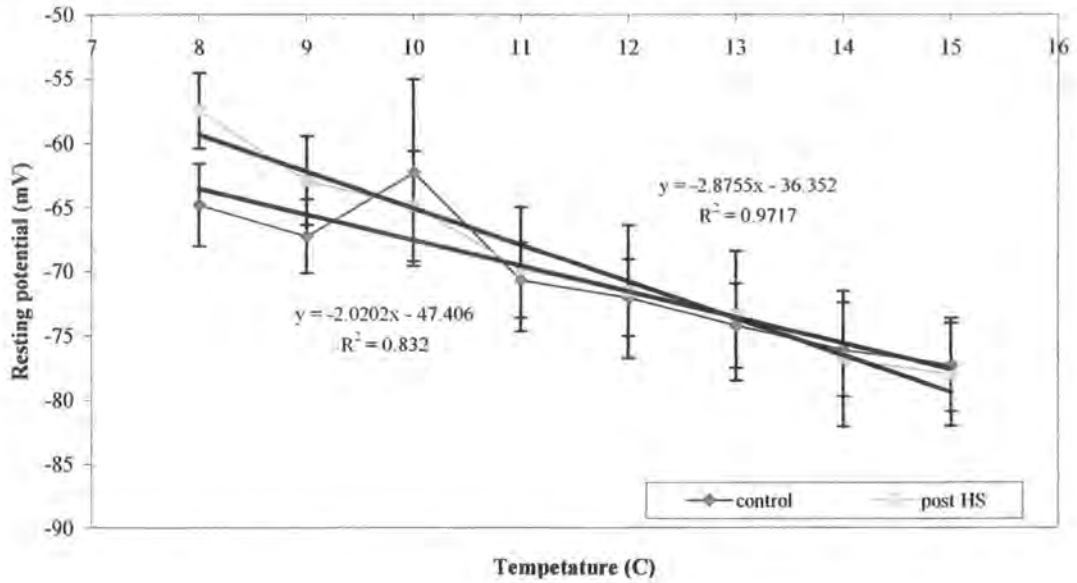
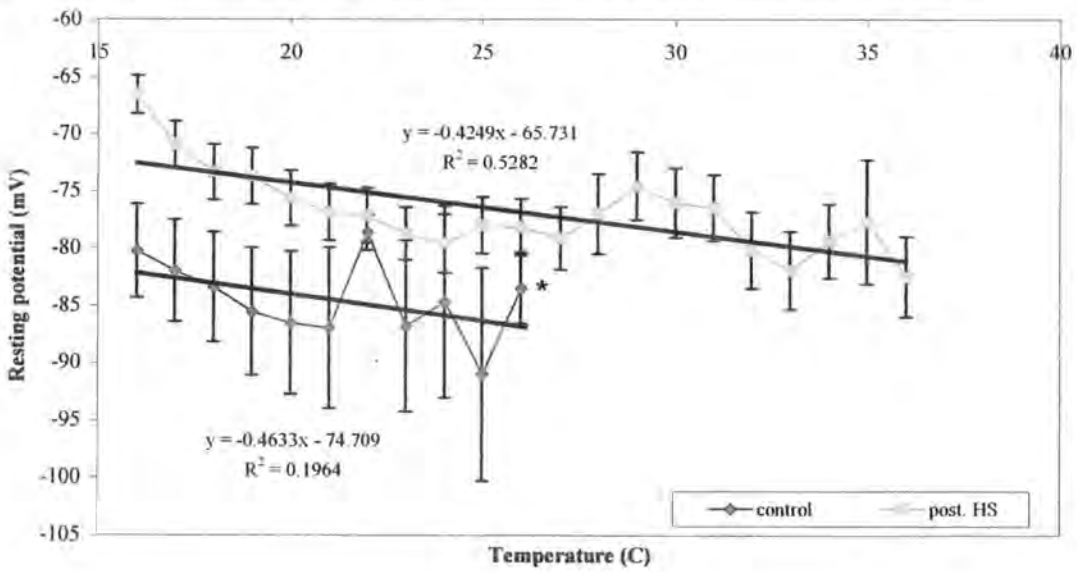


Figure 4.5: Effect of heat shock on the temperature-resting membrane potential (RP) relationship (16°C to failure) in *C. maenas* acclimated at 8°C in control and post HS.



EJP amplitude in control and after heat shock.

Figure 4.6 shows a typical set of responses and individual control and HS crabs. Over the whole of the temperature range from 8°C to 31°C. As can be seen in the control there is a progressive decline in EJP amplitude. Whereas in the HS preparations a more complex response to temperature is evident. Figure 4.7 shows the effect of temperature over 8-15°C and over 16-32°C range respectively on EJP amplitude. These were also carried out as two separate experiments. Control animals showed a decrease in EJP amplitude with increasing temperature. In heat shock animals EJP amplitude presented a more complex pattern. It increased from 8 to 18°C and then decreased at higher temperatures. Using a statistical analysis (ANOVA), there was a significant difference between control and HS animals ($p<0.05$) at 8°C to 11°C and at 17°C to 21°C. Failure of EJP occurred at lower temperatures in control animals than in heat shocked animals, which was significantly different. Thus the controls had a highest EJP at low temperatures. Whereas the HS group had much higher EJPs at temperature (between 16-19°C) but declines at still higher temperatures. But measurable EJPs can still be found at 31°C and 32°C in heat shocked crabs. Whereas in controls a progressive decline in EJP occurred up to about 21°C and then stabilized to about 26°C, beyond 26°C EJP can not be measured. HS appears to inhibit the EJP response at low temperatures; however above 15°C EJPs are all higher than the controls.

Latent period (latency).

Latency was measured from a recorded EJP (msec). It is determined as the interval between the stimulus and the initial response. Figures 4.8 and 4.9 show latent periods of control and heat shock *Carcinus maenas* acclimated at 8°C. In the controls group latency declined with increasing temperature up to the point of failure at 26°C. In the HS group a more complex response was seen. A similar fall in latency occurred between 16 and 25°C as in controls. However, above 25°C latency increased in the HS group. Statistical analysis (ANCOVA) showed no significant differences between control and heat shocked animals ($p>0.05$).

Figure 4.6A, B: Effect of heat shock (30min exposure to 32°C) on the individual EJPs recorded from leg closer muscle fibres of *Carcinus maenas*. Lower records show the effect of temperature in control animals. Upper records show the effects of temperature on the EJP following heat shock at 32°C for 30mins. Records were taken from crabs acclimated at 8°C.

Animals were placed directly from seawater at 8°C into seawater at 32°C for 30min, followed by 1hour recovery at 8°C. The time interval after heat shock with the experiment was 90min to measurement of EJP after heat shock.

Figure 4.6A: Effect of heat shock on individual EJPs recorded from leg closer muscle fibres of adult *C. maenas*. Lower records show the effect of temperature in control animals. Upper records show the effects of temperature on the EJJP following heat shock at 32°C for 30mins. Records were taken from crabs acclimated at 8°C with 16:8 L:D photoperiod.

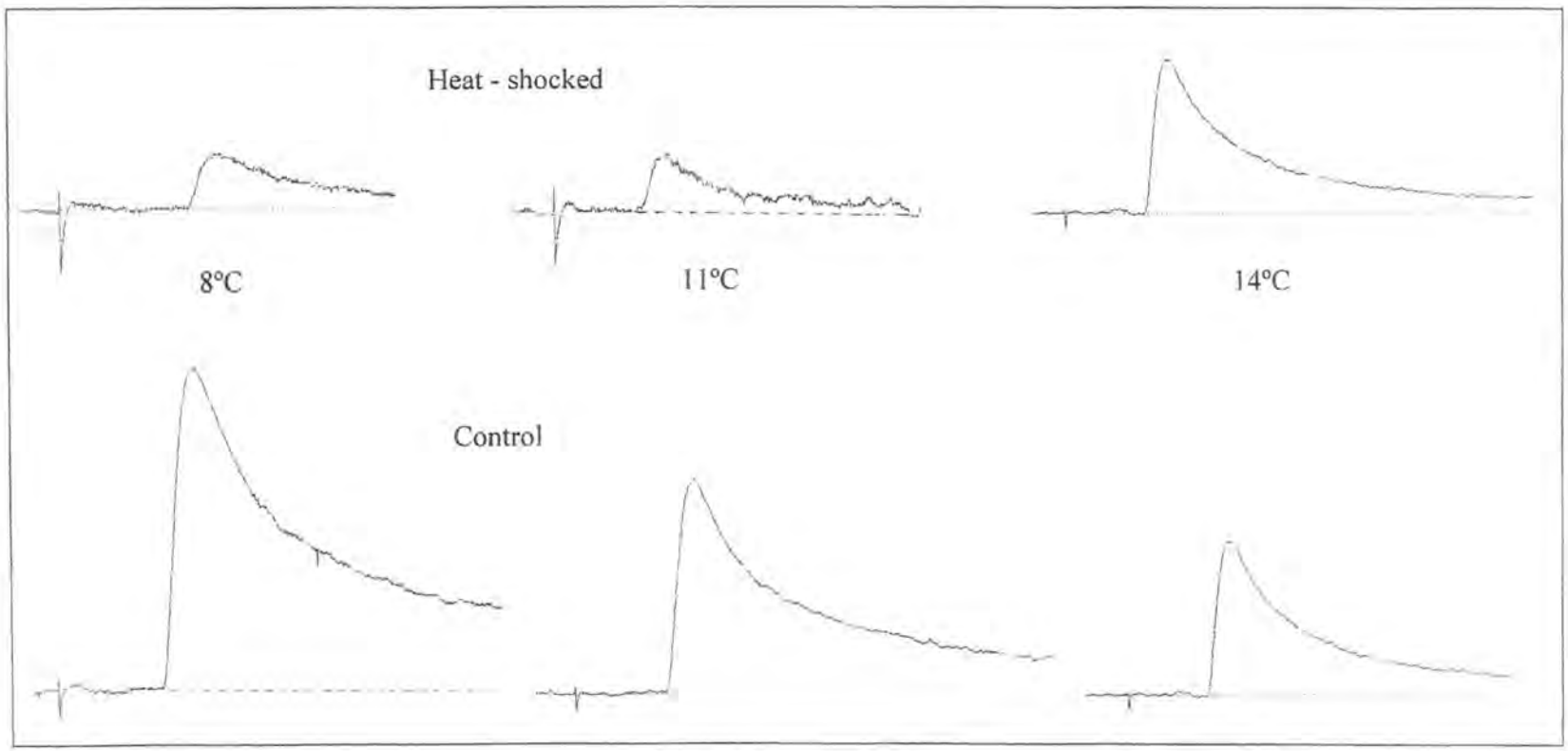


Figure 4.6B: Effect of heat shock on individual EJPs recorded from leg closer muscle fibres in *C. maenas*. Lower records show the effect of temperature in control animals. Upper records show the effects of temperature on the EJP following heat shock at 32°C for 30mins. Records were taken from crabs acclimated at 8°C with 16:8 L:D photoperiod.

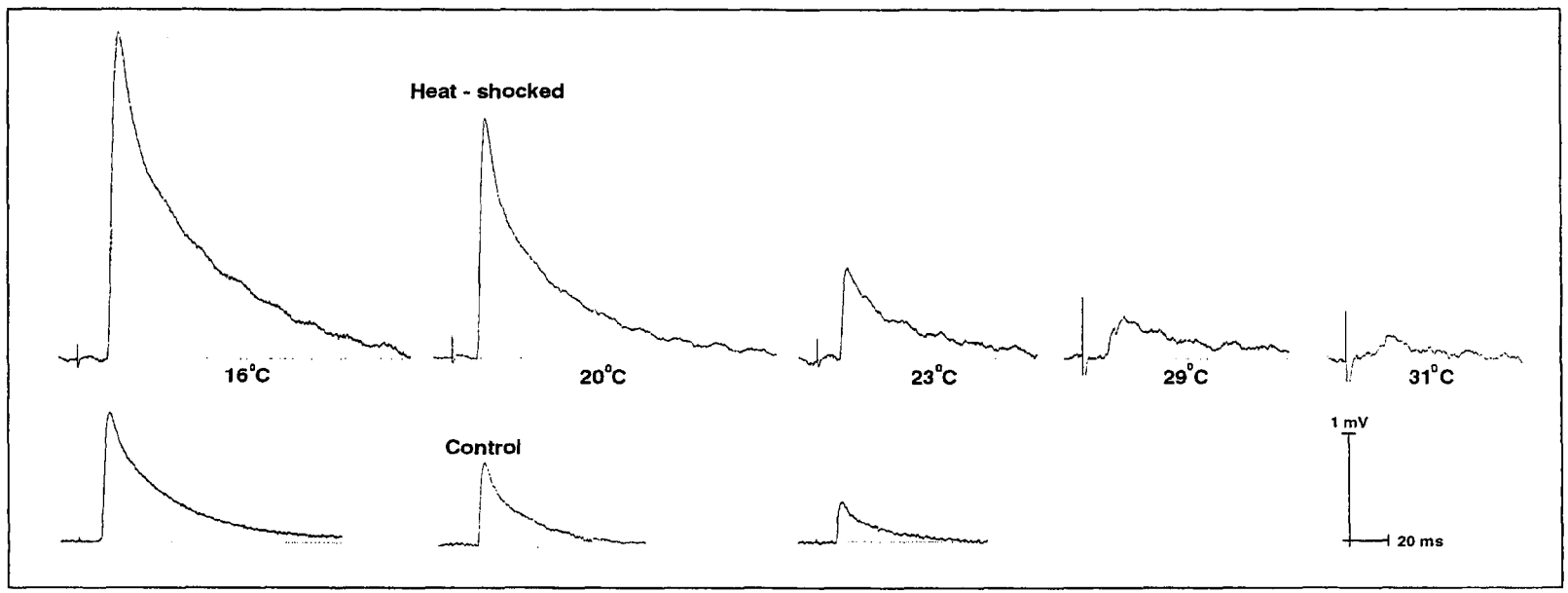


Figure 4.7: Effect of heat shock on EJP amplitude in *C. maenas* acclimated at 8°C in control and post HS.

