Adaptive responses to temperature in homogeneously and heterogeneously acclimated crabs

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ADAPTIVE RESPONSES TO TEMPERATURE IN HOMOGENEOUSLY AND HETEROGENEOUSLY ACCLIMATED CRABS.

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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.

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GLOSSARY

AP= Action potential

CN/CL= Cold central nervous system with a cold leg

CN/HL= Cold central nervous system with a hot leg

CNS= Central nervous system

C_m= Membrane capacitance

C.maenas= *Carcinus maenas*

C.maneas 8= Free *Carcinus maenas* acclimating at 8°C

C.maenas 22= Free *Carcinus maenas* acclimating at 22°C

C.pagurus= *Cancer pagurus*

C.pagurus 8= Free CP acclimating at 8°C

C.pagurus 22= Free CP acclimating at 22°C

CRO= Cathode ray oscilloscope

CTMax= Critical thermal maximum

CV= Conduction velocity

DAP= Depolarising after potential

°C= Degrees Celsius

E-CC= Excitation contraction coupling

E_{ion}= Equilibrium potential of ion x

EJP= Excitatory junctional potential

E_{junc}= Junctional potential

ER= Endoplasmic reticulum

E_{tip}= Tip potential

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

H_{II}= Inverted hexagonal phase

HN/CL= Hot central nervous system with a cold leg

HN/HL= Hot central nervous system with a hot leg

IM *C.maneas* 8= Immobilised *C.maneas* acclimating at 8°C

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ME= Microelectrode

mM= millimolar

msec= milliseconds

mV= millivolts

NMJ= Neuromuscular junction

R_m= Membrane resistance

RP= Resting potential

S.E= Standard error

SR= Sarcoplasmic reticulum

Tau= Decay time constant

TNB= Thermoneuromuscular block

$[X]_i$= Intracellular concentration of ion x

$[X]_o$= Extracellular concentration of ion x

ABSTRACT

Walking leg closer muscle neuromuscular parameters were recorded electrophysiologically from homothermally and heterothermally acclimated *Carcinus maenas* (eurythermic) and *Cancer pagurus* (stenothermic). Homothermal (and immobilised) crabs of both species were acclimated to either 8°C or 22°C, whereas heterothermally acclimated crabs were acclimated to 8°C and 22°C coincidentally, exposing the animal's central nervous system (CNS) to either the warm (22°C) or cold (8°C) acclimation temperature. Thus, heterothermal acclimation exposes the CNS/endocrine system and one set of walking legs at one acclimation temperature, the contralateral walking legs are acclimated to the other acclimation temperature. This allowed an investigation into the CNS influence on the attainment of acclimation by walking legs. Comparisons of acclimation responses of the neuromuscular function of isolated walking legs from the same animal were done with respect to the walking leg and CNS acclimation temperatures experienced.

Animals were acclimated for two weeks, recordings were taken of excitatory junctional potentials (EJP) etc., from dactylopodite closer muscle fibres when stimulated by the tonic motor axon over an experimental temperature range (6-26°C).

The acclimation responses in homothermally exposed crabs of both species resulted in partial (Precht, type III) responses in resting potential, single and double pulse stimulated excitatory junctional potential amplitudes, these were interpreted as responses that allowed the maintenance of muscle function in the new thermal condition. With respect to long term thermal acclimation other electrophysiological parameters gave equivocal compensatory responses. Capacity acclimation responses were more complete in *C.pagurus* than *C.maenas*.

In heterothermally acclimated animals resting potentials and EJP amplitudes revealed partial acclimation responses in a compensatory manner. Acclimation of heterothermally acclimated *C.maenas* and *C.pagurus* was determined to be independent of a CNS influence, indicating thermal acclimation was in response to the local tissue acclimation temperature.

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Chapter One.

Introduction.

Ectothermy.

Cowles and Bogert (1944) introduced the term ectothermy to describe those animals whose body temperatures are determined by external heat sources. Generally, they have a low metabolic rate and in consequence they generate little metabolic heat; this low metabolism is coupled to a high thermal conductance and so thermal gradients between the animal and its environment do not persist. Thus, in environments that fluctuate diurnally and/or seasonally, the body temperature of an ectotherm would vary following closely the changes in environmental temperature (Cossins and Bowler 1987). The range of environmental temperatures over which normal function in ectotherms is possible may be limited. In this respect species that are able to function over a wide temperature range are called eurythermic, whereas those species that function over a narrower temperature range are called stenothermic (Prosser and Nelson 1981). Exposure to temperatures outside the normal temperature range has consequences for the biochemical and physiological constancy of the ectothermal animal. In the face of exposure to environmental temperatures outside the normal range ectothermal animals have few options. They could migrate, as is reported for the green sunfish (Prosser and Nelson 1981), or they can adopt such avoidance strategies as torpor or diapause.

Terrestrial environments have the widest range of surface temperatures from -40°C in the Arctic to +45°C in the desert, whereas oceanic temperature ranges are less being -1.89°C (Arctic) to +35°C (Red Sea). It is pertinent to the present study that discussions are limited to aquatic animals. Animals living in deeper water experience narrower temperature fluctuations, Aagaard (1996) reported only a 2°C change in sea water temperature over a two day period in water approximately three metres deep, where animals living at depths greater than fifty metres may experience almost constant temperatures over an annual period (Nicol 1964). In contrast animals that live on the shoreline may get caught in temporary rock pools, resulting in the animal experiencing large shifts in temperature due to changes in air temperature and the effect of insolation, i.e. from 7-14°C at night to 25-30°C during the hottest period of the day (Montgomery and MacDonald 1990; Taylor and Wheatly 1979) in a temperate summer.

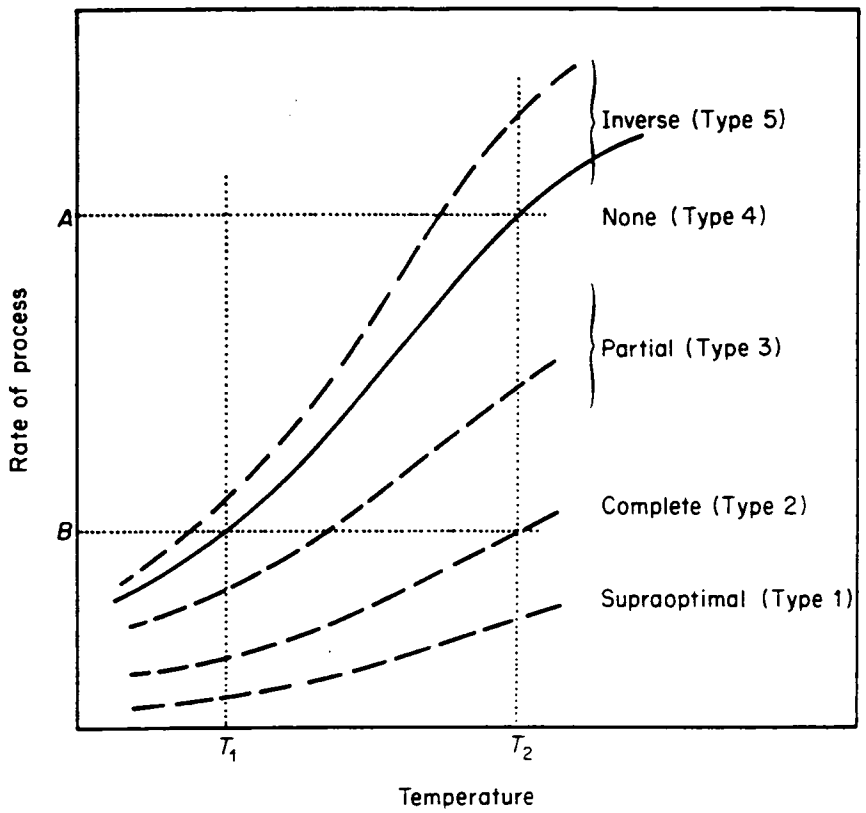
The complexity of the intertidal habitat, with respect to seasonal temperatures, is emphasised by the data presented for the Irish Sea by Southward (1958). Southward

reported average inshore sea temperatures in summer (August) and winter (November) months, sea temperatures averaged 14.6°C and 10°C respectively. The average tide pool temperature recorded over the summer and winter months was 15.9°C and 9.4°C respectively, although temperatures may be more extreme in smaller rock pools. The average shade air temperatures in summer and winter were 20.7°C and 10°C respectively, and the average body temperature of a barnacle was 25°C and 11.6°C respectively, the difference being accounted for by the effect of insolation. These temperature variations do not include short term minimum temperature extremes that may be recorded before dawn (night) when compared to maximum daytime temperatures over a year. Grainger (1968) reported *Patella* living inter-tidally over a twenty-four hour period were exposed to temperature extremes of 6-28°C, where the minimum and maximum body temperatures recorded over May to July were 5°C and 32°C respectively, sea temperature ranged between 9°C and 13°C. The temperatures reported by Southward (1958) and Grainger (1968) are examples of temperatures intertidal organisms may be exposed to over a year.

Acclimation.

Temperature is all pervasive and has profound effects on all biological processes of ectothermal animals through altering the rates of biological reactions, this may result in inappropriate function at elevated (or lowered) temperatures. In consequence, the effect of temperature is a much used experimental strategy in many studies on animals, from cells to populations (Cossins and Bowler 1987; Prosser and Nelson 1981). What is clear from many such studies is that biological processes do not merely respond to a temperature change by slavish changes in rate. Biological systems have evolved the capacity to make, in the short term, compensatory adjustments that increase fitness in response to a change in temperature. One of the most well studied is acclimation. *Acclimation* is defined as an animal's ability to adjust to a single environmental factor under controlled laboratory conditions. On the other hand, *acclimatization* is defined as an animal's ability to adjust to changes in the natural environment, and so will involve responses to changes in more than one parameter (e.g. temperature, day length, salinity etc). Environmental conditions are never constant, therefore environmental cues other than temperature may be significant in affecting acclimatization (Precht 1958). Thus, the two definitions are distinct (Montgomery and MacDonald 1990; Cossins and Bowler 1987). As temperature is all pervasive, acclimation may simply result from the combination of individual tissue responses. However, laboratory acclimation may not fully model

Figure 1.1: Schematic diagram illustrating Precht's (1958) terminology concerning rate processes compensation following transfer of an animal from temperature T1 to temperature T2. The solid line indicates the increase in rate after transfer to the higher T2 temperature. After an acclimation period the rate may be altered to give a new rate-temperature curve. The dashed lines indicate the possible new rate-temperature curves after the acclimation period (Taken from Cossins and Bowler 1987; after Precht 1958).



seasonal acclimatization responses. One source of difference could be environmental changes (day length, temperature etc.), which may be monitored by the CNS and so tissue responses may occur as directed by central nervous and/or humoral factors.

The direct effect of a change in body temperature of an ectotherm will be to alter the rate processes of cellular biochemistry and physiological function (Cossins and Bowler 1987). This has been appreciated for almost a century when Krogh (1914) demonstrated the relationship, $v=ab^T$ (where a and b are constants), for the metabolism of several animal species. Although the Krogh curve would predict an exponential increase in function as temperature rises, this may occur in response to a direct change in temperature, however it does not apply to responses of longer term temperature change. In the 1930's Precht and co-worker's produced the first evidence that the effect of temperature on ectotherms is more complex than that predicted by Krogh (1914). They showed that ectotherms were able to make long-term phenotypic changes to their physiology that compensated for the direct changes in seasonal temperature. Precht (1958) defined two forms of adaptive response to temperature; **capacity** and **resistance** acclimation.

Capacity Acclimation modifies the physiological performance of an individual or the rate of its vital processes over a normal range of temperatures. Figure 1.1 shows a schematic diagram illustrating Precht's (1958) terminology. The solid line shows the increase in rate upon transfer from T1 to a higher temperature, T2. After a period of days or weeks at T2 the rate may alter to give a new rate versus temperature curve. The broken lines represent the new possible rate versus temperature curves after the acclimation period. If the rate at T1 and T2 are the same after the acclimation period then complete or type II acclimation has occurred, if the new rate at T2 has only changed slightly to match the pre-acclimation T1 rate, partial or type III acclimation has occurred. If no acclimation has occurred then there is no rate compensation or type IV acclimation. If the new rate at T2 is above the rate at T1 inverse or type V acclimation has occurred. A decrease in rate at T2 compared to T1 is known as supraoptimal or type I acclimation (After Precht 1958; Cossins and Bowler 1987), and tabulated below in Table 1.1.

A vast literature exists on temperature experiments showing capacity acclimation. It is clear from many studies on a variety of aquatic species of ectotherms, that muscle and nervous tissue make good acclimation responses. For example, Stephens and Atwood (1982) acclimated the Pacific shore crab *Pachygrapsus crassipes* to 12°C and 21°C, temperatures this crab may experience over an annual period. *P.crassipes* shifted peak muscle depolarisation to a temperature near to the cold or warm

acclimation temperature. Similar shifts of maximal muscle depolarisation with temperature were shown for 12°C and 25°C acclimated crayfish (*Astacus leptodactylus*) by Harri and Florey (1979). Other workers have investigated compensatory (capacity acclimation) changes in plasma membrane lipid composition with respect to cold and warm acclimation. Sørensen (1993) found no changes in 2.5°C or 20°C acclimated flounder plasma membrane composition, but did report more cholesterol in warm adapted flounder membranes than cold adapted ones. In contrast Cossins *et al.,* (1977) reported increased unsaturation during cold acclimation and decreased unsaturation during warm acclimation of goldfish synaptosomes. Cuculescu (1996) reported no significant changes in overall fatty acid composition of membrane phospholipids from warm compared to cold acclimated *C.maenas* and *C.pagurus*, although a significant increase in cholesterol to phospholipid molar ratio was reported for warm acclimated crabs which resulted in increased membrane order.

Table 1.1: Acclimation definitions.

Type.	Precht (1958).
I	Supraoptimal.
II	Complete acclimation.
III	Partial acclimation.
IV	None.
V	Inverse.

Resistance Acclimation may well be a manifestation of the same phenomena as capacity acclimation. It is clear from many studies on decapods that thermal death points (LD_{50}) can be altered by acclimation. At temperature extremes, nervous systems often fail before other systems and the resulting behavioural failures occur before cellular protein inactivation can be demonstrated. Adaptations that permit continued function at environmental extremes are called resistance acclimations (Prosser and Nelson 1981).

Cuculescu *et al.*, (1997) reported significantly higher critical thermal maxima (CTMax) for warm acclimated compared to cold acclimated *C.maenas* and *C.pagurus*. Furthermore, the CTMax of winter crabs was significantly lower than the CTMax of summer caught crabs irrespective of acclimation status, which was similarly reported for cold and warm acclimated crayfish *Orconectes rusticus* (Layne *et al.*, 1987). An informative depiction of the effectiveness of acclimation on thermal limits can be best seen by use of a thermal tolerance polygon.

Figure 1.2A: Tolerance polygon for the sockeye salmon (*Onchorhyrichus nerka*). The upper incipient lethal and lower incipient lethal limits indicate the tolerance zone of salmon populations. Included are the tolerance polygons for activity and reproduction, their smaller areas indicate their increased stenothermic nature (Taken from Cossins and Bowler 1987; after Brett 1958).

Figure 1.2B: Tolerance polygons for a eurytherm bullhead (*Amieurus nebulosus*), a cold stenotherm the chum salmon (*Onchorhynchus keta*) and an extreme stenotherm the Antarctic rock perch (*Pagothenia bernachii*). The areas within each polygon represent the 'space' available to each species, the larger the space indicates a larger tolerance zone and resistance acclimation limits. The area available to each species being $1200°C^2$, for the bullhead, $650°C^2$ for the salmon and $28°C^2$ for *Pagothenia*, the larger the area the more eurythermic the species may be (Taken from Cossins and Bowler 1987).