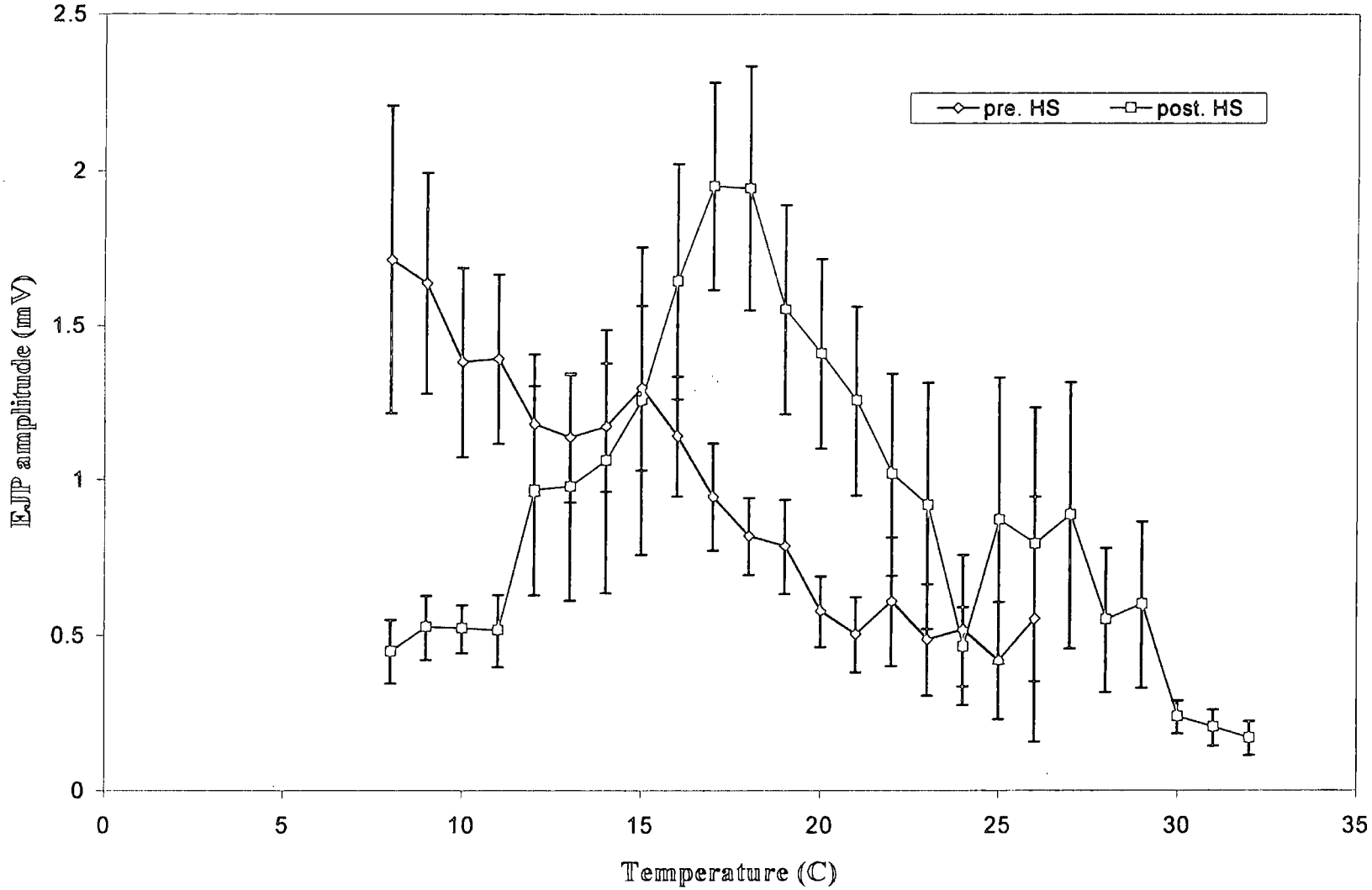


Figure 4.7: Effect of heat shock (30min exposure to 32°C) on temperature-EJP amplitude relationship in leg closer muscles of *C. maenas* acclimated to 8°C. Control groups received no heat shock treatments. In order to measure a wide range of temperatures, from 8°C to 32°C could not be a complete in the 90min period follow heat shock. Therefore two series of experiments were carried out; one in which the temperature was changed from 8-16°C within that period and another series of experiment in which the temperature was changed from 16-32°C in that period.

The experimental temperature over 8°C to 15°C. Data is presented as mean ± S.E. mean; numbers of experiments were, control (n= 17), post HS (n= 8). Statistical analysis (ANOVA) determined significant differences between control group and HS group, ($p<0.05$) over the range 8°C-11°C.

The experimental temperature over 16°C to failed. Numbers of experiments were, control (n= 17), post HS (n= 12). Data is presented as mean ± S.E. mean. Statistical analysis (ANOVA) determined significant differences between control and HS ($p< 0.05$) over the range 17°C – 21°C.

N.B In control experiments EJPs could not be recorded above 26°C denoted by a symbol (*).

Figure 4.8: Effect of heat shock (30min exposure to 32°C) on the temperature-latent period of EJP relationship (8-15°C) in leg closer muscles of *C. maenas* acclimated to 8°C. Control groups received no heat shock treatments Data is presented as mean ± S.E. mean; numbers of experiments were, control (n= 17), post HS (n= 8). Statistical analysis (ANCOVA) determined no significant differences between them, (P>0.05)

Figure 4.9: Effect of heat shock (30min exposure to 32°C) on the temperature-latent period of EJP relationship (above 16°C) in leg closer muscles of *C. maenas* acclimated to 8°C. Control groups received no heat shock treatments Changes in latent period of EJP with temperature in control and HS are shown. Numbers of experiments were, control (n= 17), post HS (n= 12). Data is presented as mean ± S.E. mean. Statistical analysis (ANCOVA) determined no significant differences between two groups, (P>0.05)

N.B In control experiments latent period could not be recorded above 26°C reflection of failure of EJP donated by a symbol (*).

Figure 4.8: Effect of heat shock on the temperature-log latent period of EJP relationship (8°C-15°C) in *C. maenas* acclimated at 8°C in control and post HS.

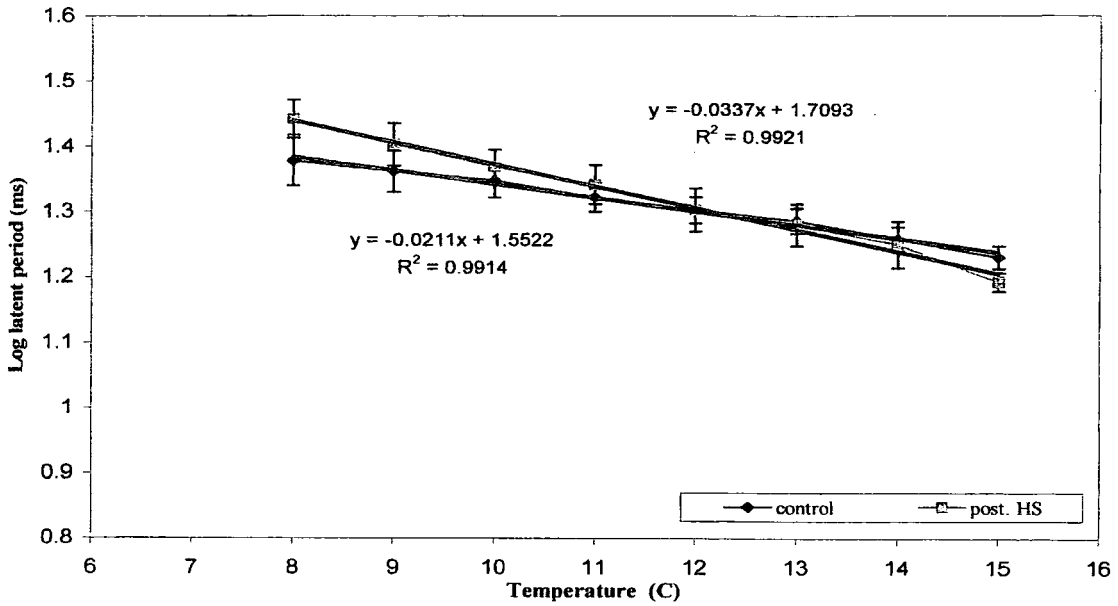
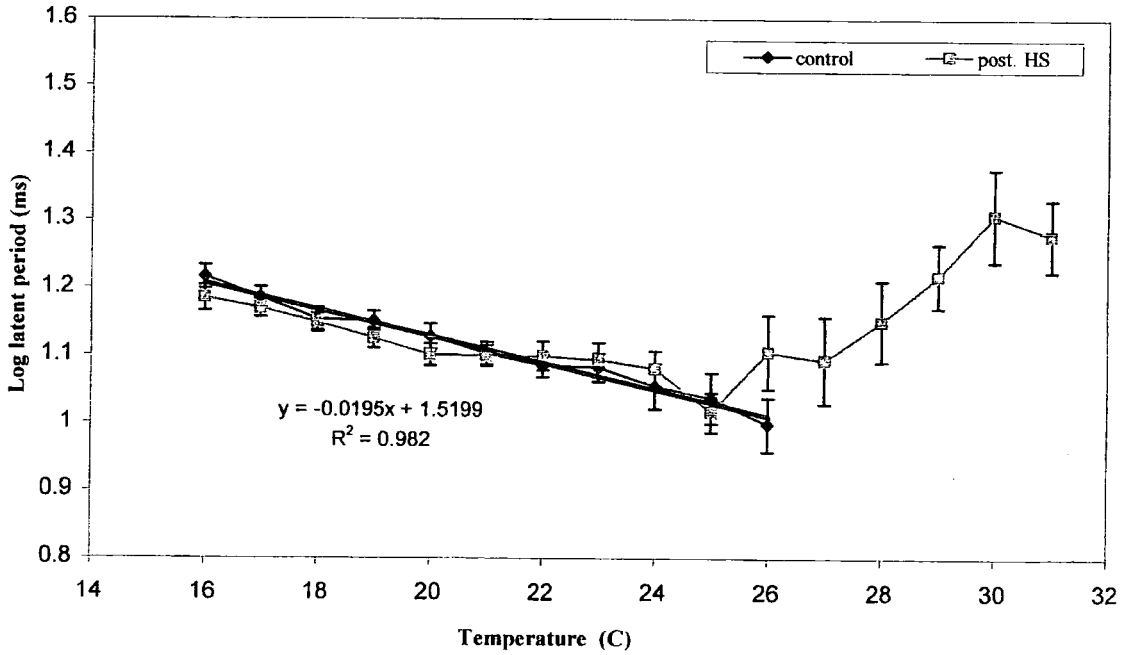


Figure 4.9: Effect of heat shock on the temperature-log latent period of EJP relationship (16°C to failure) in *C. maenas* acclimated at 8°C in control and post HS.



EJP decay time constant (Tau).

Tau is the time taken to decay to 1/e of the original amplitude; it was measured from recorded EJP (msec). Figure 4.10 shows changes in tau over an experimental temperature range of 8-15°C for control and heat shocked *Carcinus maenas.* A significant difference was found between control and heat shocked animals (ANCOVA, $p<0.01$) over 8-15°C, however there was no significant differences between control and heat shocked animals in the range above 16°C to the temperature at which EJP failed, (ANCOVA, $p>0.05$) Figure 4.11. The temperature at which tau failure occurred was increased in heat shocked animals compared with controls as is also shown in Figure 4.11. Because tau failure is part of EJP response, failure of tau was the same as failure of EJP. The failure tau was 26°C in the control and 32°C in the HS animals. Heat shock protects RP and EJP; the effects of HS on the point of the neurophysiological parameters are shown in table 4.1.

	Failure temperature (control)	n	Failure temperature (heat-shock)	n
RP	25.21°C ± 0.70	14	34.21°C ± 0.79	15
EJP	23.29°C ± 0.73	14	27.43°C ± 1.16	14

Table 4.1: Failure temperature in RP and EJP of control and post heat shocked *C. maenas* cold acclimated (8°C). Tau and latency obviously failed when EJP failed.

Expression of heat shock protein.

Figure 4.12 shows the effects of increasing heat shock temperatures on the protein isolated from *C. maenas* leg muscles. It can be seen that following heat shock at 33°C (lane 4), and at 30°C (lane6) remarkably similar bands of proteins evident between around 70-84KDa. In comparing heat shock lane4 and lane6 with control (lane7) it can be seen that the complex of bands between in the 70-84KDa region are also present in the control sample compared with heat shocked preparation. However, in this complex the four bands that can be seen in the control are of equal density whereas the band with the third largest protein is of higher density than either the equivalent control band or the other three bands

Figure 4.10: Effect of heat shock (30min exposure to 32°C) on the temperature-tau of EJP relationship (8-15°C) in leg closer muscles of *C. maenas* acclimated to 8°C. Control groups received no heat shock treatments Data is presented as mean ± S.E. mean; numbers of experiments were, control (n= 16), post HS (n= 8). Statistical analysis (ANCOVA) determine significant differences between control and HS ($p < 0.01$).

Figure 4.11: Effect of heat shock (30min exposure to 32°C) on the temperature-tau of EJP relationship (above 16°C) in leg closer muscles of *C. maenas* acclimated to 8°C. Control groups received no heat shock treatments. Changes in tau with temperature in control and HS are shown. Numbers of experiments were, control (n= 16), post HS (n= 12). Data is presented as mean ± S.E. mean. Statistical analysis (ANCOVA) determined no significant differences between two groups, ($P > 0.05$).

N.B In control experiments decay constant could not be recorded above 26°C reflection of failure of EJP donated by a symbol (⋆).

Figure 4.10: Effect of heat shock on the temperature-log decay constant of EJP relationship (8-15°C) in *C. maenas* acclimated at 8°C in control and post HS.

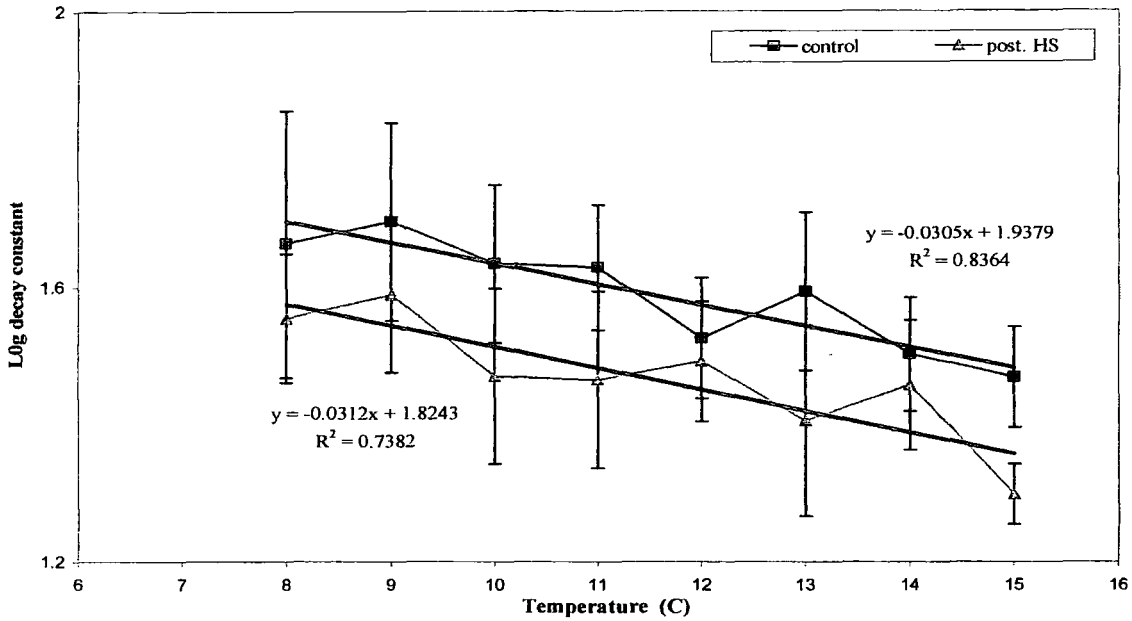


Figure 4.11: Effect of heat shock on the temperature-log decay constant of EJP relationship (16°C to failure) in *C. maenas* acclimated at 8°C control and post HS.

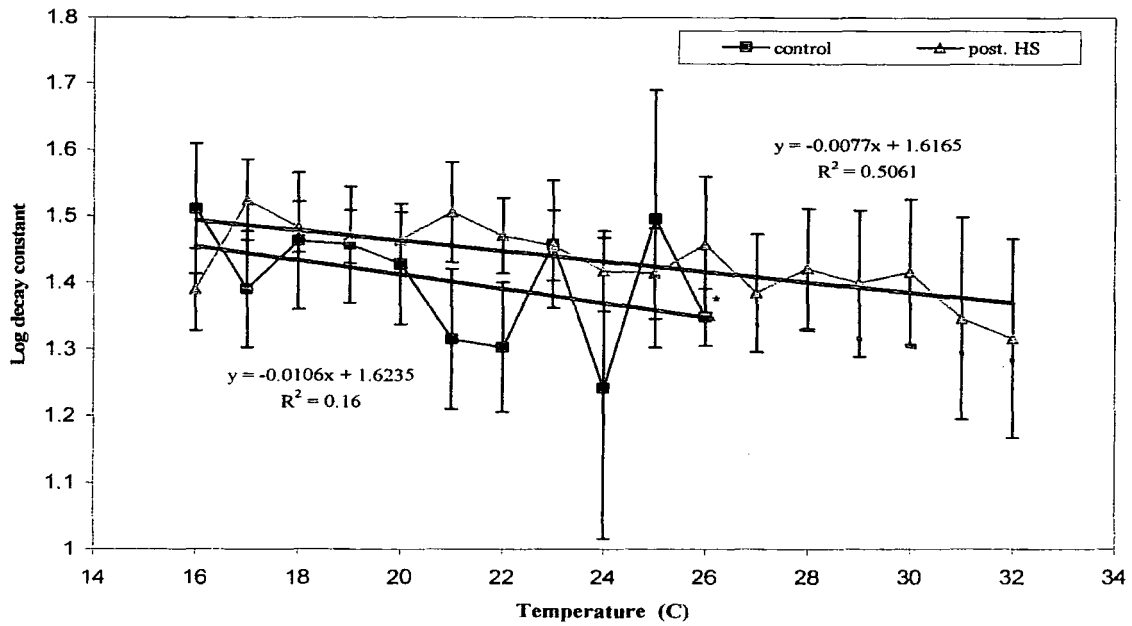


Figure 4.12: Expression of proteins in muscles of *Carcinus maenas* acclimated at 8°C following different heat shock treatments.

Lanes 1, 2 and 8, standard of molecular weight.

Lane 3 (75%seawater + 25%distilled water).

Lane 4 (heat shocked at 33°C).

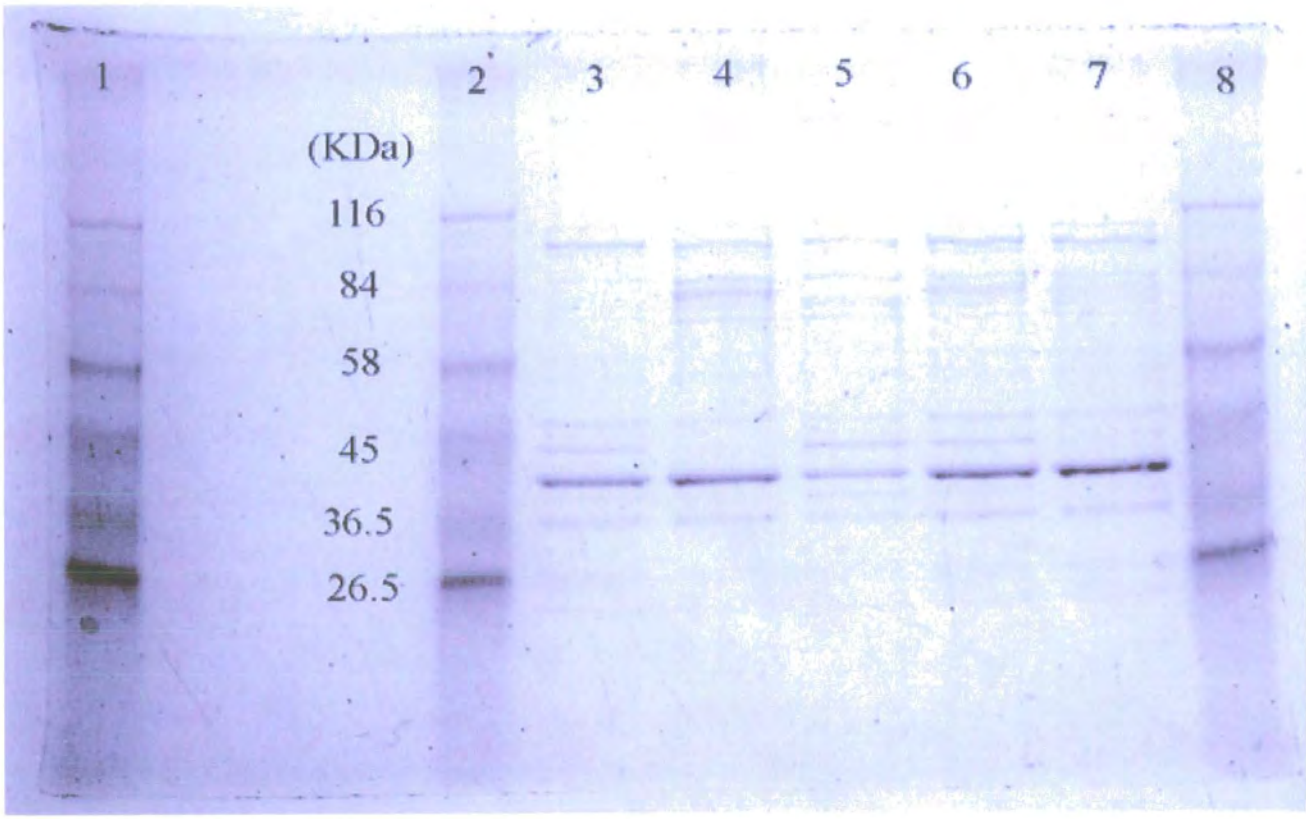
Lane 5 (heat shocked at 31.5°C).

Lane 6 (heat shocked at 30°C).

Lane 7 (control).

Animals heat shocked at 30, at 31.5°C and 75%seawater + 25%distilled water were exposed for 1hour heat shock and recovered for 1hour. Only the animals were exposed at 33°C kept for 30minut heat shock and recovered for 1hour. Each well was loaded with 10µl, and the mass of protein in each well of samples was $3.3\mu g\mu l^{-1}$.

Figure 4.12: expression of proteins in muscles of *Carcinus maenas* acclimated at 8°C and different heat shock treatments, protein concentration $3.3\mu g\mu l^{-1}$



in this complex in the lanes 4 and 6. In lane 5 of heat shock animals at 31.5°C compared with control, it might have been expected that a similar band to that present in lanes 4 and 6 would have been seen in lane5, as lane5 represents an experiment from an intermediate HS temperature. The results of osmotic stress (75% sea water) as depicted in lane 3 is relatively inconclusive in the region of interest around 70-90KDa.

The patterns of muscle proteins induced by HS close to the CTMax of *Carcinus maenas* and *Cancer pagurus* respectively is shown in Figure 4.13. Both species were acclimatized to summer condition it can be seen that there was a difference between control and heat shocked animals in both species. For *C. maenas* comparison of HS with control shows a changed pattern of bands in the region around 70-80KDa. In this region heat shock has resulted in the appearance of unresolved bands of proteins of just lower molecular weight than the prominent band at 84KDa. Whereas, no differences were observed in the intensities of the bands around between 25-58KDa which were similar in both control and heat shocked animals. For *C. pagurus* the control gel shows a series of intense unresolved bands around 70-85KDa whereas after heat shock this region is relatively devoid of protein bands above about 70KDa. Protein bands around 25-58KDa were similar in both control and heat shocked animals.

Discussion.

The critical thermal maximum (CTMax) is one accepted index and a standard for the evaluation of heat resistance of free moving animals (Hutchison, 1961). Accordingly CTMax was used as the criterion for the thermal tolerance of individual *Carcinus maenas.* Changes in thermal tolerance with acclimation temperature have been reported for many other species. Acclimation to elevated temperatures increases CTMax (Lahdes *et al.*, 1993), the high lethal temperatures are increased (Cossins and Bowler, 1987) and during the process of acclimation there is no narrowing of the inter-individual variation of CTMax (Lagerspetz and Bowler, 1993). Cuculescu (1996) have reported seasonal changes in the thermal tolerance of *C. maenas* and *C. pagurus*. But relatively little is known regarding the effect of heat shock on CTMax in crustaceans acclimated in different temperatures.

Figure 4.13: Expression of proteins in muscles of *Carcinus maenas* and *Cancer pagurus* acclimatized in summer 2002.

Lanes 1, 2 and 7, standard of molecular weight.

Lane 3 control of *C. maenas*.

Lane 4 heat shocked at 32°C of *C. maenas*.

Lanes 5 control of *C. pagurus*

Lane 6 heat shocked at 23°C of *C. pagurus*.

Each well of samples was loaded with 20µl of the samples, and the mass of protein in each well was 13.3µgµl^{-1}, whereas the standard's well was loaded with 5µl.