Figure 1.2A

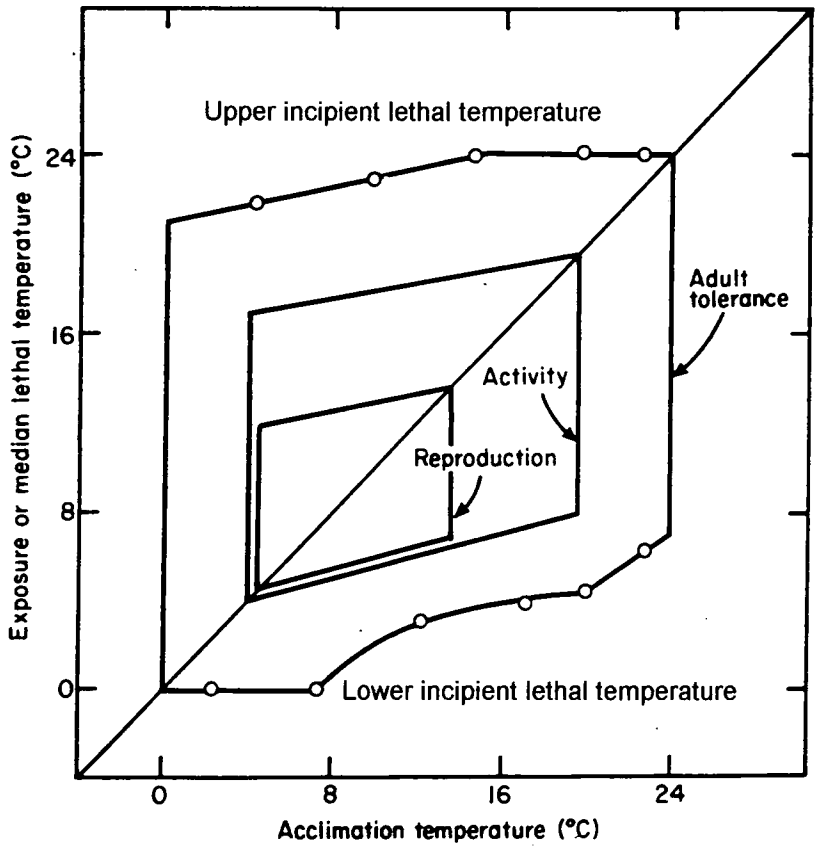
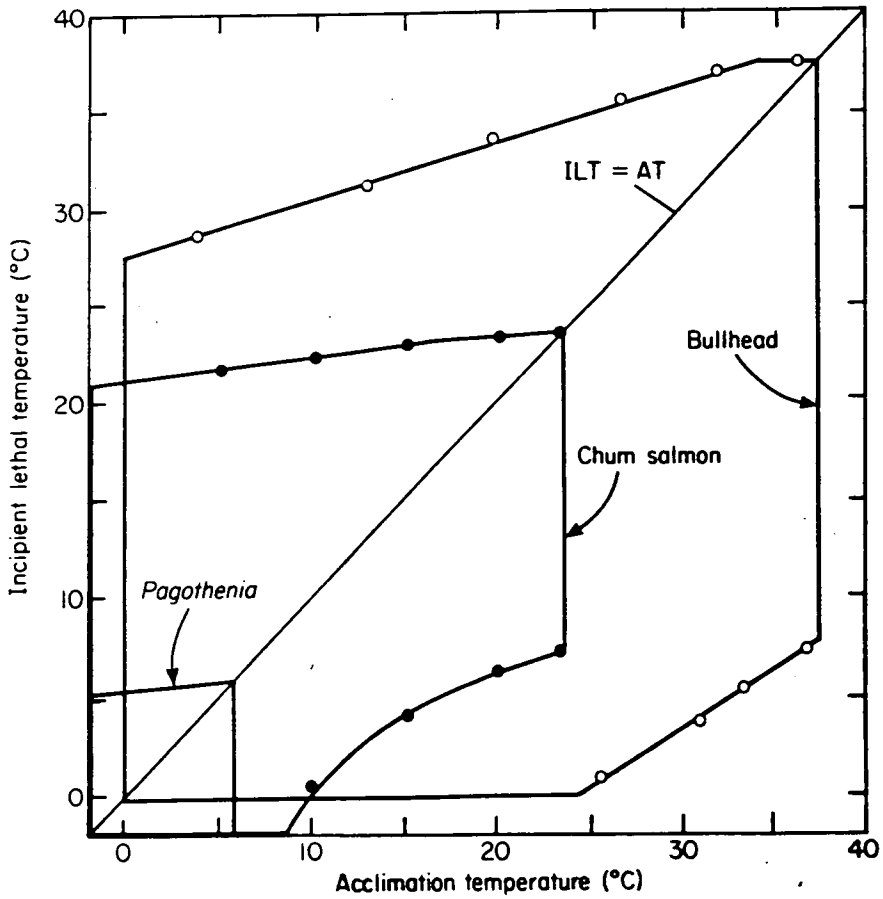


Figure 1.2B



Temperature tolerance polygons are constructed to determine the upper and lower incipient lethal temperatures in a population of animal's acclimated to a range of constant temperatures (see Figure 1.2A). The incipient high and low lethal temperatures increase with increasing acclimation temperature, the area between the high and low incipient temperatures represents a combination of acclimation and lethal temperatures that 50% of the sample population can withstand for an indefinite period (zone of tolerance). Brett (1958) extended the tolerance polygon to include the thermal limits for other processes such as growth and reproduction etc., which themselves have their own tolerance polygons (see Figure 1.2A). The tolerance polygons also reveal differences between stenothermic and eurythermic animals (see Figure 1.2B), where the eurythermal bullhead zone of tolerance and resistance acclimation limits are notably larger than the stenothermal *Pagothenia*. The area within each polygon indicates the temperature space available to each species, a eurythermal animal having a larger space available than a stenothermic animal.

It is a proper question to ask, what is changed with acclimation that brings the compensatory responses in function and the shifts in thermal tolerance?

Membrane Lipid Modelling; Animal plasma membranes contain fatty acids between 16-22 carbon units in length with typically six carbon double bonds. Phospholipids form lipid bilayers which surround and encompass every living cell and are also constituent of many organelles, hydrophobic lipid carbon chains are internalised within the bilayer (see Figure 1.3) and hydrophilic head groups are externally oriented. Intimately associated with lipid membranes are integral proteins that form channels, transporters and membrane bound proteins. The membranes and integral proteins are important in regulating ion transport, cell recognition, receptor-mediated processes, energy transduction etc (Lee and Chapman 1987).

At physiological temperatures lipids within a membrane leaflet are free to move within the plane of that leaflet, the membrane is identified as being fluid. With decreasing temperature the membrane fluidity may be reduced and at low temperatures the membrane may undergo a physical change. The physical change or phase transition (see Figure 1.3) reduces membrane fluidity resulting in more ordered packing of the fatty acids (or gel phase). Lateral diffusion of lipids in the plane of the membrane is slow, diffusion may be increased by increasing the temperature. Generally the most saturated lipids are first to solidify as the temperature drops, creating clusters or rigid lipid domains randomly dispersed in an otherwise fluid membrane. This effect increases the membrane permeability to cations and water

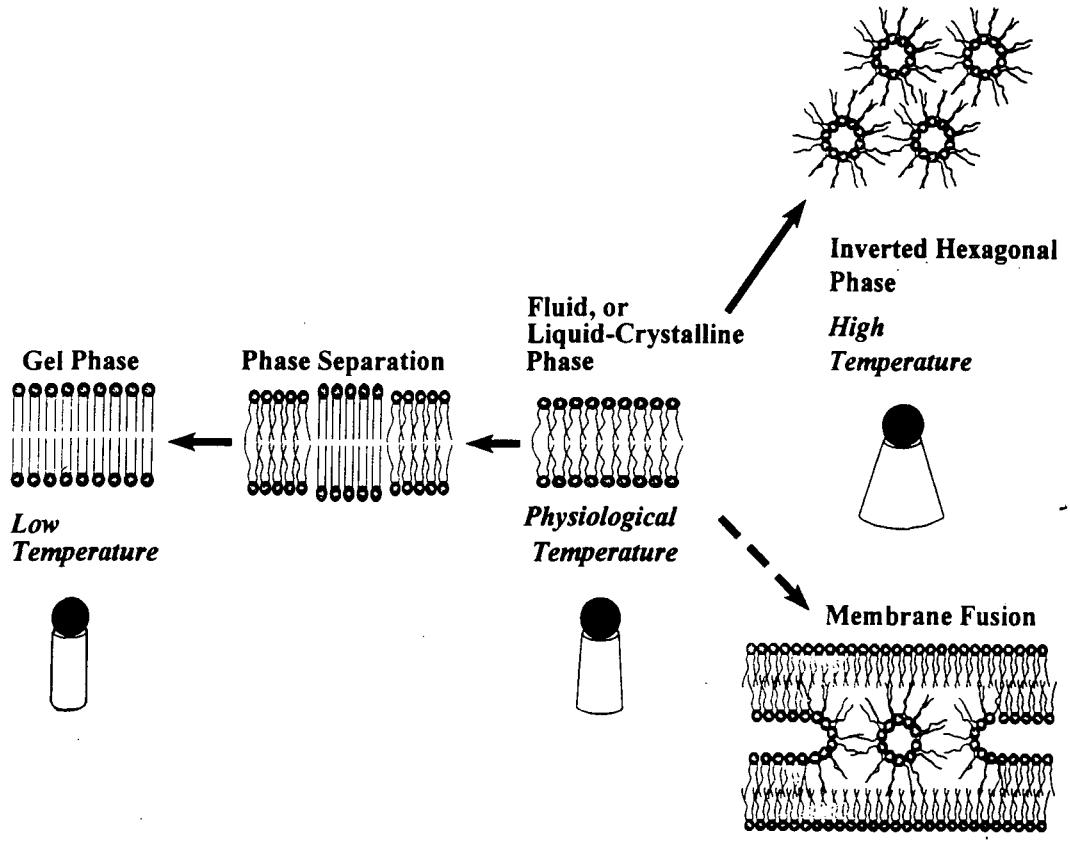


Figure 1.3

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

(Hazel 1995) between the boundaries of frozen and fluid lipid domains. Further progressive cooling increases the size of the frozen domains until all lipids are frozen. A frozen membrane leaflet exhibits noticeably reduced membrane fluidity, which is proposed to affect integral protein function. Integral proteins themselves undergo flexing movements during catalysis which is influenced by the surrounding bilayer fluidity (Cossins and Bowler 1987).

Conversely when temperature increases above the physiological range some lipids assume an inverted hexagonal phase (H_{II}), resulting in a loss of bilayer integrity (Hazel 1995; 1989, see Figure 1.3). The H_{II} transition is normally observed at high temperatures (45-70°C), although low water concentrations can cause the transition temperature to be lowered to within a physiological range (Lee and Chapman 1987). To prevent temperature profoundly affecting animal and cellular function, ectothermic animals change their membrane characteristics to maintain fluidity appropriate to the local temperature, this phenomenon has been called homeoviscous adaptation (Sinensky 1974).

In general, cold acclimation leads to an increased proportion of fatty acids containing unsaturated bonds (Cossins and Bowler 1987; Pruitt 1988). Increased unsaturation maintains membrane fluidity at lower temperatures (Hazel 1995) by allowing a more random conformation. In comparison, warm acclimated animal membrane lipid composition should be more saturated, this would provide a compensatory effect to reduce membrane fluidity in response to the increase in temperature. In most cases the membranes of cold acclimated animals (e.g. fish) are more fluid than warm acclimated animals (increased unsaturation) when measured at the same experimental temperature (Cossins and Bowler 1987).

Enzyme/Pump Activity Changes; Enzymes may be very temperature labile and exposure to an extreme (high) temperature may result in a decrease in activity, the reaction rate may decrease or become irregular, due to proteins becoming unfolded and loss of active site conformation (Somero 1995; Daniel *et al.*, 1996). Loss of function of a particular enzyme involved in the control of a step in a reaction pathway induces a rate limit i.e. excess product available from the blocked step will be utilised until exhausted. Thereafter the damaged enzyme becomes a rate limiting step, stopping that reaction pathway with potentially knock-on damaging consequences (Cossins and Bowler 1987). Membrane proteins may suffer similar molecular perturbations by high temperature with consequential effects on function. Bowler and Manning (1994) have argued that membrane proteins might be particularly susceptible because of their dependence on the physical state of membrane lipids, as discussed above, might also be subject to temperature perturbation. Many pumps require ATP

to function, loss of an energy source (heat stressed mitochondria) may limit pump activity which may affect cell integrity. Pumps such as Na^+/K^+ ATPase have been shown to be temperature sensitive (Sidell 1980), as proven by ouabain sensitive resting potential (RP) hyperpolarisation of crayfish muscle with increases in experimental temperature (White 1983). This is pertinent to the present study because pumps and membrane associated channels are proteins which may undergo temperature induced changes in conformation affecting appropriate function. Cold adapted fish maintain pump flux through increased pump densities (Hochachka 1988a) to overcome reduced enzyme activity, in contrast warm adapted animals may have reduced enzyme densities.

Changes in Cell Structure; There are a number of reports that indicate acclimation responses involve changes in cell morphology as well as function. For example warm acclimation increases the general cell size of *Aplysia* (Triestman and Grant 1993), increased muscle cell size is also reported by Rome (1990) in a fish, this may be related to the changes in muscle myosin reported by Sakurai *et al.*, (1996). Changes in myosin with warm acclimation generate greater mechanical power and faster contractions. Johnston and Lucking (1978) reported changes in the proportion and type of goldfish muscle fibres with different acclimation temperatures, they reported increases inversely related to acclimation temperature in both proportional area and number of red and pink oxidative fibres in a region of goldfish muscle. Egginton and Sidell (1989) reported marked increases (50-60%) in mitochondrial volume density in both white and red muscle fibre types in the striped bass (*Morone saxatilis*) when acclimated from 25°C to 5°C. It was suggested that expansion of organelle densities at cold body temperatures may counteract decreases in cytoplasmic diffusion coefficients of small molecules (O_2 and soluble micromolecules), a view supported by Guderley and Johnston (1996) who reported increased mitochondrial volume enhanced the activities of mitochondrial enzymes, leaving glycolytic capacities unchanged at cold body temperatures.

The question now arises as to what causes heat death and cellular heat injury?

In an aquatic animal the effects of high temperature may not be solely a thermal problem, but oxygen (O_2) availability, salinity or pH may also become additional limiting factors (Cossins and Bowler 1987). For example the following sequence of events may occur;

Increased temperature→leads to gill damage→ results in hypoxia→
causing loss of co-ordination→ resulting in death.

Gladwell *et al.*, (1975) reported K^+ leakage from cells (depolarising them) as a cause of crayfish death to a perturbing experimental temperature, he linked this to changes in the sensitivity of ionic pumps causing a failure of ionic steady state (Hochachka 1988a; 1988b). Observations from the freshwater fish *Rutilus rutilus* by Schwarzbaum *et al.* (1991) indicated that warm acclimation decreased Na^+/K^+ ATPase activity compared with the Na^+/K^+ ATPase activity in cold acclimated fish.

Other factors may be important in contributing to heat death, it has be shown that increasing temperature resulted in a decrease in pH. Stevens and Godt (1990) determined plasma pH of a bullfrog was 8.1 at 5°C which decreased to 7.7 at 25°C. The effect of decreased pH may cause a decrease in Ca^{2+} sensitivity which may result in an increased metabolic load at higher temperatures (Stevens and Godt 1990), due to Ca^{2+} pump ATP hydrolysis increasing with increasing temperature (Ushio and Watabe 1993). Increased temperature has been shown to inhibit calcium channel activity and vesicular calcium release (Hidalgo and Donoso 1995) from sarcoplasmic reticulum through changes in pH (especially at pH< 6.5; a view supported by Wolosker and Meis (1994)). Furthermore increased temperature increases muscle performance and function, this may be partially counterbalanced by decreased Ca^{2+} sensitivity which is associated with decreased pH.

In an attempt to prevent cellular damage an animal, in response to a thermal perturbation, may produce heat shock proteins (HSP) and/or chaperones that help maintain normal 3-D conformation of macromolecules, so conserving function during thermal stress (Buchner 1996). Recovery/tolerance can be dependent on the quantity and type of HSP induced (Amin *et al.*, 1995; 1996). HSPs can enable the toleration of temperature changes assuming the animal can transcribe and translate the HSP before irreparable damage is done (Mailhos *et al.*, 1994), as well as provide protection from impending thermal perturbations (Amin *et al.*, 1995). There are several families of HSP (hsp104, 90, 70, 60/GroEL and small HSPs) that are conserved between animal species (Buchner 1996; Delpino *et al.*, 1996; Kikuchi *et al.*, 1993). HSPs are proposed to act by preventing irreversible protein aggregations forming, different HSP's confer differing levels of protection to different perturbations. In dorsal root ganglion HSP70 confers protection from thermal stress more than ischemic stress (Amin *et al.*, 1996) whereas HSP90 confers little protection to either stress. Some HSP isoforms are constitutively expressed in cells, whereas others are induced only following exposure to the stress. What is not clear at present is whether the level of constitutively expressed HSPs determines the thermal tolerance of a cell.