Figure 4.13: expression of proteins in muscles of *Carcinus maenas* and *Cancer pagurus* acclimatized in summer 2002, protein concentration 13.3$\mu g \mu l^{-1}$

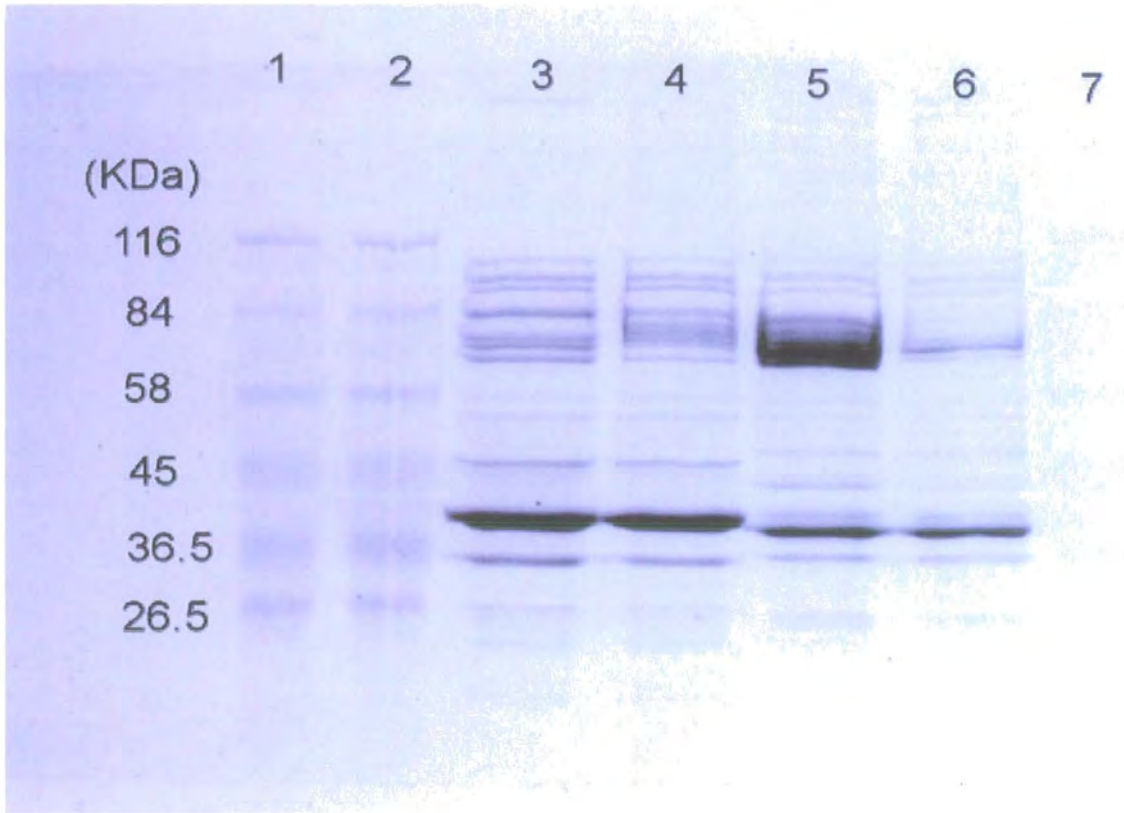


Figure 4.1 shows the CTMax values of *Carcinus maenas* acclimated for at least 2 or 3 weeks at 8°C. It was found that the CTMax in heat shocked animals were higher than in non–shocked controls by 1.13°C. Feder and Hofman (1999) have reported that the increase in CTMax following heat shock is caused by HSP expression, which can be correlated with resistance to stress. Feder *et al.*, (1996) also reported that HSP70 levels in larvae and pupae of *Drosophila melanogaster* were high after heat shock treatment and their thermotolerance significantly improved. Several studies have shown a correlation between an increased resistance to supra-optimal temperatures with the induction of HSP synthesis in various organisms such as yeast (McAlister and Finkelstein, 1980), insects (Mitchell *et al.*, 1979; Dean and Atkinson 1983; Berger and Woodward, 1983). In locust, exposure to 45°C for 3h induces thermotolerance and the expression of HSPs; it is proposed that exposure to heat shock had the adaptive value of reducing thermosensitivity of the neural circuits in the locust flight system and allowing them to operate at higher temperatures (Robertson *et al.*, 1996). It can be seen that the CTMax 1h after heat shock was significantly increased in *Carcinus maenas* in comparison with the CTMax for both 2h and 4h after heat shock. Other workers have reported a similarly increased CTMax values for *Asellus aquaticus* heat shocked at 28°C for 60mins and it has been found that 1h after heat shock the CTMax was increased by 2.2°C (Korhonen and Lagerspetz, 1996). El-Wadawi and Bowler (1995) found that the exposure of adult blowflies to a sublethal heat shock at 36°C caused a transitory increase in heat resistance. This resistance was apparent 1h after the application of the shock, was maximal 2-3h later and had disappeared 6h after exposure. When adult *Locusta migratoria* were acclimated at 28°C, photoperiod 18:6, L:D 90% of the heat shocked animals survived exposure to 55°C for 3h whereas only 40% of the non HS controls survived. The greatest thermotolerance was found in animals that were tested within 3h after heat shock (Gray and Robertson, 1998); these results indicate that the effects of heat shock on CTMax are rapid and transient (Korhonen and Lagerspetz, 1996); and the heat shock can induce thermotolerance (Gray and Robertson, 1998). Li and Laszlo (1985) and Lindquist and Craig (1988) have reported that after some stress treatments or after thermal conditioning the thermotolerance is a transient resistance to lethal temperatures induced in cells and organisms (Carretero *et al.*, 1991). Anoxia is another

kind of environmental stress, and it has been shown that also prior anoxia treatment caused increased the thermotolerance. In locust (*Locusta migratoria*) for example, prior heat shock at 45°C for 3h and anoxic for 2h in nitrogen increased the thermotolerance of locust after 0.5 h and 1 h at 53°C. Locusts that treated with heat shock and anoxia had significantly higher survival rates than controls (Wu *et al.*, 2002). This result shows that the imposition of one stressor can induce tolerance to other stressors that is cross-tolerance occurs. This emphasis the general nature and importance of the stress response mechanism.

Increasing temperature generally causes hyperpolarization of muscle membrane resting potential (RP) (Stephens and Atwood, 1982). This is shown in many other studies of different animals (Prosser and Nelson, 1981; Harri and Florey, 1979; Stephens, 1985; Fischer and Florey, 1981). Pearson (1998) pointed that RP became hyperpolarized with increasing temperature for *C. pagurus*, this hyperpolarization has two origins one is diffusion potential predicted by the Nernst equation and Goldman-Hodgkin-Katz equation, the other is an ouabain-sensitive sodium-potassium ATPase pump. In this work, a similar result has been shown as the resting membrane potential hyperpolarized with increasing temperature for both control and heat shocked animals (Figures 4.4 and 4.5). In heat shocked animals the RP was more depolarized than RP of control animals, and failed at 36°C whereas in control animals it failed at 26°C. The RP disappearance is not owing to the microelectrode failing to remain in place, but it is a collapse of the potential itself. The resting membrane potential was not significantly different between control and HS animals (from 8-15°C), but was significantly different between control and HS animals (in the range above 16°C to failure). It is possible that the HSPs could cause the RP to be reduced and that effect is greater at the higher starting temperature. Barclay and Robertson (2000) found in locust *Locusta migratoria* acclimated at 25-30°C; 16:8 L:D photoperiod that there was no significant difference between resting membrane potentials in the hindleg extensor muscle control and HS animals. Although little is known about heat shock effects on neuronal properties, Ramirez *et al.*, (1999) studied adult locust *Schistocerca americana* which were heat shocked at 45°C, maintained for 6-24h at room temperature (24°C) to recover; the control insects were maintained at 24°C. Extracellular recordings from the metathoracic ganglion indicated that, in control animals acclimated at 24C° the amplitude

of K^+ current decreased at 31-35°C and then increased at 41°C, but in animals heat-shocked at 45°C, the amplitude of K^+ current decreased over temperature range of 31-41°C and at higher temperatures the K^+ outward currents were very reduced. Thus heat shock induced alteration in K^+ conductance which they argue might contribute to increased heat tolerance. We suggest that these electrophysiological changes contribute to the ability of heat shocked animals to maintain nervous system activity during a stress.

Figure 4.6 (A and B) shows the effect of temperature on EJP amplitude. In control animals the EJP amplitude decreased as temperature increased, and failed at 26°C. This similar to the temperature at which the resting potential also failed but the EJP failure occurred before RP failure in most cases. In controls, the amplitude of EJPs decreased with increasing temperature as found previously by Pearson (1998) for cold acclimated crabs. Whereas the amplitude of EJPs following heat shock were increased up to experimental temperatures 18°C, then gradually decreased, and failed at 32°C. Thus prior heat shock resulted in electrophysiological thermotolerance as demonstrated by the increased heat induced failure temperature of EJP. Thus the heat shock induced a significant thermal protection of the EJP. Previous work has demonstrated that heat shock can induce thermotolerance of synaptic transmission (Dawson-Scully and Robertson, 1998) and neuromuscular transmission (Barclay and Robertson, 2000; Karunanithi *et al.*, 1999). Barclay and Robertson (2000) found results qualitatively similar in *Locusta migratoria*, obtained from locust kept at 25-30°C and 16:8 L:D. Locust placed at 45°C for 3h to induce heat shock. Subsequently they found that the normalized EJP amplitude from experimental temperatures of about 22°C was decreased with increasing temperature for both control and heat shock, but EJP amplitude in heat shock animals was more stable than control up to about 40°C and had larger amplitudes than controls. In control animals EJP amplitude decreased more than those heat shocked animals with increasing experimental temperature. The failure point was at about 57°C in heat shock animals and about 53°C in control. They suggested that prior exposure to heat shock protected the neuromuscular response by altering the thermosensitivity of neuromuscular transmission and reducing the thermosensitivity of EJP amplitude. Wu *et al.*, (2002) found in *Locusta migratoria* maintained at 30°C on a 16:8 L:D, were heat shocked for 3h at 45°C. EJP amplitude failed

in controls at about 35°C but the failure was about 45°C in heat shock animals. Similar results were found in *Locusta migratoria* which had been exposed to nitrogen for 2h. These results suggested that heat shock or anoxia treatment resulted in thermotolerance in locusts as demonstrated by the increased heat induced failure temperature of action potential. It was also found that exposure of locusts to anoxia had protective effects against high temperature stress but that heat shock does not induce tolerance to anoxia. In the present study the changes in RP and EJP after heat shock at constant time of temperature (15.7-16.8°C) are not function of measurement time. Barclay and Robertson (2003) showed that in heat shocked preparations, the temperature at which synaptic transmission failed was increased by 4.8°C as compared to controls. They demonstrated that increasing extracellular calcium levels in heat shocked *Drosophila* larvae synapses from single abdominal muscle fibers induced thermoprotection by increasing the upper temperature limit for synaptic transmission.

The latent period of cold and warm acclimated crabs for both *C. maenas* and *C. pagurus* decreased with increasing temperature over whole temperature range; this is shown in previous work by Pearson (1998) and Cuculescu (1996). The same result was found in this work; Figures 4.7 and 4.8 show decrease in the latent period as temperature increased. There was no significant difference between the two groups but the latency increases over the temperature range 26-31°C in the heat shock group. Thus the temperature at which failure occurred was increased by heat shock. Barclay and Robertson 2000; they found that there was no significant different in latency between control and heat shocked locusts. Gray and Robertson (1998) have reported the effects of heat stress on axonal conduction in the locust *(Locusta migratoria)* which were acclimated at 28°C, 18:6, L:D. They reported that the conduction velocity was higher in control than in heat shocked animals with increasing temperature, but the amplitude measured from control animals was lower than from heat shocked animals from 35-50°C. They conducted from this that the conduction velocity and amplitude of signals are less sensitive to temperature changes above 35°C in heat shocked animals than in controls. It has been shown that at different temperatures, axonal conduction velocity was less sensitive in heat shocked animals than control (Gray and Robertson, 1998). Cuculescu (1996) has studied the effect of

temperature on axonal conduction velocity in adult *C. maenas* that acclimated at 8°C for 3 weeks. As would be expected increasing measuring temperature increased conduction velocity up to 33°C. This would suggest that the lengthening of latent period in heat shocked preparation at temperature above 26°C is not because of decreases in axonal conduction velocity this suggest that the rise is an effect of heat shock on synaptic events.

Decay time constant (Tau) gives a measure of membrane resistance (as capacitance is constant) (Hille, 1992; Fatt and Katz, 1953). Figures 4.9, 4.10 show the decay constant of the EJP in both control and heat shock crabs. In both groups the decay constant decreased with increasing temperature up to 15°C, which indicated decreasing membrane resistance (R_m) or an increase in the number of open channels (Fatt and Katz, 1953; Nicholls *et al.*, 1992) and this suggested that the membrane resistance was high at low temperature but fell at higher temperatures. In controls the decay time constant was slower than post heat shock over 8-15°C which indicates that in control the membrane resistance (R_m) is higher than after heat shock. But over 16-26°C the result was reversed, and the decay time constant was slower in post heat shock than control preparations. Tau reached a maximum at 17°C and was relatively constant from 18C°-23C° and then decreased. For both temperature ranges 8-15°C and 16-26 there was no significant difference between control and heat shock. However, the failure of decay constant was greater than control by 6°C in the heat shocked group than in control this is simply because of EJP failure.

C. maenas largely inhabits intertidal regimes and *C. pagurus* in more subintertidal. The possible relationship of the HSP70 response and the thermal tolerance of two crabs were studied. The present study examines the effects of exposure to different temperatures on the synthesis of heat shock proteins in the muscle tissue of the shore crab (*C. maenas*). Figure 4.12, it was found a pattern of synthesized proteins in control and heat-shocked animals which were raised clearly at two temperatures 30°C, and 33°C. These heat shocked samples has a greatest intensity of HSPs around 70-84KDa and seems to be synthesized after heat-shock. Crabs heat shocked at 31.5°C represents an experiment from an intermediate HS temperature. It might have been expected that a similar band of proteins to that present in crabs heat shocked at either 30 or 33°C. But not similar bands of proteins evident between around 70- 84KDa have seen in crabs heat shocked at 31°C compare with

others heat shock temperatures. A possible explanation for this discrepancy might be the lower amount of protein used in lane5 (compare the density of the protein around 45KDa between relative lanes) despite similar amount of protein loaded each lane. It has been seen that no heat shock proteins were produced following exposure to 75%seawater + 25% distilled water which suggests did not constitute a significant stress effect on the *Carcinus*.

In comparison between control and heat shocked *C. maenas*, Figure 4.13 showed expression of proteins in muscles of *C. maenas* acclimatized in summer. *C. maenas* has a greater intensity of HSP below the band 84KDa and seems to be HSP70, induced by heat shock at 32°C (lane 4) and were absent under control conditions (lane 3). These elevated levels of HSP70 indicate a greater need for molecular chaperones. In the deep sea vent shrimp *Rimicaris exoculata* it was found that at least two proteins of the HSP70 family are present in both control and heat shocked animals. However, the heat shocked animals have more abundant HSP70 than the control animals (Ravaux *et al.*, 2003). Control and heat shocked *C. pagurus* at 23°C are compared. It can be seen that the greatest intensity of HSP around 70-84KDa is seen in controls (lane 5) whereas in heat shocked crabs no proteins are found in this region (lane 6). It might relate to varies degrees of HSPs in different organisms (Parsell and Lindquest, 1993) or the heat shock given was too great for *C. pagurus*. Nakano and Iwama, (2002) noted that the function, as well as the range of functional temperature, of constitutive and inducible forms of HSP70 may be different between two kinds of sculpins (they have distinct distribution patterns in the intertidal zone). It was proposed that the less thermally sensitive tidepool sculpin may enhance its thermal tolerance by having a large pool of cellular HSP70, thus allowing it to inhabit the upper intertidal zone with relatively large fluctuations in environmental variables. It was found that HSP70 expression can be triggered by many non thermal stresses (Feder and Hofmann, 1999), including pressure variations (Welch *et al.*, 1993). Tomanek and Somero (1999) also noted that HSP70 in gills were higher in the species of marine snails living to the higher intertidal zone, compared with the species living to the lower intertidal zone. This might be expected that heat shock response may have been modified during adaptation to different thermal habitats of these animals (Nakano and Iwama, 2002). This result is similar to recently result in this chapter as *Carcinus maenas* which live in the

intertidal zone, see Figure 4.13, lane 4 which is a sample of *C. maenas* heat shocked at 32°C and lane 6 which is a sample of *C. pagurus* heat shocked at 23°C. Miller and McLennan (1988) found that in the larva of the cryptobiotic brine shrimp *Artemia,* when heat shocked at 40°C for 5 minutes it was sufficient to enhance the subsequent expression of HSPs while 45 minutes to 1 hour at this temperature causes the strong repression of non-HSP synthesis.

The proteins are induced in different species at different temperatures, variation are also reported for the quantity of HSPs present. The changes in endogenous concentrations of stress proteins between both species, *C. maenas* and *C. pagurus* may contribute to the development of thermotolerance, which is granted by elevated endogenous levels of HSPs in cells (Hofmann and Somero, 1995). In *C. maenas,* the synthesis of an HSP70 that inducible form occurred following a heat exposure at 32°C and thus may reflect the emergence of cellular damage. Interpretations are complicated by the fact that some of the proteins are likely to play important roles in normal physiology as well as during HS. Moreover, resistance to high temperatures is probably mediated not only by the synthesis of HSPs, but by other mechanisms as well (Lindquist, 1986). Although increased concentrations of HSPs are indicative of thermal stress, they are not of themselves indicative of increased amounts of irreversible denaturation. Thus, elevated levels of HSPs may simply reflect effective rescue of heat-damaged proteins, rather than increases in irreversible protein denaturation (Hofmann and Somero, 1995). To use endogenous levels of HSP70 as an index of environmental thermal stress may require separate analyses to be made of each isoform (Tomanek and Somero, 2002).

In summary, heat shock increases thermotolerance in *C. maenas.* Thermal resistance was determined from measurements of CTMax that requires the maintenance of co-ordinated behaviour. Heat shock alters the characteristics of the response of a number of neurophysiological functions, namely the temperature dependence of RP and EJP. The temperature of EJP in particular changes in a complex manner, being small at low temperatures (8-10°C) has a maximum value at 15-18°C and declines at higher temperatures. A consistent feature of there measurements is that HS allows the continuance of function some 5-10°C higher than in control animals.

Chapter Five

Muscle tension: Effects of temperature, photoperiod and heat shock.

Introduction

The central nervous system plays a major role in controlling activity and muscle contraction as temperature changes. The electrical activities in central and peripheral nervous system may alter in a compensatory manner (Lagerspetz, 1974; Prosser and Nelson, 1981). Crustacean motor neurons are divers in their physiology and morphology (Atwood *et al*, 1994). Some of the characteristics of the crustacean neuromuscular system are: (1) Each muscle fibre has numerous motor endings (neuromuscular synaptic terminals), each nerve branches many times so the innervation of muscle is poly-neuronal or poly-terminal. (2) One efferent axon supplies many muscle fibres. (3) Each axon supplying a given muscle may result in different effects on muscle contraction (e.g. phasic or tonic). (4) Axons are divided into excitatory and inhibitory fibres (Atwood, 1976; Atwood *et al.*, 1994). Excitatory terminals have more synaptic endings than inhibitory terminals, but inhibitory synapses are larger in size than excitatory terminals. Excitatory axons are often divided into slow or tonic and fast or phasic (twitch). The difference between tonic and phasic are (1) Tonic neuron activity is involved with general movements, whereas phasic neuronal innervation is responsible for rapid and defensive actions such as fighting. (2) Tonic neurons fire at low frequency to maintain posture but phasic axons are silent most of the time until needed then fire bursts of action potential (Atwood, 1976). Phasic motor axons are larger than tonic motor axons; their synaptic terminations on the muscle fibres are very slender, threadlike and profuse. In contrast the synaptic endings of tonic motor neurons are comprised of many varicosities connected by intervening bottlenecks (Atwood *et al.*, 1994). It has been shown in crayfish abdomen muscles that fast flexor nerves branch more and innervate more muscle fibres than slow flexor nerves as neuron is involved in defensive movements and uses of all available active

muscle fibers (Arcaro and Lnenicka, 1995). Due to the different stimulation characteristics on the closer muscle it seems probable that the motor axon which is continuously active (tonic axon) would affect the phenotypic characteristics of the muscle fibre (Rathmayer and Maier, 1987).

The closer muscle in *Carcinus* to the dactylopodite is innervated by two motor axons and one common inhibitory nerve (Wiersma and Ripley, 1952); consists of four types of muscle fiber types I to IV. Type I and type II muscle fibers are innervated by both fast and slow axons. EJP characteristics of them have small variation in the decay time constant and a smaller degree of variation in facilitation (Rathmayer and Maier, 1987; Rathmayer and Erxleben, 1983; Rathmayer and Hammelsbeck, 1985). In dually innervated muscle fibers of type I and II, facilitation by fast innervating axons produces facilitating EJP's, which are smaller than the facilitating EJP's by slow innervating axons regardless of the EJP amplitude (Rathmaye and Hammelsbeck, 1985). Type III and IV muscle fibers only show successful EJP when innervated by the fast excitor closer (FEC) axon, but no response from slow excitor closer (SEC) and are not innervated by the common inhibitor (Rathmayer and Maier, 1987). Type III and IV fibers have fast time constants of EJP decay, which exhibit little to medium facilitation. At higher frequencies, the fast EJP's facilitate more than tonic EJP's (Atwood, 1976). At low frequencies of stimulation (< 10Hz) there is no variation in facilitation in a fiber type stimulated by the fast and slow axons (Rathmayer and Hammelsbexk, 1985; Rathmayer and Maier, 1987). During movement in a normal whole animal, the slow axon may fire at 250Hz, the EJP's facilitate and summate depolarizing the muscle fibre. The muscle fibers develop full strong contraction if all synapses are active. If just a limited number of synapses are activated, then a more graded contraction may occur (Hinkle *et al.*, 1971). The crabs *Carcinus maenas* and *Cancer pagurus* could modulate the dactylopodite position by activity of the closer and opener muscles through varying the extent of synaptic activation only of the closer muscle. This type of modulation occurs in all the muscle groups and it is the method employed in crabs and other crustacea to control limited movements (Rathmayer and Hammelsbeck, 1985). Generally at lower frequencies, in response to slow fibre activity, EJP's have longer time constant (τ), and summate and facilitate, increasing the EJP

amplitude and the degree of muscle depolarization. In contrast responses to fast fiber activity; at higher frequencies, fast fibers activity results in greater facilitation and may exhibit all or nothing spikes. Fast muscle fibres may show some plasticity of effect depending on its previous activity (Atwood and Nguyen, 1995). A muscle fiber perhaps classed as type Π but due to an activity change, show properties similar to a type I fibre or will be in between type Π and I. Seasonal changes are apparent in crayfish *Procambarus clarkii*, in a summer the closer muscle is more varicose and the axon innervating initiates smaller less fatigable EJP's than in winter (Lnenicka and Zhao, 1991). This dependence on the time of year may be related to the hormonal and moulting cycle (Atwood and Nguyen, 1995; Kivivuori, 1980). Muscle fibers are nearer an oval shape, and are relatively large in size (Rathmayer and Maier, 1987; Orkand, 1962; Atwood, 1976; Maier *et al.*, 1986). Due to the large size and extensive infoldings of crustacean muscle fiber membranes, the cells have low resistance (R_m) and high capacitance (C_m) (Hill, 1992; Castillo and Machue, 1953; Orkand, 1962). Time constant (τ) is proportional to (R_m) and capacitance (C_m), is effectively a measure of membrane open channels. The relation between them is $\tau = R_m \times C_m$. The decay of the EJP will reflect the time constant of the membrane (Hill, 1992; MacDonald,1990).

It has been identified that crustacean muscle and nerve action potentials (AP's) are instigated by an increase in conductance to Na^+ (Cukierman, 1996) and Ca^{2+} (Niwa and Kawai, 1982). The channel activities being different, Na^+ channels being responsible for the initial rise of the AP spike, the Ca^{2+} component appears slightly slower and contributes to the AP width. Blocking a single component of the spike decreases or prevents successful EJP generation (Stephens and Church, 1988). The AP may be blocked at a branch point (bottleneck area) of higher axoplasmic resistance or at terminal branches and therefore that less Ca^{2+} enters and consequently not enough transmitters is released to initial an EJP; temperatures of block can be modified by acclimation (White, 1983). Glutamate is a neurotransmitter for motor axons at the neuromuscular junction (NMJ) and gamma-aminobutyric acid (GABA) the inhibitory transmitter (Takeuchi and Takeuchi, 1966). It has been shown fibers producing larger EJP's have large sized excitatory synapses and

contact muscles with higher input resistance than fibers producing smaller EJP's (Atwood, 1976).

The aim of the work in this chapter was to identify the temperature and photoperiod effects on the closer muscle isometric contractions and the ability of the tonic motor axon to generate muscle contraction over an experimental temperature range. Also the aim was to investigate the contribution of facilitation to muscle function by comparison of crabs subject to long and short photoperiod having the same thermal acclimation either cold and warm, pre and post heat shocked animals.

Method.

The effect of temperature on closer muscle force development was measured in adult *Carcinus maenas*, the protocol described in Chapter 2, section 2.8 was used. Briefly, isometric tension was measured from cold (8°C) and warm (25°C) acclimated *Carcinus maenas* at different photoperiod, either short day (8:16 L:D) or long day (16:8 L:D) conditions. Animals were held in the acclimation condition for at least two weeks. For heat shock experiments, briefly, isometric contractions were measured only from 8°C acclimated crabs. Crabs were heat shocked at 32°C for 30min; following the period of heat shock the crabs were returned to their acclimation temperature for recovery. During the recovery period legs were rapidly dissected and prepared for muscle force measurements. All measurements were taken up to 90mins from the end of heat shock. A transducer unit was used for the measurement of the closer muscle isometric tension. The tip of the dactylopodite was connected to the transducer arm and the opener muscle was cut so that the force due to the closer muscle only was recorded. Care was taken to ensure constancy of the distance between the position of the transducer in relation to the joint to ensure direct comparison between different animals, animals used were of relatively constant size (between 5- 7cm). The nerve was stimulated at different frequencies of 10, 20 and 50Hz and in each case 10 stimuli were given in order to ensure that the force generated in each case was due to a total of ten EJPs. The muscle force measurements were recorded over the experimental temperature from 8°C to 26°C.

Results.