Central Influences on the Acclimation Process.

As already discussed acclimatization in nature is likely to be dependent on a variety of environmental cues, in consequence it is a complex process involving such factors as; O_2 tension, day length, salinity, temperature as well as food availability and hormonal changes. The fact that such factors as day length can influence the attainment of temperature acclimation implies that acclimation may have a centrally controlled component. The identification of whether such a controlling influence exists in the attainment of acclimatization forms the major focus of the experimental work in this thesis. The system chosen for study is the crustacean neuromuscular system because this system has been shown by numerous previous studies to be subject to change during thermal acclimation (Stephens 1985a/b, Stephens and Atwood 1982). Investigations into the control of acclimation were carried out using two different species of marine crab, the stenothermic *Cancer pagurus* and the eurythermic *Carcinus maenas*. The acclimation technique used was heterothermal acclimation, a condition where one set of walking legs and the CNS were acclimated to one temperature and the contralateral set of walking legs were acclimated to a different temperature (c.f Figure 6.0). This acclimation technique allowed comparisons of the differently acclimated walking leg neuromuscular parameters to be undertaken, allowing the CNS influence to be identified on the ipsilateral and contralateral walking legs. Because thermal acclimation may involve a hormonal influence it is important to identify the sources of hormonal factors in *C.maenas* and *C.pagurus*.

Hormonal Influences in Crustaceans.

Hormonal influences in crustaceans are particularly dramatic during the moult cycle. In crabs there are several hormone secreting organs such as the brain (possibly from the pre-optic hypothalamus), postcommissural organs (situated near to the oesophagus and involved in colour change (Fingerman 1995), Y-organ (secretory cells in the maxilla) and X-organ (in the eyestalk). These endocrine organs secrete hormones which inhibit and synergise with each other and interact with other organs principally controlling the moult and reproductive cycles, although other influences cannot be discounted (Crothers 1967).

Most crustacean hormones have a neural origin and may also act as neuroregulators that control the release of other hormones (Fingerman 1995; Dauphin-Villemant *et al.*, 1995). In crayfish it has been shown that γ-aminobutyric acid

(GABA) inhibits neurodepressing hormone (NDH) release, whereas 5-HT (serotonin) stimulates it (5-HT also stimulates ovarian and testicular maturation in crayfish). NDH when applied to isolated neurones and ganglia reduces neuronal responsiveness and spontaneous activity (Fingerman 1995), which may result in a change of tissue morphology (Lnenicka 1993). There is obviously some interaction between neurotransmitters or neuromodulators, which may be altered by changes in temperature and day length, as it has been shown in crayfish that temperature and day length control ovarian maturation (Fingerman 1995). Lagerspetz (1974) reported changes in spinal cord tonic discharge altered the enzymatic function in isolated muscles in the eel *Anguilla anguilla*. It would be interesting to know if the central discharge rate effected hormones (such as NDH) in crab?

The evidence available indicating a hormonal influence on the maintenance of cellular function with thermal acclimation was shown in cultured catfish (*Ictalurus punctatus*) hepatocytes. Serum from 15°C acclimated catfish was significantly more effective at stimulating protein synthesis than serum from 25°C acclimated catfish. Prosser *et al.*, (1991) suggested that cold acclimated catfish serum contained some stimulatory factor, maybe a hormone that had greater effectiveness in cold acclimated catfish, however, its nature is unknown (Prosser *et al.*, 1991). Silverthorn (1975) reported eyestalk extracts from cold acclimated crabs (*Uca pugilator*) significantly increased respiration in warm acclimated crabs, where eyestalk extract from warm acclimated crabs significantly decreased respiration in cold acclimated crabs. Silverthorn argued that two different hormones were involved in the regulation of respiration. These results indicate that a hormonal effect contributing to the acclimatory process may occur (Lnenicka 1993).

The two species of crab chosen for study here (*C.maenas* and *C.pagurus*) have different thermal ecologies, which might suggest that their capacities for thermal acclimation differ. Furthermore, their acclimation may be differentially subject to central/hormonal control. It is appropriate now to introduce the crustacean nervous system.

Crustacean Nervous System.

The crustacean nervous system comprises large diameter axons and ganglia (Nicol 1964; Nguyen and Atwood 1994) that are unmyelinated. The axons are surrounded by glial cells which overlap and intertwine with each other along the length of the axon (Liberman *et al.*, 1994). Large diameter axons conduct action potentials at faster velocities (3.1-5.5 ms^{-1}) than small diameter axons (Nicol 1964), in certain nerve

bundles giant axons may be present as in *C.maenas* (Quinta-Ferreira *et al.,* 1982) and crayfish (Kivivuori 1982), the giant axons are principally involved in escape responses (Shepherd 1988).

Most crustaceans have long segmented bodies, for example lobster and crayfish. The ganglia in long bodied crustaceans typically form neuropil like masses at each segment. *C.pagurus* and *C.maenas* are typical crabs, having a large ganglionic mass which is covered with a single non-segmented carapace (Shepherd 1988; Crothers 1967). Ganglia within the ganglionic mass in crabs maintain independent action despite being fused together, e.g. correct individual legs are autotomized, only one eye-stalk is retracted when touched etc (Crothers 1967). The CNS or brain of crabs is positioned ventrally and posterior to their mouth parts, the ganglionic mass being found more distal. The brain comprises a smaller mass of ganglia connected to the ganglionic mass by circumoesophageal commisures (Crothers 1967). Neural function is dependent on an axon conducting an action potential (AP) to a muscle fibre (or other axon), its response to an innervation depends on the type of axon conducting the AP (i.e. amount/type of transmitter exocytosed) and the type/number of post-synaptic receptors. Muscle fibre neuromuscular characteristics can change with acclimation or acclimatization (Atwood and Nguyen 1995).

Neuronal Action Potentials.

Previous workers have reported that crustacean APs are generated by an increase in conductance of Na^+ (Cukierman 1996) and Ca^{2+} (Niwa and Kawai 1982), the cell is repolarised by an increase in K^+ conductance. In *Pachyprapsus crassipes* neurones, the APs have been divided into Na^+ (tetrodotoxin sensitive) and Ca^{2+} (cadmium sensitive) components (Stephens and Church 1988), both ions contribute to AP initiation, although AP initiation was still seen in the presence of one channel blocker. Incubation with both Na^+ and Ca^{2+} channel blocker's prevented an AP from being generated. Na^+ channels are responsible for the initial rise of an AP, the Ca^{2+} channel component appears slightly slower and its conductance contributes to the AP width, the Na^+ and Ca^{2+} channel kinetics are different (Stephens and Church 1988). Blocking a single component of the AP did not stop action potential propagation, but did decrease or prevent a successful post-synaptic event. The decrease or block of post-synaptic events may be due to reduced AP amplitude or width altering voltage gated channel action pre-synaptically, thus reducing the amount of neurotransmitter exocytosed which therefore affected post-synaptic events. At crustacean neuromuscular junctions glutamate is the excitatory neurotransmitter, but at an

inhibitory synapse it is γ-aminobutyric acid (GABA)(Atwood 1976; Hille 1992; White 1983).

GABA stimulates ligand gated Cl^- channels known as $GABA_A$ (Hille 1992; Hinkle *et al.*, 1971; Cattaert 1994; Levitan and Kaczmarek 1992), although there is now evidence for pre-synaptic $GABA_B$ receptors in crayfish opener muscle that increase K^+ conductance, hyperpolarising the pre-synaptic membrane (Fischer and Parnas 1996). Glutamate stimulates ligand gated channels non-specifically for cations (Dudel *et al.*, 1990; Hille 1992; Marder and Paupardis-Iritsch 1978). Glutamate channels are quisqualate or α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid (AMPA) in nature and are rapidly desensitising (Moudy *et al.*, 1994). Typically two types of glutamate channel with electrical properties of Tau (channel open times) 3msec and 25msec are normally found in crayfish leg muscle fibres (Franke *et al.*, 1986). Furthermore, crayfish *Austropotamobius torrentium* muscle fibres contain a uniform population of glutamate sensitive channels, these have been separated into four types by pulse application of glutamate (Dudel *et al.*, 1990; Edmonds *et al.*, 1995).

- Type *I* Incompletely desensitising, Tau 5msec,
- Type *II* Completely desensitising, Tau 1-2msec,
- Type *III* Non-desensitising,
- Type *IV* Short opening time channels.

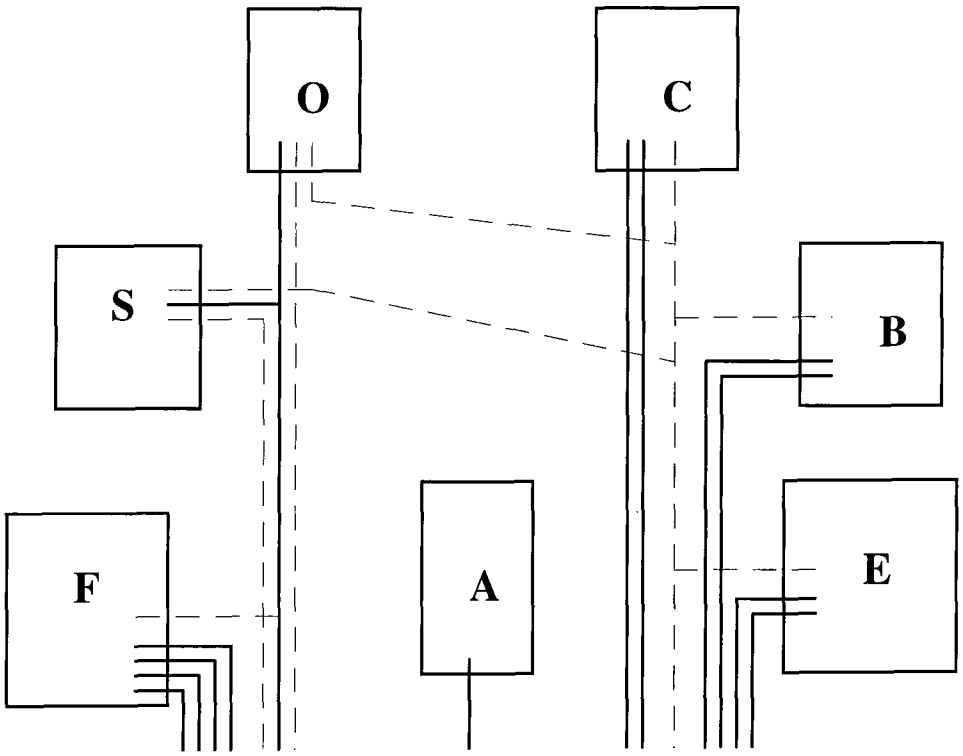
Type *I* and *II* channels are frequently isolated whereas types *III* and *IV* are rarely seen. Removal of exocytosed glutamate from the synaptic cleft is through active transport coupled to a Na^+ dependent carrier located in the plasma membrane (Kanner 1994; Wolosker *et al.*, 1996a). Glutamate is then removed from the axon terminal cytosol into synaptic vesicles by a carrier-mediated transporter driven by a electrochemical proton gradient (Wolosker *et al.*, 1996b; Xu *et al.*, 1996).

Crab Walking Leg Nervous System.

Crustacean muscle fibres are polyneuronally and polyterminally innervated, each nerve branches many times so a single muscle fibre has multiple synaptic terminals (Atwood 1976; Atwood *et al.*, 1994). Crab walking leg innervation pathways for motor and inhibitory nerves are shown in Figure 1.4. Dactylopodite article movement is controlled by the closer and opener muscles, propodite movement is controlled by

the stretcher and bender muscles, and meropodite movement is controlled by the extensor and two flexor muscles (see Figure 2.0B for article identification). The

Fig 1.4: Innervation pathway of *Brachyuran* walking legs.



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).

closer muscle to the dactylopodite is innervated by two motor axons and one common inhibitory nerve, whereas its antagonistic opener muscle is innervated by a single motor axon and two inhibitory axons (Wiersma and Ripley 1952). The two excitatory nerves innervating the closer muscle are typically termed slow (or tonic) and fast (or phasic) which relates to the EJP characteristics and not the speed of conduction. Inhibitory nerves hyperpolarise muscle fibres when stimulated through activation of Cl^- channels, the change in resting membrane potential alters the membrane's permeability and therefore the EJP characteristics change as the muscle fibres excitability changes. Pre-synaptic inhibition is seen on some motor axons (i.e. crayfish and crab opener muscle (Atwood 1976)) and has more influence at low frequencies of stimulation, whereas post synaptic inhibition is more common at stimulation frequencies higher than 30Hz (Atwood 1976).

Tonic neurones control muscle contractions involved in general movement and behaviour, whereas phasic neurones are responsible for rapid defensive actions, such

as fighting etc. (Atwood 1976). Tonic neurones fire at a low frequency background rate to maintain posture. Phasic axons are silent most of the time until needed, and then fire bursts of APs (Atwood 1976). Neuronal activity controls the contractile nature of the muscle to which it synapses i.e. phasic axons innervate fast acting glycolytically dependent muscle fibres for a burst of brief activity (fatigue easily), whereas tonic axons innervate slow acting oxidatively active muscle fibres used to control slow actions of a postural nature (Atwood *et al.,* 1994; Atwood and Nguyen 1995).

A crustacean muscle fibre may be innervated by two functionally different motor neurones (phasic and tonic), neurone activity has been shown to alter muscle phenotype to match the impulse activity levels (Wojtowicz *et al.*, 1994, Nguyen and Atwood 1994). It would be interesting to know what controls a muscle's phenotype if both axons are active?

Phasic motor neurones in crayfish have slender synaptic terminals with few mitochondria, their axons contain less metabolically active mitochondria than tonic nerves (Atwood and Nguyen 1995; Baldwin and Graubard 1995). It has been shown in crayfish abdomen muscles by Arcaro and Lnenicka (1995) that slow flexor nerve's branch less than fast flexor nerves, therefore fast flexor nerves synapse with more muscle fibres than slow flexor axons. Because the fast flexor neurone is involved in fast defensive movements, use of all available active muscle fibres to escape or fight would support this observation. The increased branching of one axon type results in it being synaptically dominant over a different axon which branches less, innervating more of a particular muscle fibre (e.g. phasic axon). That is, a muscle can be polyneuronally and polyterminally innervated and exhibit one EJP characteristic (e.g. tonic), the number of synapses from one specific axon incurs fibre dominance over another axon type, although this is dependent on axon activity. Dual motor axon innervation may aid stronger longer lasting muscle contractions i.e. tonic innervations (long lasting, repetitive, fatigue slowly) being potentiated by infrequent bursts of phasic (large depolarising, short duration, fatigue quickly) axonal activity. Because neurone impulse activity affects the muscle phenotype (Atwood and Nguyen 1995), it is important to know the type of innervation a muscle fibre receives.

Crustacean Muscle Fibre Type and Innervation.

Excitatory axons branch and innervate leg muscles to varying extents (Atwood 1976), the tonic axon is closely associated physically with the common inhibitor axon (Atwood and Nguyen 1995). The phasic flexor axon in crayfish and crab (*Eriphia spinifrons*) innervate more individual muscle fibres than the tonic flexor axon, due to

the phasic axon branching more (Arcaro and Lnenicka 1995; Atwood and Nguyen 1995). Tonic axons innervate muscle fibres with a less developed T-system than phasic axons, the sarcoplasmic reticulum (SR) is however well developed in both tonic and phasic muscle types (Atwood 1976). Fibres producing larger amplitude EJPs have bigger excitatory synapses and higher input resistances than fibres producing smaller EJPs (they have smaller synapses but more in number)(Atwood 1976). Slow contracting muscle fibres in the lobster *Homarus americanus* have longer sarcomeres than fast contracting fibres (Silverman *et al.,* 1987), this is also seen in the crustacean *Hutchinsoniella macracantta* (Read *et al.,* 1994) and the crayfish *Procambarus clarkii* (Sakurai *et al.*, 1996).

Crab closer muscle is composed of four functionally different muscle fibre types, the EJP characteristics of types I and II are very similar when stimulated by both phasic and tonic axons (Rathmayer and Maier 1987; Rathmayer and Erxleben 1983; Rathmayer and Hammelsbeck 1985). Type I fibres have slower EJP decay time constants than type II fibres as seen in the crab *Eriphia spinifrons*. Type III and IV muscle fibres only show successful EJP propagation when innervated by the phasic closer excitor axon. Typically, phasically stimulated EJP's facilitate more than tonic EJPs at higher stimulation frequencies (Atwood 1976). At low stimulation frequencies (<10Hz) no variation in facilitation may be seen in a fibre type stimulated alternately by the phasic and tonic axons (Rathmayer and Hammelsbeck 1985; Rathmayer and Maier 1987).

In an animal the axons conduct trains of APs which stimulate the muscle during movement (i.e. the tonic axon may fire at 250Hz). EJP's facilitate and summate together depolarising the muscle fibre and T-system, initiating Ca^{2+} release from ryanodine sensitive SR stores (Lea and Ashley 1993; Meissner 1994). The released calcium binds to troponin-C and initiates muscle contraction (Hille 1992), at the same time further calcium is released through calcium induced calcium release (CICR) as shown in crayfish (Györke and Palade 1994) or carp (Ushio and Watabe 1994). Muscle contraction is dependent on muscle fibre depolarisation, commonly known as excitation contraction coupling (E-CC) which incorporates electromechanical coupling (Carl *et al.*, 1996). Muscle relaxation is initiated through active removal of the Ca^{2+} out of the muscle or back into internal stores (Meis *et al.*, 1996). Ca^{2+} is removed from crayfish (*Astacus fluviatilis*) striated muscle by a Na^{+}-Ca^{2+} exchanger, highest activity was reported in the transverse-tubule plasma membranes (Ruščák *et al.,* 1987).

Due to axon branching, the extremely large muscle fibres have multiple synapses on their surfaces. If all synapses are active, a full strong contraction of that muscle fibre would occur, due to the large amount of transmitter released. If a limited number

of synapses are successfully activated, achieved by modulating the frequency of APs, then a more graded contraction of the muscle fibre may be observed. Graded contractions have been seen in crayfish *Procambarus clarkii* (Hinkle *et al.,* 1971) and the pyloric motor circuit of lobster *Panulinus interruptus* (Johnson *et al.,* 1991). Graded contractions are modulated in crayfish *Procambarus clarkii* by the calcium dependent potassium channel current ($I_{K(Ca)}$), which acts in a maxi-k (or BK) manner. BK channels are voltage dependent fast activating with a large conductance when compared to small-k (or SK) channels (Araque and Buño 1995; Hille 1992). In dually innervated muscle fibres (i.e. type I and II), phasic axons ultimately produce facilitating EJPs that are smaller than the facilitating EJP's generated by tonic axons, regardless of the EJP amplitude (Rathmayer and Hammelsbeck 1985). Generally, as tonic EJPs have longer decay time constants (Tau), their EJP's summate and facilitate together increasing the successive EJP amplitude and the degree of muscle depolarisation (till tetani) at lower frequencies than that exhibited by phasic fibres. At higher frequencies, phasic muscle fibre's facilitate more and may even exhibit all or nothing spikes (Atwood 1976). Phasic axons require higher stimulation voltages experimentally than tonic axons to generate a post synaptic event (Harri and Florey 1979), this occurs so that no matter the frequency of innervation tonic muscle fibres are always stimulated first, then the phasically innervated muscle fibres. This sequence of depolarisation initiates larger muscle contractions (Atwood 1976). Summation is the contribution to an EJP's amplitude from a previous EJP not having fully decayed, it is a post-synaptic event. Facilitation, however is the contribution of latent Ca^{2+} from a previous AP/EJP in a muscle or synapse synergising with a following AP/EJP's Ca^{2+}, leading to the following EJPs increased amplitude (Baldo *et al.,* 1983). Observations from *Procambarus clarkii* have (Blundon *et al.,* 1993) determined that facilitation is attributed more to bound Ca^{2+} than free Ca^{2+} pre-synaptically. Pre-synaptic Ca^{2+} channels have been identified as P-type (Uchitel and Protti 1994), and post synaptic Ca^{2+} channels as L-type in the crayfish *Procambarus clarkii* (Araque *et al.,* 1994).

Neural and Muscle Fibre Adaptation.

In crayfish (*Procambarus clarkii*; Lnenicka and Zhao 1991) it has been shown that phasic motor neurones are more plastic than previously thought (Wojtowicz *et al.,* 1994), they can modify muscle phenotype in response to changes in axon activity (Atwood and Nguyen 1995; Silverman *et al.,* 1987). In mammals and other vertebrates, muscle fibre characteristics are governed by the innervating motor axon.

Muscle fibre function can be modified by altering the impulse activity, by artificially stimulating the muscle or re-innervating the muscle with a different axon. These protocols modify the metabolic properties of the muscle fibre to match the axon's activity level (Eisenberg 1985). Changes in an axonal ability to innervate confer a change in the muscle fibre biochemistry and physiology (Atwood and Nguyen 1995). A muscle fibre may be classed as type II, but due to an axon activity change it will become similar to a type I muscle fibre. The change is never total and it may form a different class of fibre in-between type I and II. It has been shown in *Procambarus clarkii* that after ganglionectomy an increase in excitability of the tonic flexor abdominal muscle occurs. The abdominal muscle being mainly composed of slower fibres, then increased spike activity several weeks later is seen i.e. a more phasic situation (Lehoueller *et al.,* 1983). The increase in spike activity is related to denervation supersensitivity. Denervation supersensitivity occurs after ganglionectomy and is due to an increase in post-synaptic Na^+ channels and neurotransmitter receptors (Hille 1992), these result in increased muscle sensitivity and response to exocytosed neurotransmitter.

These areas of adaptation highlight the extent of EJP diversity, no specific type of muscle fibre can be strictly categorised, a dually innervated muscle fibre could be more type I than type II or somewhere in-between. Seasonal changes in EJP characteristics are apparent in the crayfish *Procambarus clarkii*, summer crayfish chelae closer muscles were more varicose and the innervating axon initiated smaller fatigue resistant EJPs when compared to winter crayfish (Lnenicka and Zhao 1991). This reversible cycle is dependent on the time of year, hormonal and moulting cycle influences and could possibly be age dependent (Atwood and Nguyen 1995; Kivivuori 1980).

To limit the intrinsic variability between experimental preparations, work on the crab *Eriphia spinifrons* has determined that conserving the loci of investigation (dissect same area of muscle), that individual muscle fibres with characteristic properties can be located at identical positions from one preparation to the next (Rathmayer and Maier 1987; Rathmayer and Erxleben 1983) in a homogenous population. Therefore, investigating a small group of muscle fibres in a known area of a larger muscle mass reduces the amount of muscle fibre type variation, the results therefore are more homogenous.

The response of a muscle fibre to a depolarisation depends on its electrical properties. Muscle fibres in crustaceans are not perfectly circular, in fact they are nearer a squashed oval shape, and are relatively giant in size (i.e. can be >200μm in diameter and the order of mm in length; Rathmayer and Maier 1987; Orkand 1962; Atwood 1976; Maier *et al.,* 1986), this has implications on accurate input resistance

measurements. It is not known if there are changes in fibre length, or position and number of specific synapse types when crustaceans are thermally acclimated. Due to the large size and extensive infoldings of crustacean muscle fibre membranes (T-system), the muscle fibres are of typically low resistance (R_m) and high capacitance (C_m). Most biological membranes have capacitance values of $1\mu F/cm^2$ (Hille 1992; Castillo and Machne 1953; Orkand 1962), whereas Fatt and Katz (1953) calculated crustacean muscle fibre membrane capacitance to be $40\mu F/cm^2$. However, capacitance remains constant over varying temperatures and it has been shown that homeoviscous adaptation has only a slight impact on the electrical capacitance of a cell, increased unsaturation increasing C_m (MacDonald 1990).

Decay of an EJP (Tau) is dependent on the capacitance (C_m) and resistance (R_m) properties of the muscle cell (see 1.1). It has been shown at a frog endplate that nerve evoked current decay rate constant's decreased more with increasing temperature and depolarising resting potential values (clamped) than in more hyperpolarised preparations (Magelby and Stevens 1972). Capacitance is effectively a membrane constant and as such τ (Tau) is proportional to membrane resistance (R_m), which gives a measure of the number of open channels (Hille 1992; MacDonald 1990). High resistance fibres have longer EJP decay time constants, which at higher stimulation frequencies maintain muscle depolarisation and therefore muscle function (Adams 1987).

$$\tau = R_m \times C_m \qquad (1.1)$$

The work presented in this thesis was carried out on two species of ectothermal crabs, investigations included the effect of experimental temperature on neuromuscular performance in crabs acclimated to 8°C and 22°C. The technique of heterothermal acclimation enabled the control site of thermal acclimation to be investigated by use of electrophysiology. The question arises, *how might temperature effect neuromuscular parameters?*

Electrophysiology.

Electrophysiology allows recordings of excitable cell electrical activity, these reveal cellular changes in ionic conductances, the ionic conductance determines the resting potential (RP) and therefore cell activity. Accurate recording of the RP is critical, the RP can be calculated using the Nernst equation (1.2), or the Goldman-Hodgkin-Katz equation (1.3) if ion concentrations (including any contribution to ionic

concentration changes by electrogenic pump activity) and selective permeabilities are known.

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^+ (Hille 1992), as shown in abdominal muscle of the crayfish *Procambarus clarkii* (Hinkle *et al.*, 1971). Therefore the RP moves toward the equilibrium potential for potassium (E_{K^+}), RP changes are mainly K^+ driven changes, where Cl^- generally stabilises the muscle fibre RP (Hille 1992). The RP is therefore dependent on the movement of different ions with different membrane permeabilities. To account for membranes permeable to more than one ion the Goldman-Hodgkin-Katz equation (1.3) should be used (Levitan and Kaczmarek 1991; Hille 1992).

Nernst equation.

$$E_x = \frac{RT}{zF} \log_e \frac{[X^+]_o}{[X^+]_i} \qquad (1.2)$$

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.

$$V_m = \frac{RT}{zF} \log_e \frac{K_o + [P_{Na}/P_K]\, Na_o + [P_{Cl}/P_K]\, Cl_i}{K_i + [P_{Na}/P_K]\, Na_i + [P_{Cl}/P_K]\, Cl_o} \qquad (1.3)$$

It can be seen that there is a temperature component to equations (1.2) and (1.3), temperature affects the calculated RP potential of the ions. See Table 1.2 for calculated RP values with changing temperature using Nernst (equation 1.2) assuming $[K]_i$= 400mM and $[K]_o$= 10mM. The calculated coefficient RP change with temperature was 0.3163 mV/°C. Because the RP changes with temperature, the response of an axon or muscle to a stimulation may be different at different experimental temperatures.

At rest the ionic concentration gradients are kept relatively steady by active transport (Na^+/K^+ ATPase). The equilibrium potential for each ion controls the RP depending on membrane permeability, changes in permeability move the RP in the direction of the equilibrium potential of that ion, this then affects the membrane permeability of other ions and therefore cell function (Shepherd 1988). Examples of intracellular and extracellular ion concentrations in squid and crab axon are shown in Table 1.3.

Table 1.2: Change in RP with temperature.

°C	Calculated equilibrium potential.
5	-88.4
10	-90.02
15	-91.11
20	-94.26
25	-96.347

It can be seen in Table 1.3 that there is a difference between intracellular and extracellular ionic concentrations reported by different workers, this is attributed to different sea water ionic composition. Because of $[Ion]_i$ and $[Ion]_o$ differences it is not possible to calculate an exact RP change with temperature, but Nernst coefficients can still be estimated.

Table 1.3: Examples of ionic cellular concentrations of marine animals.

Aidley (1989) Squid axon [mM].			Hodgkin (1964) Squid axon [mM].		Bendall (1969) Crab axon [mM].	
Ion	$[Ion]_i$	$[Ion]_o$	$[Ion]_i$	$[Ion]_o$	$[Ion]_i$	$[Ion]_o$
K^+	400	10	400	10	410	12
Na^+	50	460	50	460	52	510
Cl^-	40-150	560	60	540	26	540
E_{ion}	K^+=-89.35; Na^+=+53.75; Cl^- =-63.9 to -31.9		K^+=-89.35; Na^+=+53.75; Cl^-=-53.2		K^+=-85.83; Na^+=+55.3; Cl^-=-73.47	

$[Ion]_o$=haemolymph≙sea water of variable composition. The equilibrium potentials were calculated using Nernst at a temperature of 8°C.

The typical events occurring in neuromuscular transmission are; an AP is conducted along an axon to the terminal, where the RP change opens pre-synaptic channels which subsequently initiates exocytosis. The neurotransmitter diffuses across the synaptic cleft and interacts with post-synaptic receptors, depolarising the post-synaptic membrane, which subsequently initiates an EJP. This complex system of events is subject to change in response to a change in direct temperature, the question arises, *does temperature have an effect on pre- and post-synaptic function?*

Effect of Temperature on Axonal Conduction.

Action potentials (AP) in crayfish and crab incorporate Na^+ and Ca^{2+} components (Niwa *et al.,* 1982; Stephens and Church 1988), decreasing temperature reduces the

amplitude and broadens the AP. The AP width is dependent on Ca^{2+} channel activity and the AP amplitude is dependent on Na^{+} channel activity. AP rise times in 5°C and 20°C acclimated *Astacus astacus* were not significantly different from each other, but the rise time did decrease with increasing experimental temperature (Kivivuori and Lagerspetz 1982). Action potential conduction velocity is dependent on membrane resistance (R_m), the number of Na^{+} channels and axon diameter (Stephens 1985b; Hille 1992), at higher temperatures membrane resistance decreases and ion shunts occur potentially blocking AP conduction (Fatt and Katz 1953; Orkand 1962; Adams 1987; Castillo and Machne 1953). However, once an AP is successfully initiated conduction is relatively assured, the speed of conduction is linearly related to temperature in non-myelinated neurones (Montgomery and MacDonald 1990).

Decreased temperature prolongs the absolute refractory period, which can decrease the frequency of conduction of successive APs. In contrast increases in temperature potentiate the depolarising after potential (DAP); following an AP normally there is a K^{+} dependent hyperpolarisation. A DAP is caused by failure of the K^{+} hyperpolarisation, the axon remains slightly depolarised for 2-3 msec, this DAP is caused by the Ca^{2+} component of the AP (Stephens 1983) and may facilitate axonal hyperexcitability. This hyperexcitability may be responsible for peripheral generation of APs (Stephens *et al.,* 1983; 1988; Lazarus *et al.,* 1982) i.e. at a site of increased R_m (due to a narrowing of an axon or at a branch point), a single AP may produce multiple EJPs which may lead to erratic movements in crayfish (White 1983) and crabs (Stephens 1985a).

Effect of Temperature on Nervous System Pre-Synaptic Events.

Ectotherms exposed to perturbing temperatures die before metabolic function fails, this is generally due to nervous system failure (Stephens 1985a; White 1983). It is known that;

- Inhibitory synapses are more thermosensitive than excitatory synapses (Friedlander *et al.*, 1976; White 1983), which may result in peripheral generation of action potentials at warm temperature extremes (Lazarus *et al.*, 1982).
- Polysynaptic pathways are more labile than monosynaptic routes (Friedlander *et al.*, 1976).
- AP initiation blocks with less cooling than synaptic potentials (Prosser and Nelson 1981; MacDonald 1990).

A model of sensitivities to cold or heat extremes may be:- neuromuscular (NMJ) junctions (i.e. synapses) are more thermolabile than contraction in muscle (direct stimulation) or nerve conduction (Montgomery and MacDonald 1990).

The nervous system fails at extreme temperatures (synaptic block (White 1983)) before other systems when maintained at the new temperature long enough (Cossins and Bowler 1987). The key to the attainment of successful resistance acclimation is an animal's ability to maintain neurophysiological integrity (White 1983; Stephens 1985a/b; Stephens and Atwood 1982; MacDonald 1990). It would be predicted therefore that the ability of an animal's CNS to respond to a new thermal regime would determine the capacity of the acclimation response at the level of the organism. Neural acclimation is reported to occur earlier than in other tissues (Prosser and Nelson 1981; Montgomery and MacDonald 1990; Kivivuori and Lagerspetz 1982).