Figure 5.1 shows the muscle tension in cold (8°C) acclimated *C. maenas* with nerve stimulation at 10Hz in both short and long photoperiod groups. It can be seen that in both groups the tension decreased with increasing experimental temperature, but the tension was larger and decreased dramatically for the long day acclimated group compared with the short day acclimated crabs. Little measurable force was generated at temperature more than about 23°C in cold acclimated animals at the long day photoperiod. There was a significant difference between long and short day photoperiod in cold acclimated animals (Regression, $P<0.001$). It can be noticed that there was a very large difference in EJP amplitudes of animals from short and long day photoperiod. In long day acclimated groups, the muscle force was maximum at about 160mN whereas in short day maximum tension was about 80mN at the acclimation temperature (8°C). In Figure 5.2 with acclimation at higher temperature (25°C) it can be seen that there was a decrease in force with increasing temperature in both short and long day acclimated animals. Over this range the force generated by short day group was consistently larger that of the long day group but the two relationships were parallel. Comparisons of the effects of temperature on the cold acclimated group (Fig. 5.1) with the warm acclimated group (Figure 5.2) show:

1. Tension in both groups reduced with acclimation temperature but in the warm acclimated group tensions were measurable up to at least 26°C compared, but only about 23°C in the cold acclimated group. There was relatively little effect of photoperiod on this upper temperature limit.

2. Comparisons of the rate of decline of tension with experimental temperature shows that in the warm acclimated group the temperature amplitude relationship for both long and short day were parallel. In contrast, with low temperature acclimation there was a marked difference in the reduction of tension with experimental temperature. This difference is due to the fact that for animals acclimated at 8°C the long day photoperiod group showed a much higher tension (160mN) at the acclimation temperature than the short day group (80mN).

Further experiments were carried out using stimulus frequencies of 20Hz and 50Hz for both photoperiodic groups. The results are presented in Figures 5.3 and 5.4 (8°C and

Figure 5.1: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 8°C with photoperiods of short day (8:16 L:D) and long day (16:8 L:D). Stimulus frequency was 10Hz. Data is presented as mean ± S.E. mean; numbers of experiments were, 8:16 L:D (n= 8), 16:8 L:D (n= 6). For compare using comparison of the slopes were found to be significant different (Regression, $p<0.001$)

Figure 5.2: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 25°C with photoperiods of short day (8:16 L:D) and long day (16:8 L:D). Stimulus frequency was 10Hz. Data is presented as mean ± S.E. mean; numbers of experiments were, 8:16 L:D (n= 6), 16:8 L:D (n= 6). For compare using comparison of the slopes were found to be not significant different (Regression, $p>0.05$)

Figure 5.1: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 8°C with photoperiods of short day (8:16 L:D) and long day (16:8 L:D). Stimulus frequency was 10Hz.

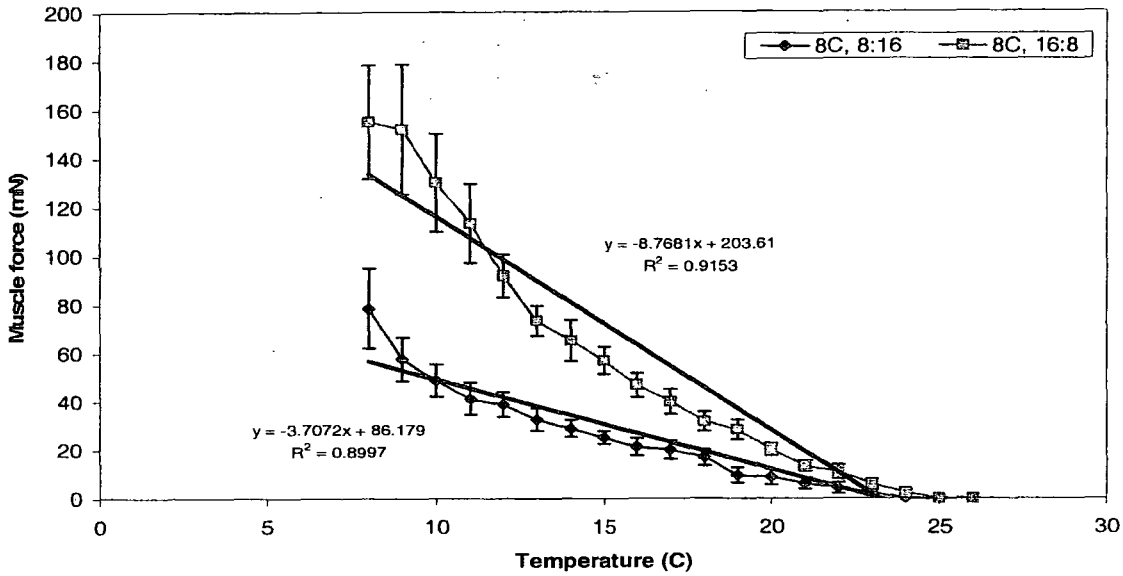


Figure 5.2: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 25°C with photoperiods of short day (8:16 L:D) and long day (16:8 L:D). Stimulus frequency was 10Hz.

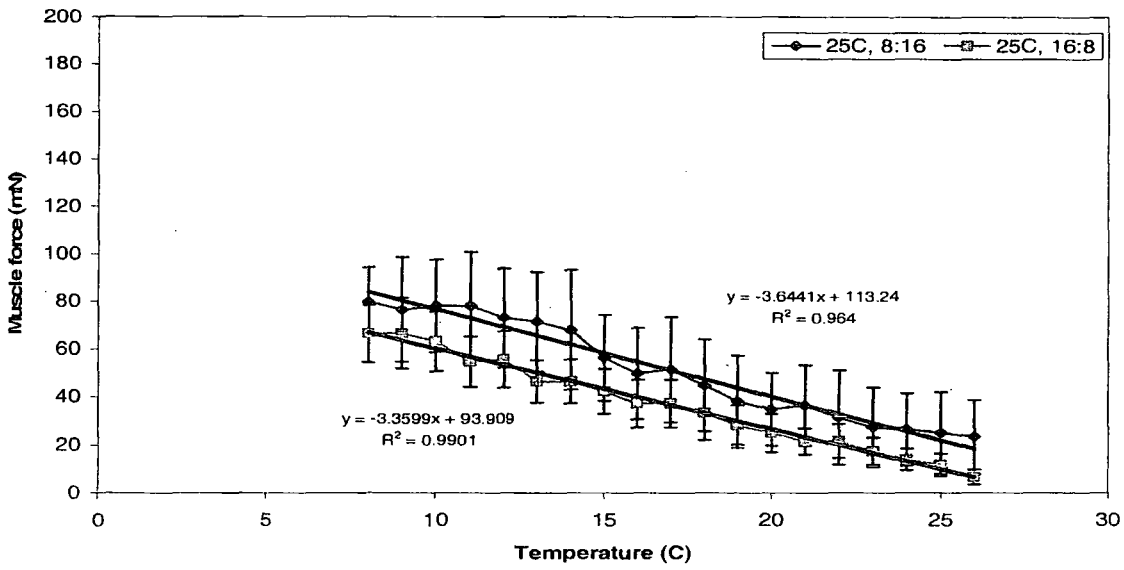


Figure 5.3: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 8°C with photoperiods of short day (8:16 L:D) and long day (16:8 L:D). Stimulus frequency was 20Hz. Data is presented as mean ± S.E. mean; numbers of experiments were, 8:16 L:D (n= 8), 16:8 L:D (n= 6). For compare using comparison of the slopes were found to be significant different (Regression, $p<0.001$)

Figure 5.4: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 25°C with photoperiods of short day (8:16 L:D) and long day (16:8 L:D). Stimulus frequency was 20Hz. Data is presented as mean ± S.E. mean; numbers of experiments were, 8:16 L:D (n= 6), 16:8 L:D (n= 6). For compare using comparison of the slopes were found to be significant different (Regression, $p<0.01$)

Figure 5.3: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 8°C with photoperiods of short day (8:16 L:D) and long day (16:8 L:D). Stimulus frequency was 20Hz.

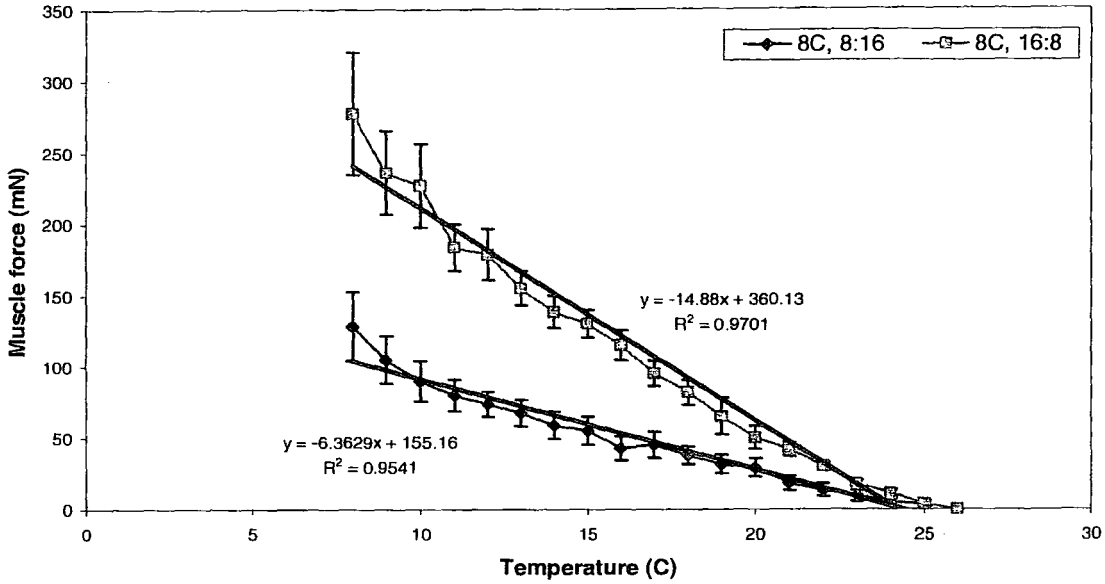


Figure 5.4: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 25°C with photoperiods of short day (8:16 L:D) and long day (16:8 L:D). Stimulus frequency was 20Hz.

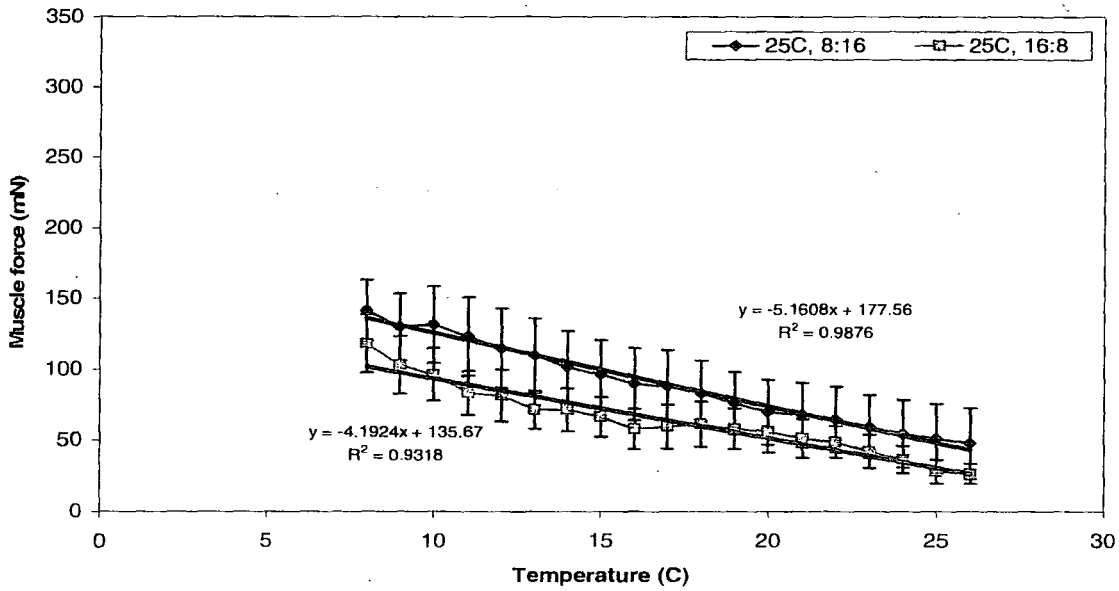


Figure 5.5: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 8°C with photoperiods of short day (8:16 L:D) and long day (16:8 L:D). Stimulus frequency was 50Hz. Data is presented as mean ± S.E. mean; numbers of experiments were, 8:16 L:D (n= 8), 16:8 L:D (n= 6). For compare using comparison of the slopes were found to be significant different (Regression, $p<0.001$)

Figure 5.6: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 25°C with photoperiods of short day (8:16 L:D) and long day (16:8 L:D). Stimulus frequency was 50Hz Data is presented as mean ± S.E. mean; numbers of experiments were, 8:16 L:D (n= 6), 16:8 L:D (n= 6). For compare using comparison of the slopes were found to be significant different (Regression, $p<0.05$)

Figure 5.5: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 8°C with photoperiods of short day (8:16 L:D) and long day (16:8 L:D). Stimulus frequency was 50Hz.

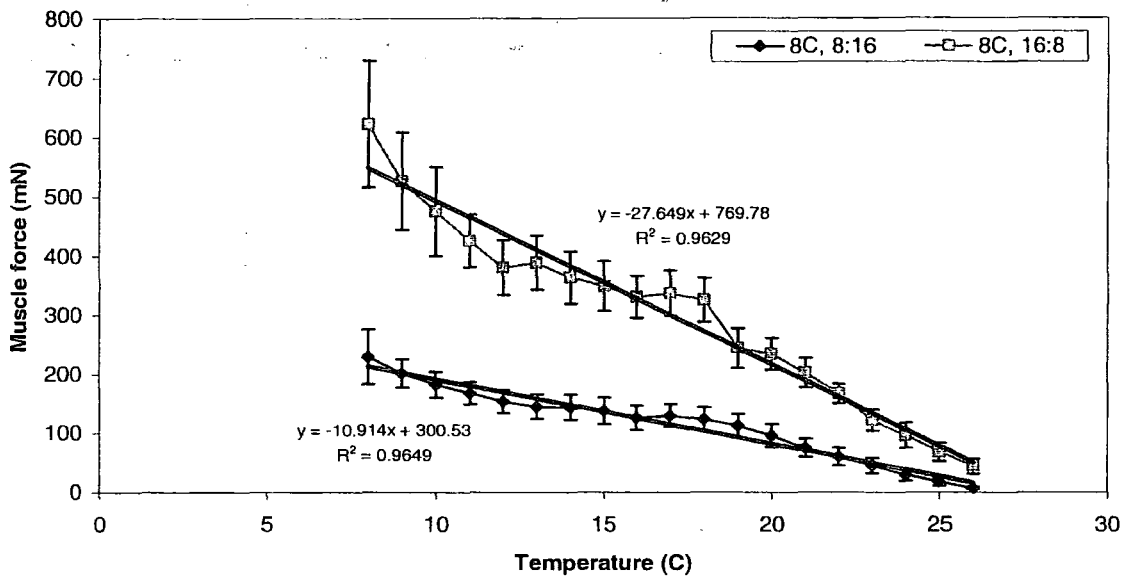
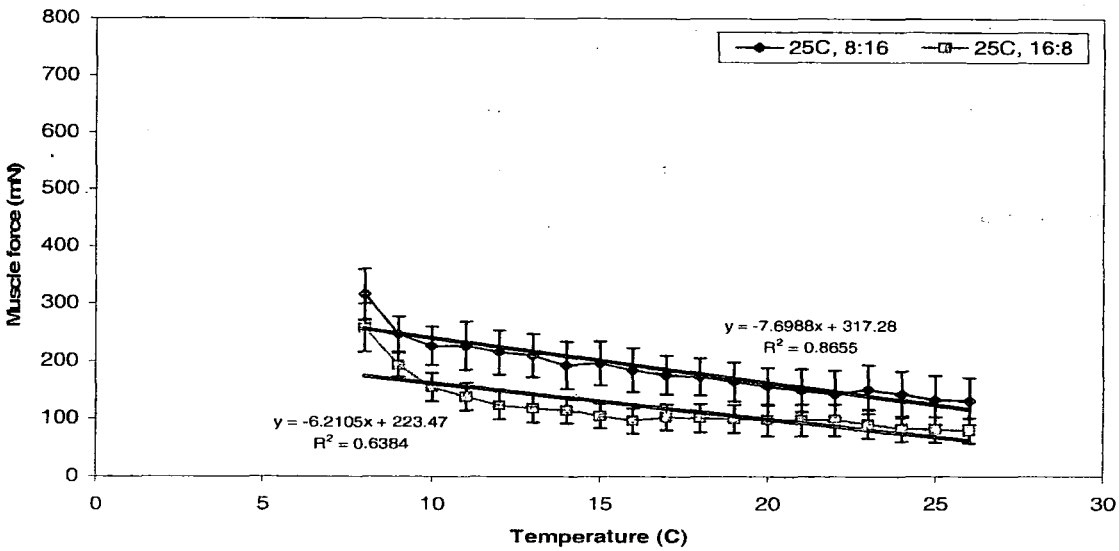


Figure 5.6: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 25°C with photoperiods of short day (8:16 L:D) and long day (16:8 L:D). Stimulus frequency was 50Hz.



25°C acclimated respectively, 20Hz) and in Figures 5.5 and 5.6 (8°C and 25°C acclimated respectively, 50Hz). The results obtained for the higher stimulation frequencies were qualitatively similar to those found with stimulus frequencies of 10Hz. With higher stimulation frequencies the tensions produced by the closer muscle were generally larger over the experimental temperature range. But, as for the results produced by 10Hz stimulation, there was a large difference in the tension and its decline with temperature between short day and long day acclimation in cold acclimated groups. Such a difference was not apparent in warm acclimated groups. The data from these experiments are shown in Table 5.1.

Heat shock

Figure 5.7 shows the leg closer muscle tension developed in response to nerve stimulation at 10Hz in pre heat shock (control) and post heat shocked cold acclimated *C. maenas*. In control animals there was a linear decline in the tension-temperature relationship from a value of about 120mN at the acclimation temperature (8°C) to no measurable force developed above 25°C. After heat shock there was a large reduction in tension developed over the whole of this temperature range. So, at 8°C post heat shock the tension was reduced to about 42mN compared with 120mN in controls. The temperature-tension relationship in post heat shock animals also declined and no tension was measurable above 21°C. The difference between the two sets of data was significantly different (Regression, $P<.001$). Qualitatively similar results were found for stimulus frequencies of 20Hz and 50Hz from these acclimated animals. With higher stimulation frequencies the tensions produced by the closer muscle were generally larger over the experimental temperature range. The data from these experiments are shown in Table 5.2.

Discussion.

Under all combinations of conditions of acclimation, temperature, photoperiod and stimulus frequency there was a decrease in muscle force with increasing experimental

Table 5.1 shows a summary of variation in closer muscle tension with different stimulation frequencies over a range (8-26°C) of acute experimental temperatures from *Carcinus maenas* acclimated to cold (8°C) and warm (25°C) temperatures with short day (8:16 L:D) and long day (16:8 L:D) photoperiods.

Temperature-tension relationships were fitted by linear regression (Figures 5.1 to 5.6) and the slopes of these relationships under different conditions tabulated above. Also tabulated are the average tensions produced by the closer muscle at the acclimation temperature at each photoperiod. The table also records whether tension was present (+) or absent (-) at 25°C.

Table 5.1: Summary of experimental temperature characteristic of muscle tension in *Carcinus maenas* leg closer muscles in cold (8°C) and warm (25°C) acclimated animals at short day (8:16 L:D) and long day (16:8 L:D) photoperiods.

	10 Hz				**20Hz**				**50Hz**			
	Short day		Long day		Short day		Long day		Short day		Long day	
	8°C	**25°C**	**8°C**	**25°C**	**8°C**	**25°C**	**8°C**	**25°C**	**8°C**	**25°C**	**8°C**	**25°C**
Slopes	-3.71	-3.64	-8.77	-3.36	-6.36	-5.16	-14.9	-4.19	-10.9	-7.70	-27.6	-6.21
Tension at acclimated temperature, (mN).	80	85	160	70	125	145	275	115	230	330	620	270
Tension at 25°C	-	+	-	+	-	+	+	+	+	+	+	+

Figure 5.7: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 8°C in pre (control) and post heat shock. Stimulus frequency was 10Hz. Data is presented as mean ± S.E. mean; numbers of experiments were, pre HS (n= 7), post HS (n= 6). For compare using comparison of the slopes were found to be significant different (Regression, $p<0.001$)

Figure 5.8: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 8°C in pre (control) and post heat shock. Stimulus frequency was 20Hz. Data is presented as mean ± S.E. mean; numbers of experiments were, pre HS (n= 7), post HS (n= 8). For compare using comparison of the slopes were found to be significant different (Regression, $p<0.001$)

Figure 5.7: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 8°C in pre (control) and post heat shocked crabs. Stimulus frequency was 10Hz.

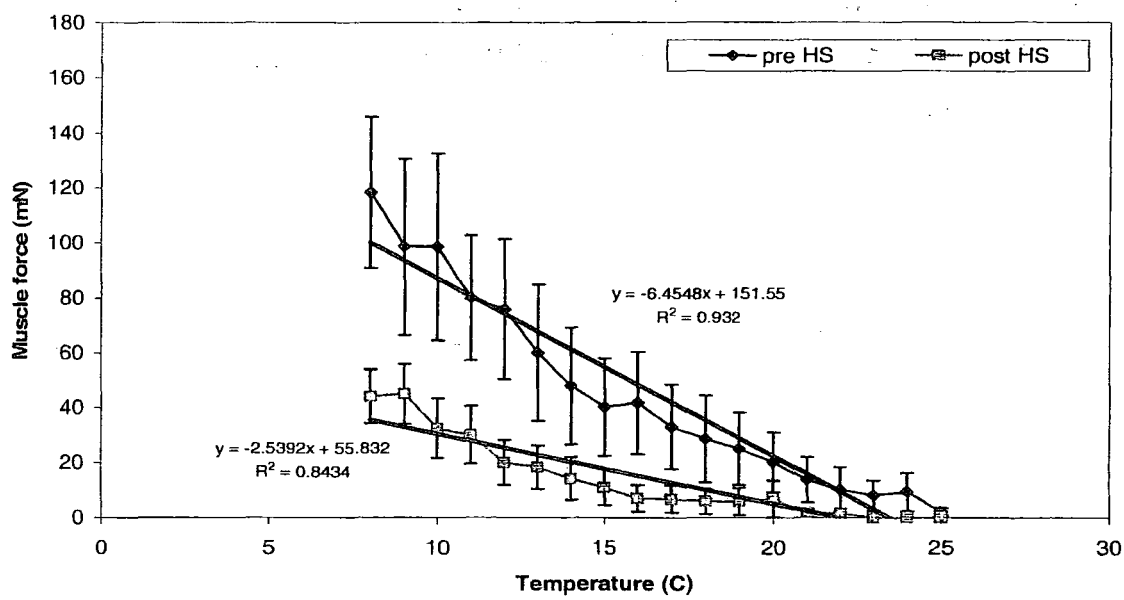


Figure 5.8: Muscle tension developed leg closer muscles of *Carcinus maenas* acclimated to 8°C in pre (control) and post heat shocked crabs. Stimulus frequency was 20Hz.

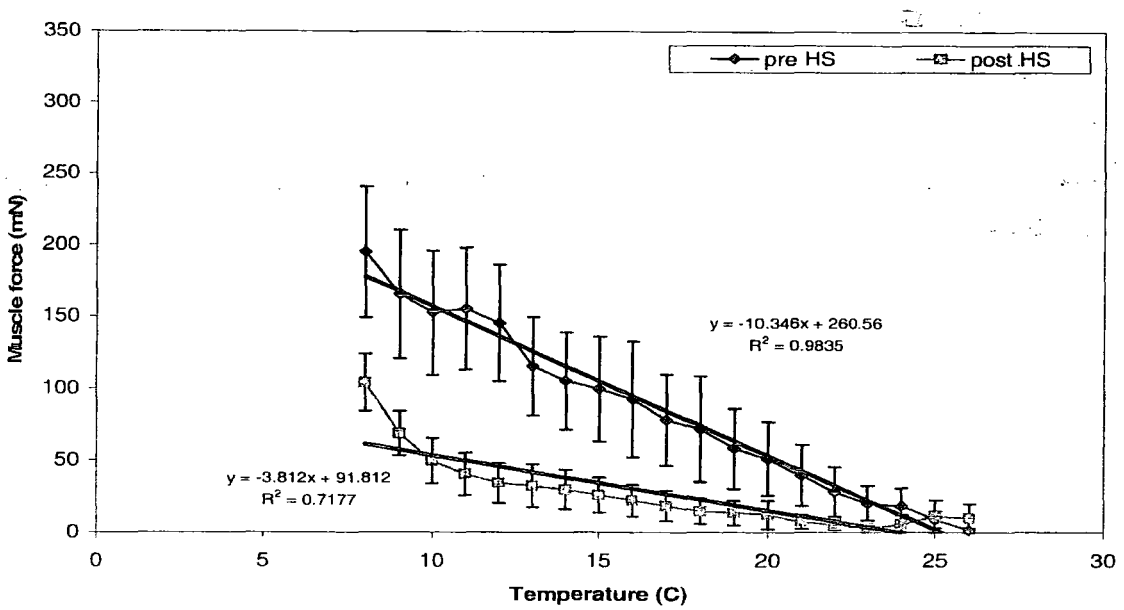


Figure 5.9: Muscle tension developed by leg closer muscles of *Carcinus maenas* acclimated to 8°C in pre (control) and post heat shock. Stimulus frequency was 50Hz. Data is presented as mean ± S.E. mean; numbers of experiments were, pre HS (n= 7), post HS (n= 8). For compare using comparison of the slopes were found to be significant different (Regression, $p<0.01$)

Figure 5.9: Muscle tension developed leg closer muscles of *Carcinus maenas* acclimated to 8°C in pre (control) and post heat shocked crabs. Stimulus frequency was 50Hz.