The reason for increased inhibitory synapse thermosensitivity may be due to loss of transmitter from intracellular stores after sudden cooling. Loss of glutamate decarboxylase is seen in rat brain (Acosta *et al.,* 1993) after sudden cooling, and a fall of 10-80% in GABA levels of some ectotherms has been reported (Prosser and Nelson 1981). However, no information is available on GABA levels after sudden heating in ectotherms.

Synaptic block may also involve decreased Ca^{2+} entry presynaptically which would reduce neurotransmitter vesicle release leading to insufficient ligand gated post-synaptic channel activation, resulting in excitatory junctional potential (EJP) or AP block (White 1983). Frogs acclimated to 10°C and 25°C successfully alter the temperatures of nervous system cold block, cold block occurred at 1°C and 4°C respectively (Jensen 1972). Additionally the NMJ cold blocking temperature was lowered with decreasing $[Ca^{2+}]_o$ (White 1976).

On warming, a cell's resting potential (RP) hyperpolarises, the hyperpolarisation may be greater than that predicted by Nernst (Montgomery and MacDonald 1990; Kivivuori *et al.,* 1990; Florey and Hoyle 1976). However, in the presence of ouabain the change in RP with temperature was reduced to that more closely predicted by Nernst (White 1983; Montgomery and MacDonald 1990). The ouabain sensitive Na^+/K^+ pump contribution to RP change with temperature in crayfish was 1.5mV/°C, in the protozoan *Paramecium caudatum* it was 0.2mV/°C (MacDonald 1990). The RP controls axon and muscle fibre excitability, different animal's RPs show different sensitivities to temperature. See Table 1.4 for examples of other animal RP changes with temperature, noting the RP change predicted by the Nernst equation was 0.3163mV/°C.

Table 1.4: RP changes with temperature in different animals.

RP change mV/°C	Animal.	Temperature range.	Ref.
2	*Balanus nubilus* muscle.	10-13	Fischbarg (1972).
1.1	*Ocypode ceratophthalma* closer walking muscle.	12+	Florey and Hoyle (1976).
1.5	*Procambarus clarkii* abductor muscle walking leg.	16	White (1983).
Constant	*Astacus leptodactylus* walking leg muscle.	10-26	Fischer and Florey (1981).
0.6	*Homarus americanus* giant axon.	2-10	Dalton and Hendrix (1962).
2-3	*Homarus americanus* walking leg muscle.	0-9	Colton and Freeman (1975).
1	*Homarus americanus* walking leg muscle.	9-20	Colton and Freeman (1975).

It can be seen (Table 1.4) that there are differences in the RP change with temperature in different species, the RP dependency on temperature also changes with the temperature range investigated, this further indicates the complexity of changes that occur in the nervous system when exposed to different experimental or acclimation temperatures.

Effect of Temperature on Post-Synaptic Events or Muscle Function.

The previous sections indicated that the direct effect of temperature on RP is complex, in consequence acclimation responses to temperature may affect those complex events differentially. Muscle fibre RP's hyperpolarise with increasing temperature due to thermal activation of membrane Na^+/K^+ ATPase (Fatt and Katz 1953). However, in warm and cold acclimated animals the RP may be quite different at different experimental temperatures, which may be due to changes in the activity or density of Na^+/K^+ ATPase (Schwarzbaum *et al.*, 1991, Hochachka 1988a). The difference in RP between warm and cold acclimated animals maintains appropriate excitability, it is not clear if the excitability is maintained by re-alignment of specific channel type conduction kinetics as the relative equilibrium potentials do not change. The RP at a given acclimation temperature may be shifted correctly in an adaptive manner (i.e. partial or complete acclimation (type III/II)) toward that of a different

(new) acclimation temperature, so that the RPs are the same at the two acclimation temperatures.

Changes in K^+ leak has been identified as a mechanism involved in AP conduction impairment of neural and particularly muscle function (Hochachka 1988b). Increases in $[K^+]_o$ at axon branch points may occur due to changes in pump activity leading to selective conduction block (Atwood 1976; Atwood and Nguyen 1995; White 1983) even in cells that are relatively depolarised. At increased temperatures Na^+/K^+ ATPase function may be impaired due to phase transitions of the membrane (Bowler and Manning 1994) and with increased K^+ leak may act synergistically to depolarise the cell with concomitant loss of function (Gladwell *et al.,* 1975; Stephens 1985a; Lazarus *et al.,* 1982).

In isolated experiments all muscle fibres are maximally recruited by direct electrical stimulation, whereas in whole animal experiments, which incorporate axonal stimulation, not all muscle fibres are recruited at the same time, due to different axons having different excitatory thresholds. Innervation pathways of axons which stimulate crayfish or crab skeletal muscle (see Figure 1.4) also reveal certain muscle fibre types are innervated by specific axons, due to axonal branching (Rathmayer and Maier 1987). In the closer muscle of the crab *Eriphia spinifrons* only muscle fibre types I and II are dually innervated by both motor axons whereas muscle fibre types III and IV are phasically innervated only (Rathmayer and Maier 1987). In whole animals the recruitment of more muscle fibres (such as types III and IV) with increased temperature may occur to compensate for changes in muscle function (Rome 1990; Atwood and Nguyen 1995; Lnenicka 1993; Lnenicka and Zhao 1991).

Increased force of muscle contraction with increased temperature is also seen due to faster shortening times of muscle fibres; force however decreases at temperature extremes (Rall and Woledge 1990). The force produced depends on the number of attached cross bridges and the force per cross-bridge. The force generating capacity of a cross bridge increases with increased temperature, the explanation for increased tetanic force is thought to be augmented force per cross bridge, rather than an increase in cross bridge cycling (Rome 1990; Rall and Woledge 1990). The permeability for Ca^{2+} required to generate 50% of maximum force is inversely related to absolute temperature, the affinity of Ca^{2+} specific troponin sites decreases with increased temperature. There is a balance between fibre shortening and the efficacy of Ca^{2+} to troponin-C (Rome 1990). An increase in mechanical power can be achieved by;

1. Changing the myosin isoforms of muscle so that each fibre can shorten faster and generate greater mechanical power.

2. Adding on more muscle (hypertrophy of a fibre not increased number of muscle fibres).
3. Enhanced number of synapses and therefore altered electrical activity (plasticity).

Calcium plays an important role in muscle function, therefore the regulation of internal calcium concentration ($[Ca^{2+}]_i$) is critical (Meldolesi 1993; Hille 1992). The restoration of low $[Ca^{2+}]$ in a muscle fibre after a depolarisation is by a Ca^{2+}ATPase pump which transports calcium into the sarcoplasmic reticulum (SR) (Meis *et al.*, 1996). Calcium buffering failure may cause cellular damage if $[Ca^{2+}]_i$ rises too high (Hochachka 1988a), buffering failure may be due to a reduction in Ca^{2+}ATPase activity (Giemonen *et al.*, 1994). Ushio and Watabe (1993) found that Ca^{2+}ATPase activities of carp SR were decreased with lowering temperatures, but through acclimation their activity improved toward normal levels. Furthermore it has been shown in male Muscovy ducklings that four weeks of cold acclimation increased the Ca^{2+}ATPase and Ca^{2+} release channel contents in SR membranes (Dumonteil *et al.*, 1995), indicating similar acclimation effects on Ca^{2+}ATPase as that previously reported for Na^+/K^+ ATPase. Furthermore, short term heating of rabbit skeletal muscle SR vesicles to 42°C quickly decreased the Ca^{2+}- transporting efficiency with no effect on ATPase activity (Geimonen *et al.*, 1994). Uncoupling of Ca^{2+}-transporting ATPase activity may be responsible for increased facilitation or DAP at high experimental temperatures due to calcium buffering failure. For a review on calcium homeostasis in crustaceans see Wheatly (1996).

The amplitudes of end plate potentials (EPP) in frog and excitatory junctional potentials (EJP) in crayfish or crab show a mixed response to temperature (Florey and Hoyle 1976; Harri and Florey 1979). In frog sartorious muscle, EPP amplitudes were maximal at 15-18°C and minimal at 22-26°C, but rapidly increased in amplitude up to a thermal blocking temperature of 36°C (Jensen 1972). Facilitation was maximal near the frog acclimation temperature (Jensen 1972); similarly reported for crayfish by Fischer and Florey (1981). However in some ectotherms facilitation was minimal near the acclimation temperature, as shown in crab by Stephens (1985a). The effect of temperature on facilitation and EJP amplitude are dependent on the acclimation temperature, membrane resistance, differences in Ca^{2+} buffering, neurotransmitter release etc. Acclimating an animal to a new regime may realign the temperature tolerances of the contributing components in neurone/muscle excitability (MacDonald 1990).

The next question was to determine the most useful function to allow the study of acclimation and the responses of *C.maenas* and *C.pagurus* to different experimental temperatures. Investigations into thermal acclimation here will involve examining

temperature induced changes in electrophysiological parameters when recorded intracellularly from crab walking leg muscle fibres. Tiiska and Lagerspetz (1994) acclimated the frog *Rana temporaria* for two weeks during their neuromuscular investigation, other workers such as Harri and Florey (1979), Cuculescu (1996), Kivivuori *et al.*, (1980) acclimated their ectothermal crustaceans for two weeks. As neuronal acclimatory changes take place faster than metabolic acclimation, they are effectively complete after two weeks (Prosser and Nelson 1981, Logue *et al.*, 1995), therefore a two week acclimation protocol will be used to acclimate *C.maenas* and *C.pagurus*. The electrophysiological investigations here will be related to work done by Stephens *et al.*, (see 1981; 1982; 1985a; 1985b; 1988 etc.) and Atwood *et al.*, (see 1995; 1976; 1994; 1971 etc).

Chapter Two.

General materials and methods.

Animal information.

Decapod crabs, *Carcinus maenas* (shore crab) and *Cancer pagurus* (edible crab; see Figure 2.0) were obtained from a fisherman at Hartlepool, they were caught in the North Sea off the Hartlepool coast. The method of capture was baited lobster pots (Crothers 1968). This enabled crabs of various sizes and both sexes to be caught without injury, and stored in environmental conditions until collected. The depth of capture was from 1-20 fathoms (1.83-36.6 metres), at shallower depths most of the crabs caught were shore crabs whereas in deeper water mainly edible crabs were caught (personal communication from Mr F. Allen (Hartlepool fisheries); although no systematic data were obtained). Crabs of appropriate (see Table 2.1) size were selected from the catch and transported fresh from the lobster pots directly to the laboratory at Durham. Undersized *C.pagurus* were caught under licence from MAFF.

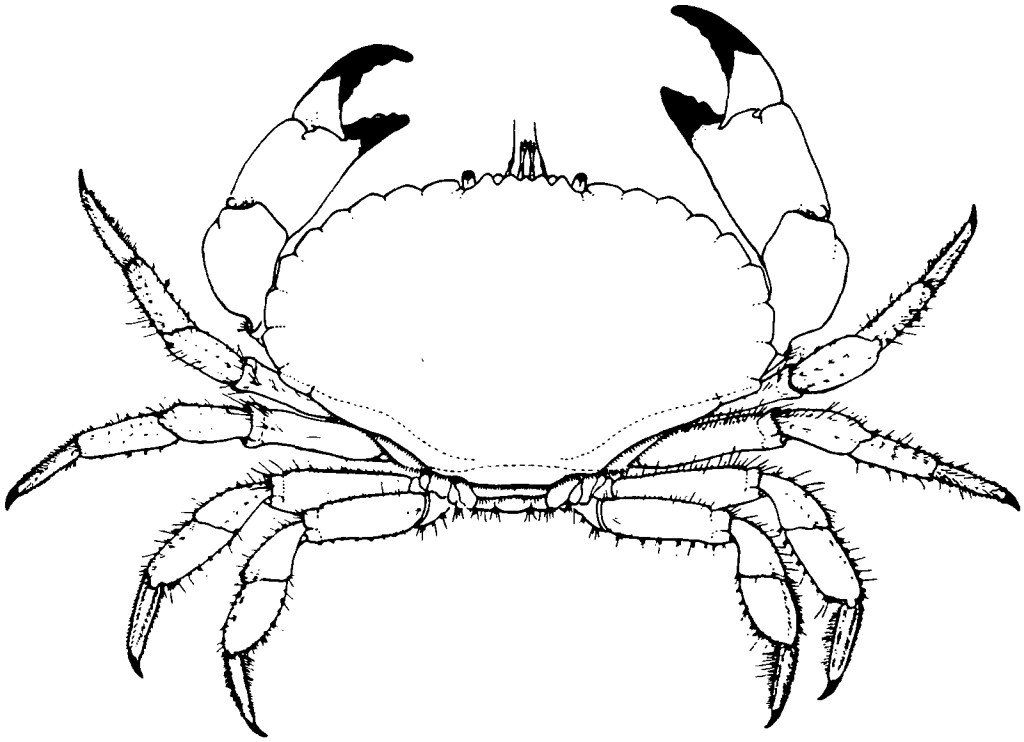
Table 2.1 Crab data.

Species.	Wet Weight (g).	Carapace width (mm).	Sex.	n
Carcinus maenas	102.3 ±4.0	69.7 ±0.7	75% male.	48
Cancer pagurus	196.3 ±7.8	106.9 ±0.2	52.8% male.	36

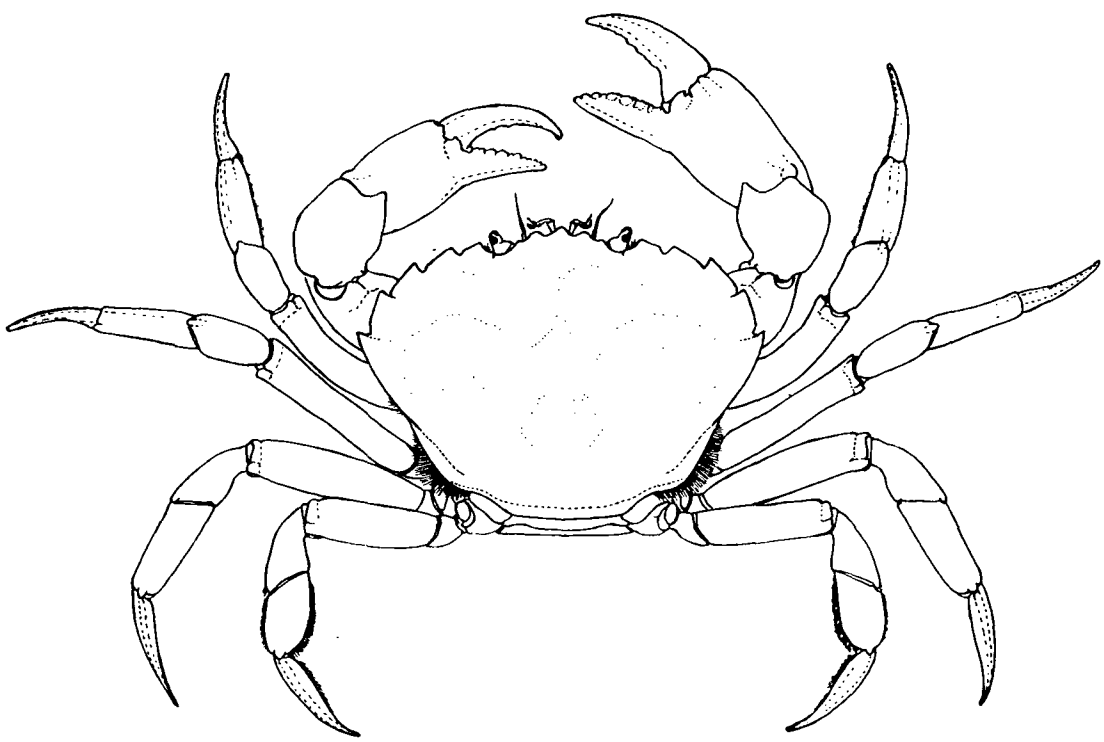
Similar sized *C.maenas* and *C.pagurus* were used due to the physical constraints of the heterothermal apparatus (see Figure 6.0), therefore younger *C.pagurus* were probably used throughout. In both species the typical life span is approximately four years, but the ages of the animals cannot be accurately determined from their size as an animal may persist in terminal anecdysis for sometime or be prevented from moulting due to parasitization. Females of both species were less aggressive than the males, female *C.pagurus* adopt a defensive posture when handled (curled legs under body i.e. Eisenchutreflex; Crothers 1967).

Throughout the experimental period crabs of an inter-moult stage were used, typically crabs moult between the beginning of September to mid-October depending on their age and size (Crothers 1967). *C.maenas* showing any parasitic infestation

Figure 2.0A



Cancer pagurus (edible crab): carapace 130 mm across



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

Taken from Crothers and Crothers 1983

were avoided as their capacity to survive in the laboratory was reduced, ovigorous *C.maenas* were also avoided. No berried *C.pagurus* were ever observed, although some crabs were infested with a parasite, observed around the hypodermis of their mouth parts. Animals that appeared diseased or covered in mucus were excluded, as were animals covered in industrial waste (bitumen). Parasitic infections have a detrimental effect on the animal's physiology, such that the animal may be weakened to miss a moult, typified by the presence of exterior encrusting organisms, which may also be present on an animal in terminal anecdysis. Animals were kept in fresh aerated sea water for a period of 24 hours immediately after capture in a temperature controlled room (8°C) with a 12 hour light to dark cycle. This enabled the animals to clean their gills of any particulate matter. Thereafter the animals were put into fresh sea water at 8°C and maintained there until an acclimation protocol was decided. Crabs were separated by species for ease of handling, a maximum of two *Cancer pagurus* per tank and three *Carcinus maenas* per tank. The acclimation temperatures investigated were 8°C and 22°C.

Sea water was collected when required from Redland Aggregates at Hartlepool (courtesy of Mr Tony Fergusson), Redland Aggregates pump fresh sea water from the North Sea in the process filtering the water through sand bags to clean it of any solid matter or vegetation. Sea water was stored in a reservoir facility in an environmental room set to 7°C, it was recirculated and filtered by Eheim pumps until used.

Glassware.

All glassware was Pyrex and cleaned thoroughly using a weak detergent (Teepol), rinsed several times using distilled water and allowed to dry before re-use, as was any plastic used.

Crab saline and microelectrode solution.

Crab saline (Stephens 1985a) consisted of NaCl 470mM, $CaCl_2$ 20mM, $MgCl_2.6H_2O$ 10mM, KCl 8mM, HEPES 10mM. The saline was pH titrated using NaOH to pH 7.4 (approximately 13°C) and aerated prior to use.

Microelectrode (ME) solution consisted of 3M KCH_3COO^-, 100mM KCl. The KCl was added to reduce polarization potentials. The potassium acetate was used in preference to KCl to reduce changes in the muscle cell's internal ionic medium, the acetate being metabolised by the muscle fibre if the electrode solution leaked from the ME. All chemicals were supplied by Sigma-Aldrich unless otherwise stated.

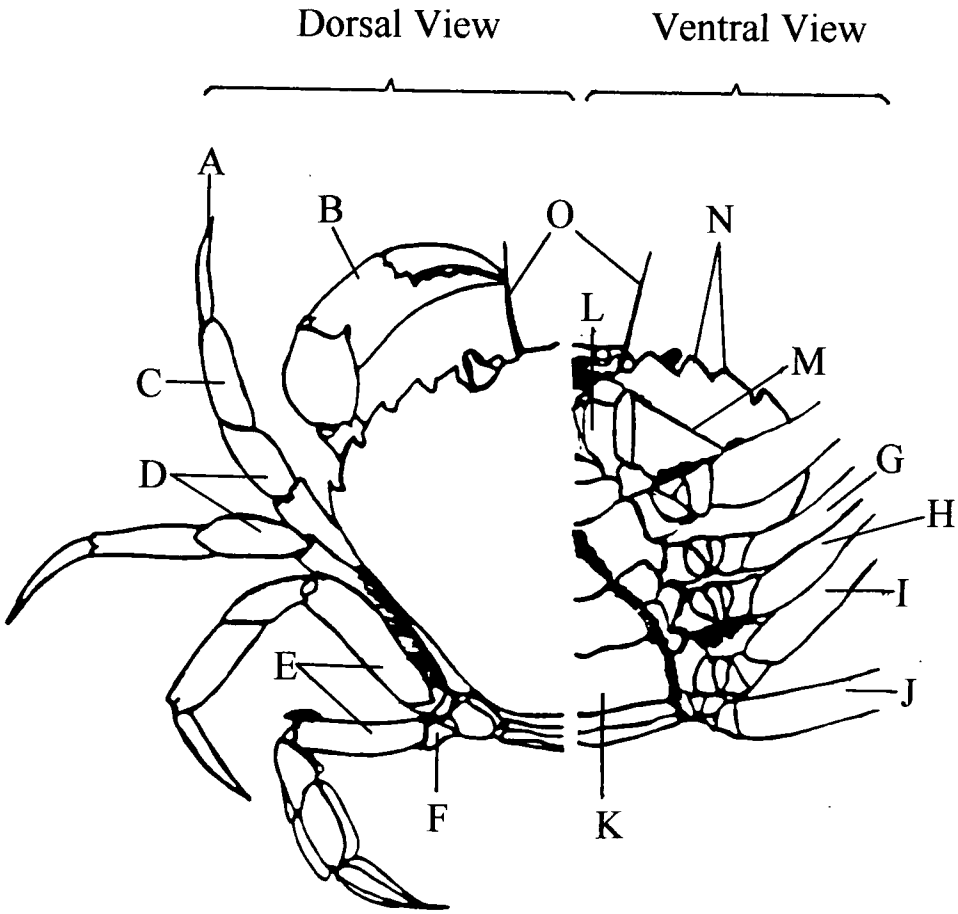


Figure 2.0B

Female *Carcinus maenas.*

A Dactylopodite,
B Chela,
C Propodite,
D Carpopodite,
E Meropodite,
F Basipodite,
G 1st walking leg,
H 2nd walking leg,
I 3rd walking leg,
J Swimming leg,
K Abdomen,
L 3rd Maxilliped,
M Pleural ridge,
N Antero-lateral teeth,
O Antennae.

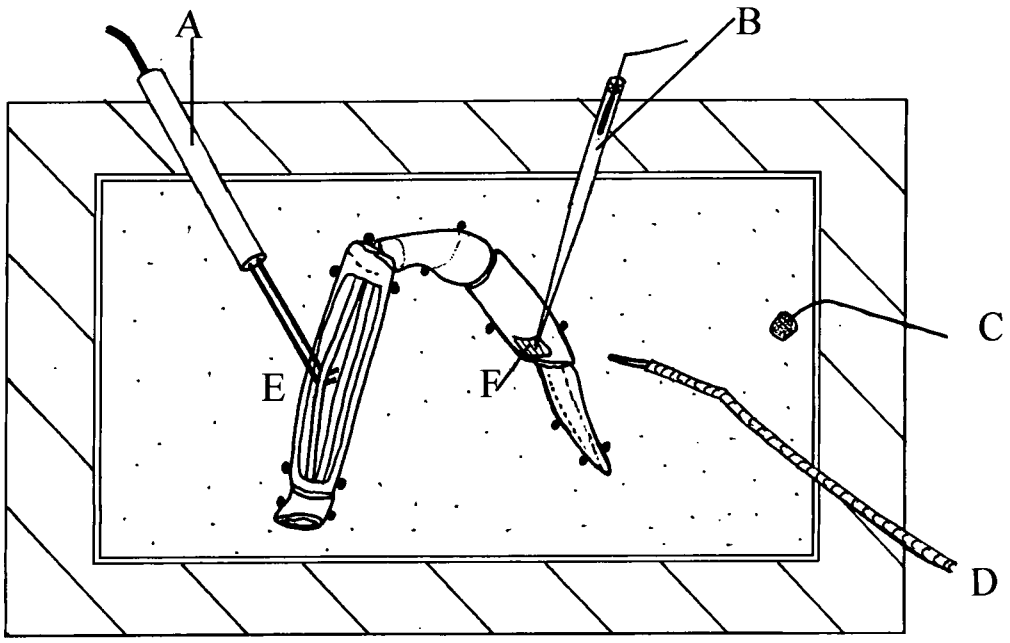


Fig 2.1: Experimental chamber.

A= Stimulating hook electrode,
B= Microelectrode,
C= Reference electrode,
D= Thermocouple probe,
E= Crab saline,
F= Muscle window cut in the propodite.

Dissection.

Walking leg acquisition.

After acclimation a walking leg was forced to autotomize by clipping with a pair of scissors the basipodite near the propodite joint of the crab walking leg (see Figure 2.0B), swimming legs were not investigated. Autotomization was initiated by cutting between the joint of the propodite and the autotomization break point which is visible as a feint line. Autotomization or leg shedding is a defence mechanism used by the crabs to escape a potential predator, damage to the leg has to occur more proximal than the dactylopodite to initiate autotomization. If allowed, the crab would commence limb regeneration after the next moult period.

Autotomization was not consistently implemented, it occurred in most dissections but not all. If autotomization was not successful the leg was cut off. Autotomization was the preferred way of obtaining legs because the animal sheds its leg, in the process sealing the wound site with a membrane, this successfully prevents haemolymph leakage and helps maintain the crab's integrity (Crothers 1967).

Walking leg dissection.

During dissection the autotomized leg was temporarily glued to a dissection board using a cyanoacrylate glue and viewed under a Zeiss dissection microscope. Areas of the cuticle were removed from the leg, specifically the anterior surface the full length of the meropodite, and a small window was cut in the anterior surface towards the distal tip of the propodite near to the ventral surface (see Figure 2.1). Removal of the meropodite carapace revealed a coloured membrane, generally yellow in *C.maenas* and orange in *C.pagurus*. The coloured membrane was pealed away to expose the carpopodite extensor muscle, the apodemes were cut at their most proximal and distal points, this muscle was removed without damaging the nerve bundle underneath. The nerve bundle was identified and washed with crab saline, and released from underlying muscle (carpopodite flexor muscle) using a pair of blunt hand made glass seekers. The glass seekers were then used to split the nerve bundle longitudinally down the centre, this was done as the closer muscle to the dactylopodite is innervated by two motor and one inhibitory nerves (see Figure 1.4), splitting the nerve bundle at the site of stimulation allowed accurate identification of the specific motor nerve required (see Chapter Three). While observing the nerve bundle in some preparations it could be identified as being in two bundles in close proximity, although previous

studies on *Brachyuran* animals have determined this to be a single bundle (Wiersma and Ripley 1952). Separation of the bundle into two equal halves was achieved on all occasions.

The muscle window cut in the distal tip of the propodite also had an underlying coloured protective membrane that was removed to reveal muscle fibres. Damage to the muscle fibres was found to result in a colour change from colourless to opaque and usually signalled future experimental problems. All exposed tissue was washed again using crab saline, the leg was removed from the dissection board and pinned within the experimental chamber See Figure 2.1. Most crab legs were slightly too long to fit appropriately into the chamber so a 5mm length of the dactylopodite was chopped off (distal tip), this had no adverse effects on the experimental protocol, it may also enable fresh saline to enter the muscle window from a different source. The chamber had a sylgard base which enabled easy pinning of the leg to an appropriate position to best facilitate electrode access, the chamber was filled with aerated crab saline to just cover exposed tissue surfaces (see Figure 2.1).

Temperature changes.

Once pinned in the experimental chamber, the preparation was placed within the Faraday cage and secured on top of a Peltier block where it would remain for the remainder of the electrophysiological investigation. A thermocouple probe (Digitron T200KC Type K) was placed near to the exposed muscle fibres to follow accurately temperature changes (±0.1°C). A sintered reference electrode was placed within the experimental chamber (see Figure 2.1) to reduce electrode drift. The Peltier block was a brass heat exchanger, by changing the d.c. current with a frigistor unit (Mectron Ltd model 1501) the amount of heat or cooling produced by the Peltier block could be manipulated to raise or lower the experimental chamber temperature. To prevent the block overheating it had cooling water running through it acting as a counter current heat exchanger. The rate of experimental temperature change was 0.4°C per minute. The experimental range investigated was 6-26°C, the frigistor unit was capable of heating to 28°C and cooling to 5°C depending on the temperature of the cooling water running through it.

Electrophysiology.

A microelectrode (ME) was used for intracellular recordings, the capillary glass was pulled into two pieces after heating the middle section, typically the ME tip produced being 1μm or less in diameter (Shepherd 1988; Standen *et al.*, 1987)(see

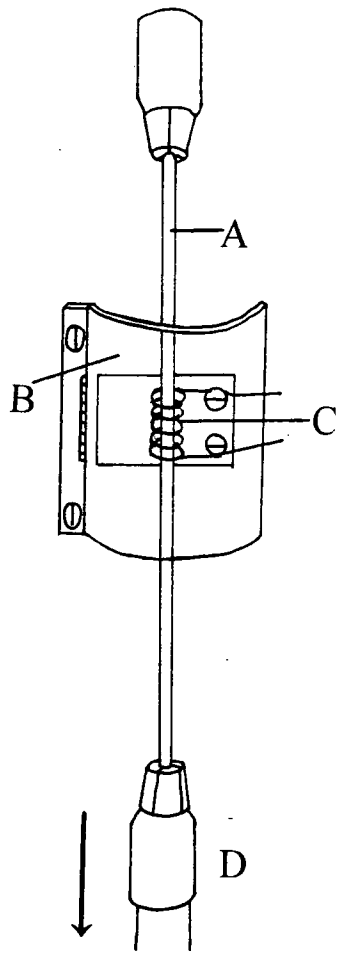


Fig 2.2: Microelectrode puller.

A= Microelectrode glass,
B= Wind shield,
C= Heating coil,
D= Locking chuck, and direction of solenoid pull.

Figure 2.2). The electrode tip is so fine that when penetrating a cell membrane little cellular damage is done to the muscle membrane or intracellular organelles. The ME was filled with a salt solution using the glass-fibre filling method (Stamford 1992; Okada *et al.,* 1975). When successful penetration of a cell membrane occurred the salt solution acted as an electrical conductor between the intracellular compartment and the wire in the (large) end of the ME which was connected to the electronic recording equipment. Penetration of a cell was confirmed by a sudden deflection on the cathode ray oscilloscope (CRO: Philips PM3206; 15MHz) from the zero level to a typically negative value of -70mV, the membrane sealed itself around the ME tip, and this stable set-up enabled experiments of several hours to be achieved (Standen *et al.,* 1987; Stamford 1992). The observed deflection to the resting potential is determined to be the ionic potential difference across the membrane, i.e. internal compartment with respect to the external medium (zeroed with respect to a reference electrode; low resistance to earth). If the RP moved toward the zero line the RP was depolarising, if the RP moved away from the zero line it was hyperpolarising (Levitan and Kaczmarek 1991; Hille 1992; Shepherd 1988). Accurate measurement of the RP of a cell during membrane permeability changes were therefore possible. However, as these changes are measured by a glass ME filled with a salt solution, junction and tip potentials (E_{junc} and E_{tip} respectively) may affect accurate measurement of the RP (Okada *et al.,* 1975; Stamford 1992). A junction potential occurs at the interface between two different solutions, due to different ion diffusion mobilities and concentration gradients (i.e. occur between salt solution in ME and intracellular or extracellular medium), or at metal-electrolyte interface (between salt solution in ME and Ag/AgCl wire or at the reference electrode between the Ag/AgCl wire and the external bathing medium). Junction potentials at electrolyte-wire interfaces are reduced as much as possible by using a Ag/AgCl sintered wire, as E_{junc} at this interface are dependent on Cl^- ion activity. The Ag/AgCl is stable to light and non-polarisable as passage of current in either direction does not alter the potential difference between the metal and solution (Standen *et al.,* 1987; Stamford 1992).

Tip potentials are a ME phenomenon and are believed to be due to the ME tip acquiring a layer of negative charge while the adjacent solution acquires a layer of positive charge, this ion plug therefore hinders the movement of anions (-ve). E_{tip} increases with increased ME resistance (i.e. >40MΩ), using electrodes with comparatively low resistance (e.g. 10-20 MΩ) and filling the ME using the inner-fibre filling method have been shown to reduce E_{tip} to a satisfactory level (Okada *et al.,* 1975). E_{junc} and E_{tip} are eliminated at the start of an experiment, after placing the ME in the extracellular solution by zeroing any potential difference by using the offset control on the amplifier (ME resistance was also measured at this point)(Stamford

1992). This assumes that once cell penetration has occurred the RP measured does not incorporate any changes in E_{junc} and E_{tip} even though the ME is now in a new (intracellular) medium and the tip may have been damaged or partially blocked during cell penetration (Stamford 1992; Standen *et al.*, 1987).