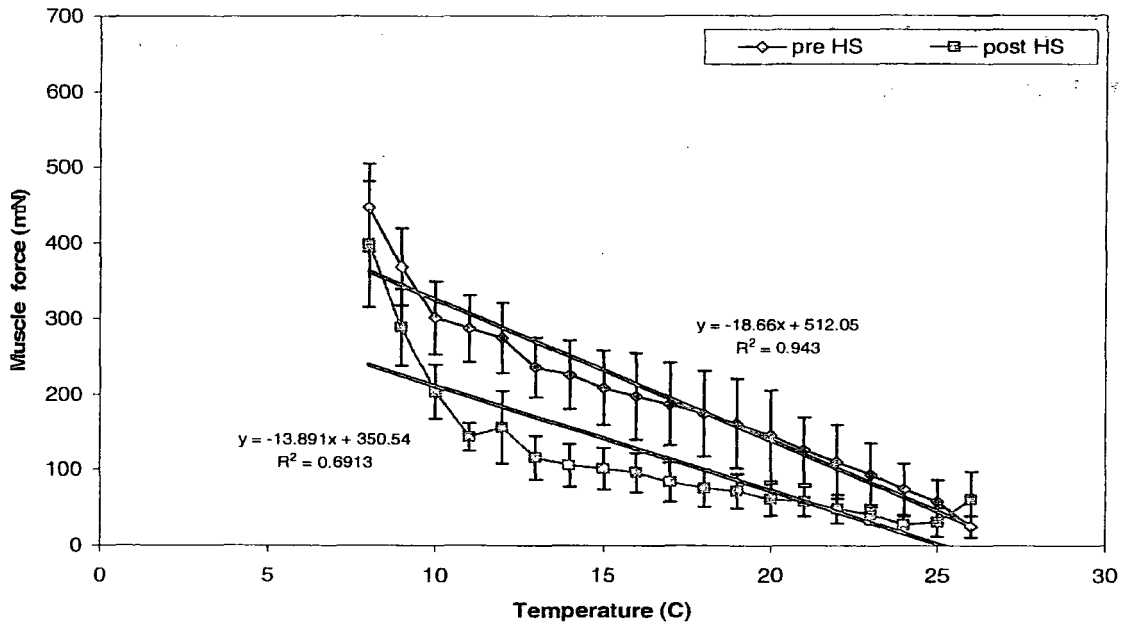


Table 5.2 shows a summary of variation in closer muscle tension with different stimulation frequencies over a range (8-26°C) of acute experimental temperatures from *Carcinus maenas* acclimated to cold (8°C) temperatures in either pre (control) or post heat shocked animals.

Temperature-tension relationships were fitted by linear regression (Figures 5.7 to 5.9), and the slopes of these relationships under different conditions tabulated above. Also tabulated are the average tensions produced by the closer muscle at the acclimation temperature at pre (control) and post heat shock. The table also records the upper temperature limit for both conditions.

Table 5.2: Summary of experimental temperature characteristic of muscle tension in *C. maenas* leg closer muscles in cold (8°C) acclimated animals between pre (control) and post heat shocked crabs.

	10 Hz		**20Hz**		**50Hz**	
	Pre-HS	**Post-HS**	**Pre-HS**	**Post-HS**	**Pre-HS**	**Post-HS**
Tension at 8°C	120	42	190	110	450	400
Upper temperature limit	26	21	26	22	26	26
Slopes	-6.45	-2.54	-10.35	-3.81	-18.66	-13.89

temperature (Figures 5.1 to 5.6). A variety of factors could be responsible for this decrease with increased temperature. Firstly it was noted in Chapter 3 that muscle resting potential was increased (more hyperpolarized) with temperature. Since the extent of muscle contraction in crustacean is determined directly by the membrane potential the decline in muscle force with temperature could be a reflection of the temperature induced muscle membrane hyperpolarization. This might not be the complete explanation, however, as the slopes of the decrease in muscle force. With temperature are different under different conditions, especially that of photoperiod. No such marked differences were evident in the changes of resting potential with temperature.

A second possible explanation for the decrease in muscle force with temperature could result from a reduction in EJP amplitude as a function of temperature. Figures 3.5 and 3.6 in Chapter 3 show the changes in EJP amplitude as a function of experimental temperature for cold and warm acclimated crabs under the two different conditions of photoperiod. With warm acclimation, either at short or long day photoperiods, EJP amplitudes were relatively constant over the experimental temperature ranges of 8 to 26°C. But in cold acclimated crabs EJP amplitudes declined over this temperature range and therefore this reduction might be relate to the reduction in force generation in cold acclimated crabs. However, it cannot account for the reduction in force in warm acclimated crabs. Pearson (1998) noted that the force changes with experimental temperature in cold and warm acclimated *C. maenas* were similar to their EJP amplitude changes with temperature. Similar links between EJP amplitude and tension have been reported in crayfish by Orkand (1962), Hawaiian ghost crab by Florey and Hoyle (1976) and in crab by Stephens and Atwood (1982). A third possibility is that a change in facilitation with experimental temperature might be a factor in the reduction of force generated using multiple stimuli. Indeed it is evident that force generation is stimulus frequency dependent as the force generated under all conditions increases with an increase in stimulus frequency from 10Hz to 20Hz to 50Hz. This increase is non-linear, the higher stimulus frequency producing a disproportionate increase in force as would be expected from the combined effects of summation and facilitation. However, the results obtained in Figures 3.19 and 3.20 in Chapter 3 on the effects of temperature with respect to facilitation show that

facilitation is relatively constant for both temperature acclimation and photoperiod, except at higher experimental temperatures where there is a tendency for facilitation to increase rather than a decrease that would be expected to explain the force results. Increasing force with increasing stimulation frequency in all groups has been shown in similar systems by Harri and Florey (1979) and maximal force was recorded at 50Hz for both warm and cold acclimated *Pachygrapsus crassipes* by Stephens and Atwood (1982). The results reported here are similar to those reported by Pearson (1998) except that in the latter report the force generated with higher stimulus frequencies (50Hz) showed a maximum at about 7°C for crabs acclimated to 8°C and a maximum at about 11°C for crabs acclimated to 22°C. Pearson (1998) suggested that these results indicated a compensatory acclimation of muscle force with temperature acclimation but from his records the maximum force generated with warm acclimation was still lower that than in cold acclimated animals

Two questions remain: does muscle force show an acclimatory shift with temperatures and does photoperiod have any effect on the force-temperature relationship? To answer the first question it is necessary to compare the force-temperature relationships in animals acclimated to 8°C and 25°C. Comparison of cold acclimate crabs using warm acclimated crabs from short day photoperiods (Figures 5.1 and 5.2) show that the slopes and intercepts of the two lines are very similar (-0.371 and -0.336; 8.618 and 11.324 respectively). Such similarities are also found at higher stimulus frequency for cold and warm acclimated crabs from short-day groups. For short day acclimated crabs there is however a difference between cold acclimated and warm acclimated animals. The muscle tension on cold acclimated crabs decreases with temperature until no tension is recoded at temperatures above 23°C with a stimulus frequency of 10Hz not about above 26°C with a stimulus frequency of 50Hz. By contrast, in warm acclimated crabs from short day photoperiods, muscle tension is still significant at 26°C at these stimulus frequencies. Warm acclimation therefore enables the leg closer muscle to maintain its ability to produce significant tension at higher temperatures. A marked effect on muscle tension is seen with photoperiod. By contrast with short day, cold acclimated crabs in which the temperature-tension relationship is not much different from warm acclimated crabs, the long-day, cold acclimated crabs display markedly increased tensions at the acclimation temperature

(90mN versus 160mN at 8°C). Such an elevation in force generation is not seen in long-day acclimated crabs at the higher acclimation temperature. In fact the force generated at the higher acclimation temperature group with long day photoperiods is somewhat decreased compared with the short-day photoperiod group at the same acclimation temperature. These differences are apparent with experiments from all stimulus frequencies.

Thus, long day acclimation results in a change in the force production at low temperatures of acclimation which is not seen in animals acclimated to higher temperatures. Nevertheless, the increased force in long day cold acclimated animals declines with increased experimental temperatures such that little measurable force is evidence at about 24-26°C. This coincides with the disappearance of force generation with experimental temperatures in the short day cold acclimated groups. Therefore long day photoperiods do not protect the force generation at higher temperatures compared with short day photoperiods-force generation disappearing at roughly the same elevated experimental temperatures.

Closer muscle force generation was stimulated at different frequencies by tonic axon which was selectively stimulated and all force measurements were determined from muscle fibre types I and II only, no force was generated by muscle fibre types III and IV (Rathmayer and Maier, 1987). Throughout the force measurement experiments, crabs acclimated at 25°C either at short or long day photoperiods generated decreased force being linked to increased lability of excitation contraction couple (E-CC) (Harri and Florey, 1979). Fischer and Florey (1981) reported E-CC in *Astacus leptodactyulus* became more effective with increasing experimental temperature, but nerve-evoked tension decreased with increasing experimental temperature. This was linked to changes in EJP summation as the threshold for E-CC was shown to be almost independent of temperature (Fischer and Florey, 1981). Thus the changes in force with experimental temperature are might be related to muscle fibre depolarization or electromechanical coupling. Under control and heat shock *C. maenas* acclimated at 8°C and stimulus frequency also there was a decrease in muscle force with increasing experimental temperature (Figures 5.7 to 5.9). The decline in muscle force with temperature could be a reflection of the temperature

induced muscle membrane hyperpolarization. It was noted in Chapter (4) that muscle resting potential was increased (more hyperpolarized) with temperature under both conditions control and heat shock crabs. However, as the slopes of the decrease in muscle force, no such marked differences were evident in the changes of RP with temperature. Other possibility for the decrease in muscle force with increasing temperature in control and heat shocked animals could result from a reduction in EJP amplitude. It was found in Chapter (4), Figure (4.7) with heat shock animals EJP amplitude were relatively constant over the experimental temperature range of 8°C to 11°C. From 12°C to 18°C EJP amplitude were increased then gradually decreased and failed at 32°C where there is a tendency for EJP amplitude to increase rather than a decrease that would be expected to explain the force results. However, in control animals EJP amplitude decreased as temperature increased, and failed at 26°C. Therefore this reduction of EJP amplitude might be related to the reduction in force generation. However, it can not account for the reduction in force in heat shock group. Thus the changes in force with experimental temperature are might be related to muscle fibre depolarization or electromechanical coupling. In fact the force generated in heat shock group acclimated at 8°C is somewhat decreased compared with control group at the same acclimation temperature. These differences are apparent with experiments from all stimulus frequencies. The muscle tension on control group decreases with temperature until no tension is recorded at temperatures above 25°C with a stimulus frequency of 10Hz. Whereas in heat shock group no tension was measurable above 21°C. Thus heat shock does not protect the force generation at higher temperatures compared with control-force generation disappearing at roughly the same elevated experimental temperatures.

Chapter Six

General Discussion

The aim of the study reported here was to address the question of whether there was any interaction of photoperiod with the attainment of acclimation to temperature in an ectothermic animal. The reasons for carrying out this study were the observation that average temperature and photoperiod covary with latitude. Very few studies have been carried out previously to investigate the relationship between temperature acclimation and photoperiod.

Furthermore temperature acclimation may be due to direct effects of temperature at the tissue level, whereas photoperiod would be expected to act through photoreceptors and transduction mediated centrally by CNS/ hormonal effects. Pearson (1999) attempted to separate the roles of central process (CNS/hormonal) from local ones in an ingeneous experimental paradigm in which peripheral leg muscles were acclimated to different temperature that the CNS. The results of these experiments using heterothermal acclimation showed that the attainment of compensatory temperature acclimation in leg neuromuscular properties was a response to peripheral, leg temperatures with no effects attributed separate acclimation of central systems at low temperature. However, with warm acclimation of central systems the attainment of compensatory acclimation at the peripheral leg locus was affected.

The experimental procedures adopted here was to homothermally acclimate crabs (*Carcinus maenas*) under combinations of conditions of temperature and photoperiod and examine leg muscle neurophysiology. Chapter 3 reports microelectrode studies of neuromuscular transmission in the leg closer muscle in animals acclimated at 8°C or 25°C with either short day photoperiods (8hr:16hr, L/D) or long day photoperiods (16hr:8hr, L/D). The main findings were that photoperiod had no effects on EJP latency, time constant or EJP amplitude. Whereas there was a significant difference with photoperiod for acclimation in the resting potential and EJP facilitation. In addition, it was found that

photoperiod had a marked effect on force generation of the closer muscle. This was evident in the difference in force-temperature relationship in cold acclimated groups whereas muscle force was consistent by much grater for animals from long day photoperiods compared with short day photoperiods. In warm acclimated animals this difference was reduced such that photoperiod had little effect on the temperature-force relationship.

A second line of enquiry was pursued in the role of heat shock in affecting thermotolerance to high temperatures. Thermotolerance as measured by CTMax was increased by a prior heat shock which also resulted in the induction of heat shock proteins. Heat shock also affected the neurophysiological parameters of neuromuscular activity. EJP amplitudes were reduced by prior heat shock but the upper temperatures limits of the EJP was increased significantly compared with non heat shock. Prior heat shock and post heat shock also reduced force generation in the closer muscle.

In this work we found a strong effect of photoperiods on acclimation to temperature, it might be interesting to know the effect of photoperiods with another species like *C. pagurus*, which in many respects shows better acclimatory responses than *C. maenas*; and are there any differences the effect of photoperiod on acclimation between these two species. Other line of enquiry that how does photoperiod act? This suggests doing different experiments in different ways i.e. does photoperiod effect on the central nervous system or it is a hormonal effect. In future work also it should be interesting to look at HSPs. As shown in this work there is a different pattern of synthesized HSPs in comparison between *C. maenas* and *C. pagurus*. This suggests doing experiments to determine more identification of HSPs by using anti bodies of heat shock proteins plus doing two dimension gels. Also it might be useful using different stresses rather than the heat to look at heat shock response.

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