The neuromuscular parameters investigated were latency, resting membrane potential, facilitation, single and double pulse stimulated excitatory junctional potential (EJP) amplitudes, single EJP decay time constants.

Making Microelectrodes.

Microelectrode (ME) glass type GC200F-10 (Clark Electromedical Instruments) was used throughout. The ME glass was pulled in a Harvard vertical single barrel puller (See Figure 2.2). By altering the heat produced by the heating coil or pull of the solenoid, electrodes of required resistances were reproducibly made. The resistance of a ME was determined by passing a current (1nA) across the ME tip (when in a wet circuit), then noting the voltage deflection, the resistance could be calculated using Ohm's law (V=I×R). The electrode was then zeroed to remove E_{tip} and E_{junc} by passing an equivalent current to zero the ME with respect to its resistance. The electrodes determined to be most suitable were typically of resistance 20.44 MΩ ±0.87 (n=25). The microelectrodes were filled using a syringe (containing ME solution) this was aided by the electrode glass having an inner filament which is known to facilitate quick filling and reduce E_{tip} (Okada *et al.*, 1975). Electrodes were filled just before use as a means of maintaining their resistance properties and discarded at the end of an experiment. Immediately after filling the ME it was gently tapped as a means of releasing air bubble's trapped toward the electrode tip, bubbles would not alter the electrode' properties unless they built up around the Ag/AgCl amplifier wire preventing a full circuit.

Experimental protocol.

The chamber containing the (see Figure 2.1) autotomized leg was viewed in the Faraday cage using an Olympus dissection microscope. The Faraday cage was used as a means of blocking electrical interference (50Hz (mains) and 100Hz (magnetic) interference). Any cables used were electrically screened, the microscope base-plate and other metal apparatus (capable of conducting) were earthed to the Faraday cage at

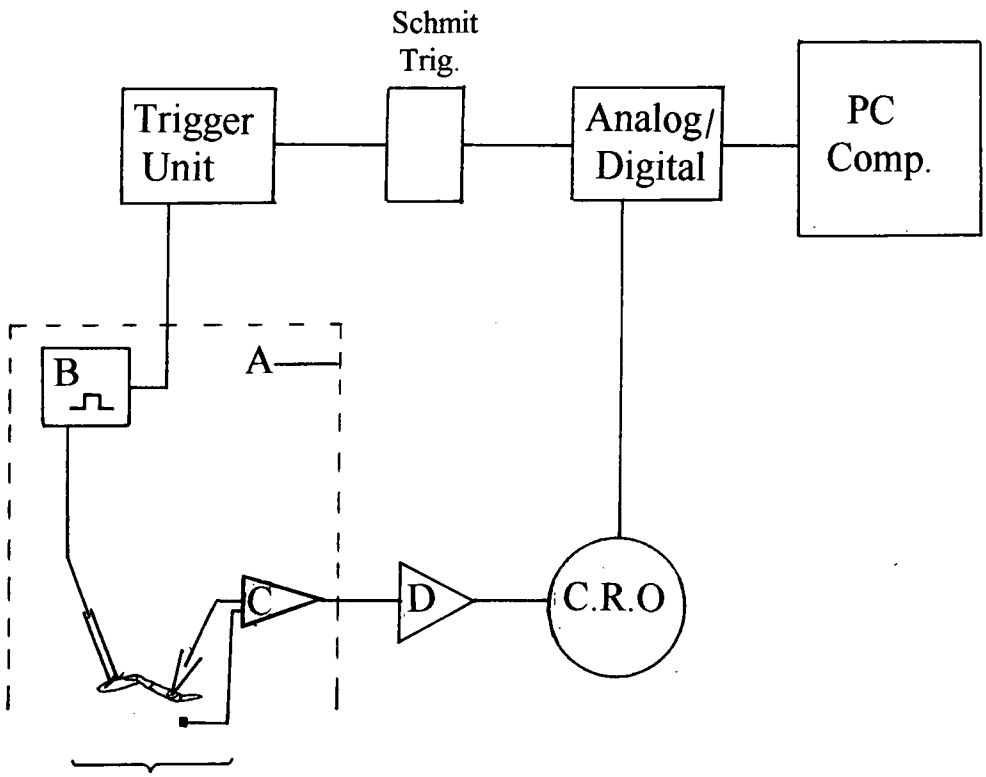
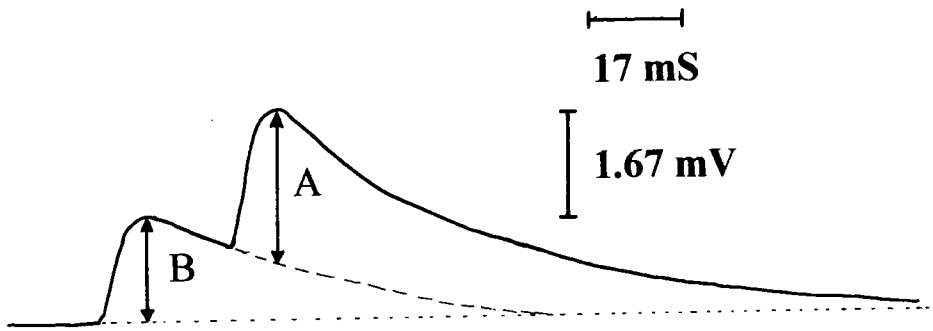


Fig 2.3: Apparatus set-up.

A= Faraday cage,
B= Digitimer isolated stimulator unit (Type D52A),
C= Headstage,
D= Amplifier (linked to Cathode Ray Oscilloscope).



Where $\frac{(A-B)}{B}$ = Facilitation.

Fig 2.4

a central point by a low resistance route. Experiments were carried out in a room with an isolated earth to reduce interference. Half the axon bundle was laid across the stimulating hook electrodes (platinum, 0.33mm diameter, Fisons). A ME was then clamped into a plastic electrode holder and secured in a micromanipulator (Prior: smallest step 10μm). The experimental set-up was now ready for muscle fibre impalement (see Figure 2.3).

Muscle fibre impalement and membrane potential measurement.

Viewing the muscle fibre window through the dissection microscope gave a clear view of approximately nine to fourteen muscle fibres. The micromanipulator holding the ME was adjusted to place the ME tip close to a muscle fibre membrane, by gently tapping the base plate and observing the cathode ray oscilloscope (CRO) it could be determined if successful impalement had occurred. Muscle impalement was proven by a sudden decrease of the CRO beam away from zero to approximately -70mV. Once impalement was successful the preparation was left for 5-10 minutes to equilibrate, typically the time taken for the Peltier block to reach the required experimental start temperature. Results were recorded from surface muscle fibres only.

Neuromuscular parameters.

All electrophysiological experimental data was recorded on 3½" floppy disks using a SCAN software program from the University of Strathclyde, sixteen records were taken per temperature change. From a single recorded EJP it was possible to determine latency (msec), decay (msec) (i.e. Tau, time taken to decay to 1/e of the original amplitude or 63%), single EJP amplitude and by recording two EJP's stimulated by a double pulse (40 msec inter-stimulus interval (I.S.I) between pulses) facilitation could be determined (see Figure 2.4). A facilitation value of zero denoted no facilitation, a value greater than zero denoted facilitation and a value less than zero denoted de-facilitation (probable inhibitory component). EJP's were recorded to disk over the experimental temperature range (6-26°C), averaged and analysed later.

It is not possible to record successfully from a muscle fibre with exactly the same characteristics, determining muscle fibre type may only be done accurately histologically (measure sarcomere lengths etc., a slow fibre having a long sarcomere (Silverman *et al.*, 1987)) and biochemically. At the start of an experiment EJP's that

gave an initial EJP amplitude <6mV were approximated to be one fibre type, whereas EJP's of >6mV were identified as a different fibre type and excluded, it was observed that almost all EJP amplitudes were <6mV. Acclimatory group data were then compared to determine differences in the acclimatory process between species and acclimation temperature.

Thermoneuromuscular block (TNB).

TNB was considered to have occurred in a preparation when the amplitude of a recorded EJP fell below the resolution of the SCAN program (i.e. <0.05mV)(White 1983), also when the stimulating voltage had to be increased greatly to initiate an EJP (stimulating voltage increases were necessary to a certain degree due to selective axon branch block and decreasing input resistance as the experimental temperature increased (White 1983)).

Data handling.

Recorded EJP data was averaged in groups of sixteen, and the mean latency, EJP amplitudes etc. were noted. The SCAN program fitted exponential decay curves (using least squares fit) to the single EJP's to determine Tau values (see equation 1.1). Data from responding animals only were then averaged using the SPSS statistical software package and analysed for significance (2-tailed) using appropriate statistical tests (one way analysis of variance, Paired t-test and Student's t-test). Mean data comparisons were determined to be significant if $p<0.05$ when using t-tables (Swinscow 1976).

Results were graphed using the Cricket Graph software package. Numbers of experiments shown in figure legends are maximal values and indicate the number of walking leg preparations used, these values varied over the experimental temperature range depending when an individual experiment was thermally blocked (TNB).

Chapter Three.

Motor axon identification, facilitation and EJP amplitude changes with temperature and inter-stimulus interval.

Introduction.

The walking leg closer muscles of *Brachyuran* crabs are innervated by two excitatory motor axons, and one inhibitory axon (see Figure 1.4 and Wiersma and Ripley 1952). The innervation of muscles in crustaceans is polyneuronal, each nerve branches many times so a single muscle fibre has multiple neuromuscular synaptic terminals (Atwood 1976; Atwood *et al.*, 1994). The two excitatory nerves to the closer muscle are typically termed slow (or tonic) and fast (or phasic). These terms describe the excitatory junctional potential (EJP) properties when each nerve is stimulated (Stephens 1985b; Stephens *et al.*, 1983; Arcaro and Lnenicka 1995) separately and not the speed of axon conduction. Excitatory terminals have more synapses than inhibitory terminals although inhibitory synapses are larger than excitatory terminals (Atwood 1976). The polyterminal nature of muscle fibre innervation may produce graded contractions due to selective conduction block of some of the neuronal terminal branches and the utilisation of functionally different synapses (Atwood *et al.*, 1994; Atwood and Nguyen 1995; Wojtowicz *et al.*, 1994). Graded contractions are dependent on the amount of transmitter released and the electrical properties of the muscle fibre. Phasic axons are normally larger diameter than tonic motor axons, but phasic synaptic terminations are particularly fine and threadlike and profuse in number. Higher muscle fibre input resistances account for 25% of the difference between EJP amplitudes generated through phasic or tonic stimulation (Cooper *et al.*, 1993). It has been shown that phasic terminals have a higher proportion of multiple active zones than tonic axons (King *et al.*, 1996), which accounts for increased transmitter release from phasic axons. Atwood *et al.*, (1994) has shown that crayfish leg opener muscle have high and low output synapses from a single innervating neurone. High output synapses recruit target muscle fibres at low impulse frequencies (large amount of transmitter released). On the other hand low output synapses show marked facilitation with increasing impulse frequency resulting in the recruitment of more muscle fibres, which results in increased depolarisation and tension with increased stimulation frequencies (Atwood *et al.*, 1994). The difference between high and low output synapses has also been identified in walking legs of

10°C acclimated lobster *Homarus americanus* (Golan *et al.*, 1996), the output of the synapse was linked to the capacity of their muscles to generate graded potentials.

Tonically innervated muscle fibres are slow oxidatively active, with a higher content of mitochondria, which correlates with their sustained muscle activity (Atwood *et al.*, 1994). Phasic axons typically innervate fast acting, glycolytically dependent muscle fibres with few mitochondria. The phenotype of a fast muscle can be changed to be more tonic simply by altering the impulse frequency (Atwood and Nguyen 1995). This can also occur under natural conditions, the claw muscle of *Procambarus clarkii* became more tonic in its physiology in summer animals, this occurred in response to a change in the capacity to release transmitter in the phasic axon (Lnenicka and Zhao 1991), but the change in muscle properties was never complete. Fast muscle fibres have short sarcomeres (3-4μm) whereas those in slow muscle fibres are long (~10μm), as shown in the crayfish *Procambarus clarkii* (Sakurai *et al.*, 1996).

The closer muscle of *Eriphia spinifrons* is composed of four functionally different muscle fibre types, the EJP characteristics of types I and II are very similar when stimulated by both phasic and tonic axons, showing small variations in the decay time constant and a smaller degree of variation in facilitation (Rathmayer and Maier 1987; Rathmayer and Erxleben 1983). Type I and II fibres are innervated by both motor axons and the common inhibitor axon (Rathmayer and Hammelsbeck 1985; Rathmayer and Erxleben 1983). Type III and IV muscle fibres only show successful EJP propagation when innervated by the phasic excitor axon, muscle fibre types III and IV are not innervated by the tonic motor axon (Rathmayer and Maier 1987). Type IV fibres may comprise as much as 50% of the total closer muscle mass in *Eriphia spinifrons* (Rathmayer and Maier 1987). Type I fibres have slower decay time constants than type II fibres as seen in the crab *Eriphia spinifrons* (Maier *et al.*, 1986). Type III and IV fibres have fast EJP decay time constants which exhibit little to medium facilitation. Typically, phasically stimulated EJP's facilitate more than tonic EJPs at higher frequencies (Atwood 1976). At low frequencies of stimulation (<10Hz) no variation in facilitation may be seen in a fibre type stimulated alternately by the phasic and tonic axons (Rathmayer and Hammelsbeck 1985; Rathmayer and Maier 1987), see Figure 2.4A showing a typical double EJP. In comparison crayfish (*Pacifastacus leniusculus*) muscle fibres are more homogenous than *Brachyuran* crabs, having three muscle fibre types, known as A, B and C, with a similar innervation as crab (Günzel *et al.*, 1993). The muscle fibre type, or composition of a muscle directly affects that muscles function, muscle contraction is mainly controlled by excitation-contraction coupling (E-CC). E-CC is a combination of electromechanical coupling i.e. control of muscle contraction by membrane potential,

and pharmacomechanical coupling i.e. control of muscle contraction independent of membrane potential but not necessarily independent of the internal calcium concentration ($[Ca^{2+}]_i$) (see Carl *et al.*, 1996).

Throughout this chapter only homothermally acclimated crabs were studied. Investigations included the affect of temperature on motor axon stimulation thresholds and the difference between EJP characteristics of muscle fibres in the closer muscle, the affect of varying the inter-stimulus interval (I.S.I) on facilitation and EJP amplitude, and the affect of thermal acclimation on closer muscle isometric contractions over the 6-26°C experimental temperature range.

The purpose of these experiments was to identify the tonic motor axon, its ability to generate muscle contraction over an experimental temperature range and to investigate the contribution of facilitation to muscle function.

Methods.

Motor axon identification.

See general methods (Chapter Two) for dissection and muscle fibre microelectrode (ME) impalement protocol. The axon bundle was ribbon like in appearance, the nerve bundle supplying the closer muscle was divided into two equal halves by splitting it longitudinally. After muscle fibre impalement, it was important to determine which half of the axon bundle (one half resting on the hook electrodes) contained the required tonic motor axon. Stimulating the axon bundle on the hook electrodes was done with a square pulse (0.2msec duration, Digitimer Ltd isolated stimulator model DS2A), initially at the lowest voltage available, if no EJP was seen on the cathode ray oscilloscope then the voltage was increased until an EJP was observed and the voltage threshold noted. This was repeated for the other axon bundle, and was the method used to identify the low threshold or tonic motor axon throughout this thesis. By examining the EJP at the start of an experiment it could be determined if any inhibitory component was present, normally denoted by a hyperpolarisation. If any hyperpolarisation was present a different muscle fibre was chosen. Inhibitory synapses are reported to be more thermolabile than excitatory synapses, changes in inhibitory synapse function may alter an EJP's characteristics (White 1983) over the experimental temperature range. The common inhibitory axon in the closer muscle is closely associated with the tonic motor axon (Günzel *et al.*, 1993), therefore presence of any hyperpolarisation of an EJP was a useful indication of the axon type.

To compare the affect of temperature on axon voltage thresholds and any differences in EJP neuromuscular parameters, both motor axons were stimulated and results recorded (see general methods, Chapter Two) from 8°C and 22°C acclimated *C.maenas* and *C.pagurus* over the experimental temperature range chosen.

Variation in closer muscle fibre electrophysiological characteristics.

In order to measure the electrophysiological characteristics of the surface muscle fibres in the closer muscle, impalement of each visible muscle fibre was carried out and EJP amplitudes recorded. Data was obtained at an experimental temperature range of 6-10°C. These measurements were taken from five 8°C acclimated *C.maenas* only. Previous studies have determined that muscle fibre types in crab (Rathmayer and Erxleben 1983) and crayfish (Günzel *et al.*, 1993) are homogeneously located from one preparation to the next. The results would reveal the differences between individual muscle fibres that make up the distal portion of the closer muscle. For each muscle fibre, resting potential, latency, double pulse EJP amplitude and double pulse decay time constant were recorded.

Walking leg closer muscle force development.

The electrophysiological apparatus shown in Figure 2.3 was used to record force measurements. In these experiments however the ME was removed and in its place a 50g isometric force transducer was used. After identification of the low threshold motor axon the leg was securely pinned in the experimental chamber using thirty 15mm steel pins, the dactylopodite article was then hooked to the force transducer after the apodeme to the opener muscle was cut. The voltage required to just initiate an EJP was increased by fifty percent, this was the suprathreshold stimulatory voltage used throughout each individual force measurement. The isometric muscle contraction generated after five seconds of a six second stimulation at 10, 20 and 50Hz over 6-26°C was recorded for the whole closer muscle. Isometric contractions were measured from 8°C and 22°C homothermally acclimated *Carcinus maenas* to determine the affect of temperature on force development and its relationship to EJP amplitude and other EJP characteristics.

Facilitation and frequency of stimulation.

After impalement of a muscle fibre with a ME the affect of a square stimulus pulse (0.2msec duration) at 0.5, 1, 2, 5, 10, 20 and 50Hz was investigated. Impalement of a

Figure 3.1A: Determination of voltage threshold in 8°C acclimated *Carcinus meanas*. The voltage required to just initiate an EJP in low and high threshold motor axons are shown; numbers of experiments were, *C.maenas* 8 Low n=5, *C.maenas* 8 High n=5. The data is presented as mean ±S.E.mean, and was analysed using paired t-test ($p<0.05$). Significant differences between low and high threshold axons are denoted by asterisks on the figures.

Figure 3.1B: Determination of voltage threshold in 22°C acclimated *Carcinus maenas*. Data is presented as mean ±S.E.mean; numbers of experiments were, *C.maenas* 22 Low n=7, *C.maenas* 22 High n=7. Significant differences between low and high threshold axons are denoted by asterisks (Paired t-test) on the figures.

Figure 3.1A: Determination of voltage threshold in 8°C acclimated Carcinus maenas.

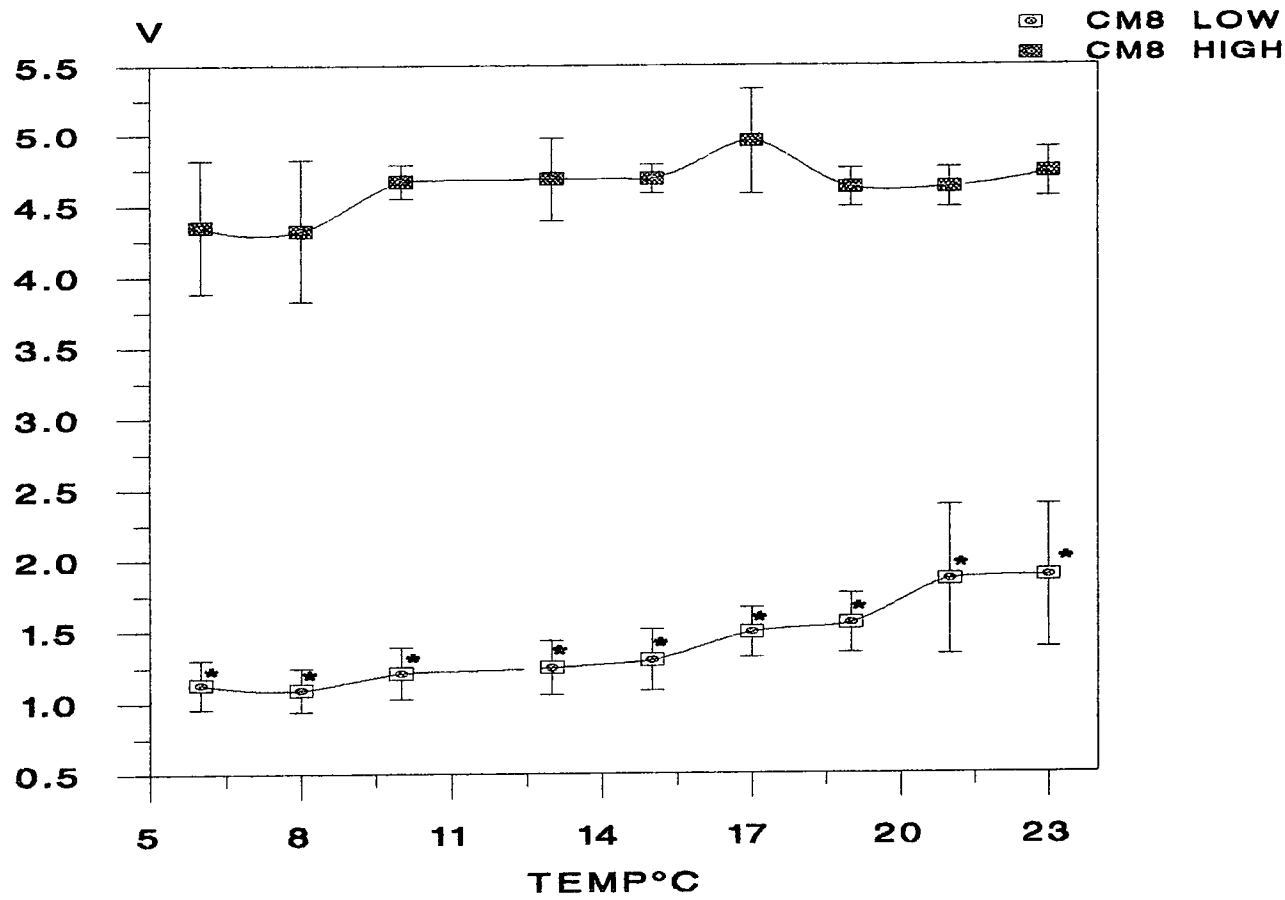
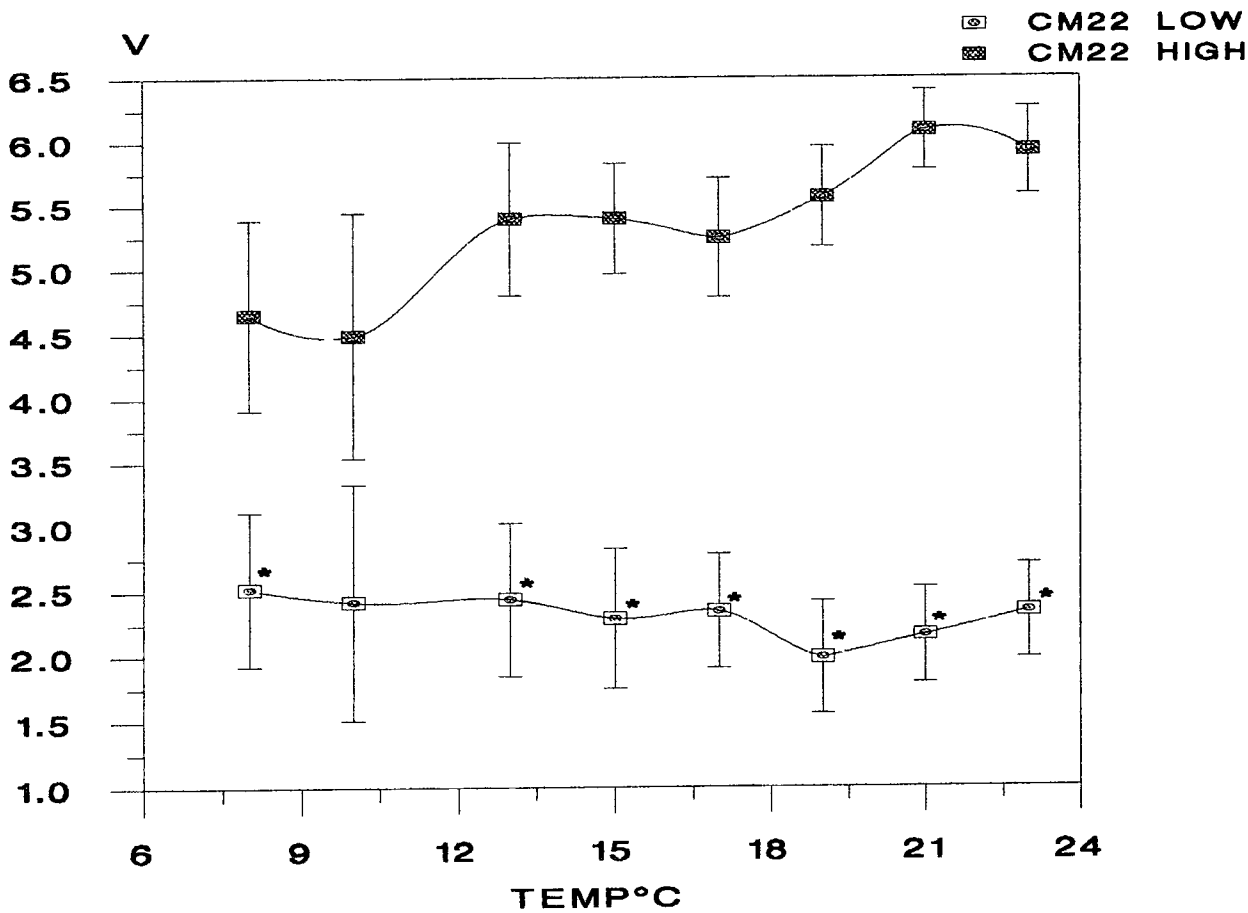


Figure 3.1B: Determination of voltage threshold in 22°C acclimated Carcinus maenas.



single muscle fibre was maintained throughout. Muscle fibre impalement was carried out on 8°C acclimated *C.maenas*, recordings were taken from a closer muscle fibre at different stimulation frequencies using both motor axons by alternately swapping the axon bundle to be stimulated. Data was recorded from five animals at an experimental temperature of approximately 6-8°C. In all experiments some leg movement occurred at the bender article. This leg movement may have affected axon stimulation as the leg moved away from the hook electrodes, this therefore may cause differences between experiments, in consequence statistical comparisons between experiments were not attempted although general comparisons of EJP amplitude and facilitation in the same animal was done to a limited extent.

To identify better the changes in facilitation and EJP amplitude with temperature, double pulse EJPs were recorded at experimental temperatures of 8, 15 and 22°C. At these temperatures the inter-stimulus interval (I.S.I) was altered to 10, 20, 40, 70 and 100msec, EJPs were recorded from 8°C and 22°C homothermally acclimated *C.maenas* and *C.pagurus*.

Results.

Motor axon identification.

Carcinus maenas.

Figure 3.1A and 3.1B show voltage thresholds required to initiate an EJP in 8°C and 22°C acclimated *C.maenas* respectively. Both motor neurones innervating the walking leg closer muscle were investigated over the 6-23°C experimental temperature range. It can be seen that in cold acclimated *C.maenas* (Figure 3.1A) the voltage required to initiate an EJP in both motor axons increased slightly. The low threshold axon response in this case was more temperature sensitive than the high threshold axon, as its threshold increased more over the experimental temperature range. Statistical analysis (Paired t-test $P<0.05$) determined significant differences between the two motor axon voltage thresholds, denoted on the figure by asterisks.

Figure 3.1B shows the data from warm acclimated *C.maenas*, in which voltage thresholds were significantly different (Paired t-test) over most of the experimental temperature range, the only exception being between the two motor axons at 10°C. Motor axon voltage thresholds in warm acclimated crabs were more variable than those of cold acclimated crabs, the warm acclimated crabs also generally required increased stimulation voltages to both motor axons to initiate an EJP. Additionally it

Figure 3.2A: Determination of voltage threshold in 8°C acclimated *Cancer pagurus*. The voltage required to just initiate an EJP in low and high threshold motor axons are plotted; numbers of experiments were, *C.pagurus* 8 Low n=5, *C.pagurus* 8 High n=5. Data is presented as mean ±S.E.mean, and was statistically compared using Paired t-test ($p<0.05$). Significant differences between low and high threshold axons are denoted by asterisks on the figures.

Figure 3.2B: Determination of voltage threshold in 22°C acclimated *Cancer pagurus*. Data is presented as mean ±S.E.mean, numbers of experiments were, *C.pagurus* 22 Low n=7, *C.pagurus* 22 High n=5. Significant differences between low and high threshold axons are denoted by asterisks on the figures.

Figure 3.2A: Determination of voltage threshold in 8°C acclimated Cancer pagurus.

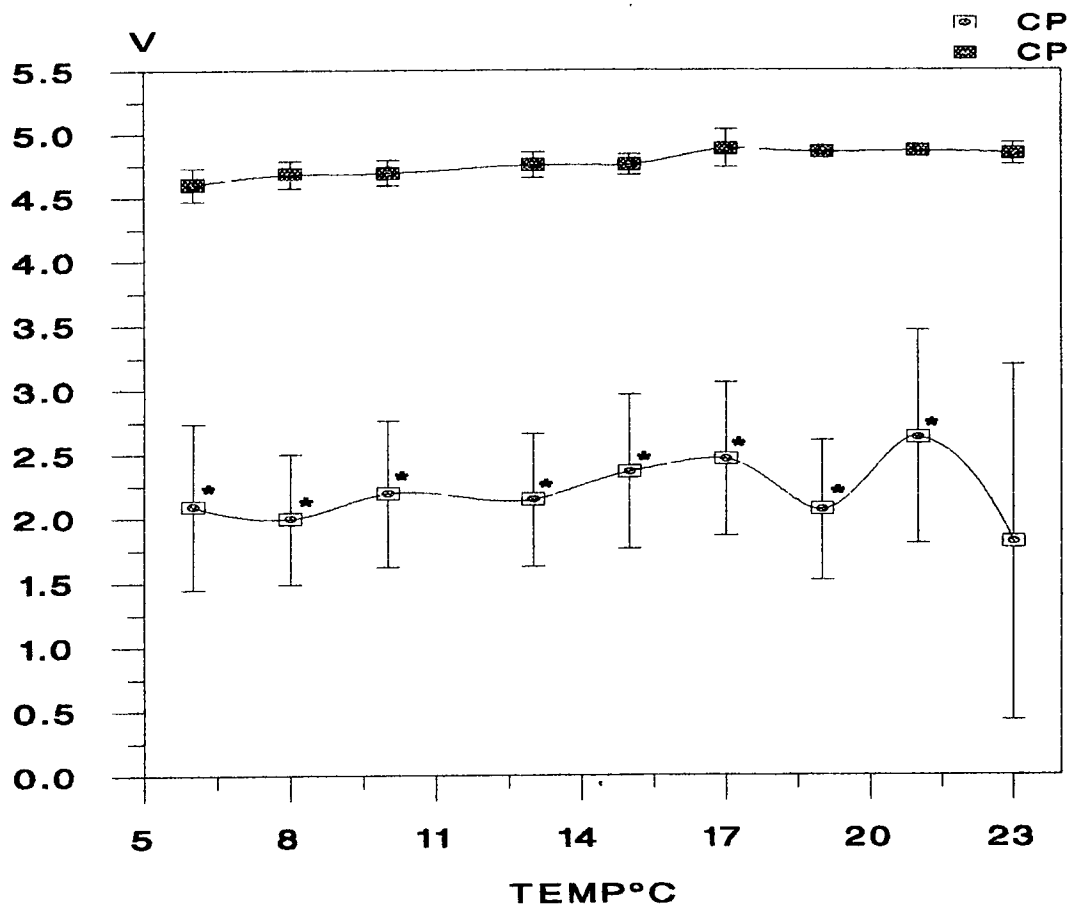
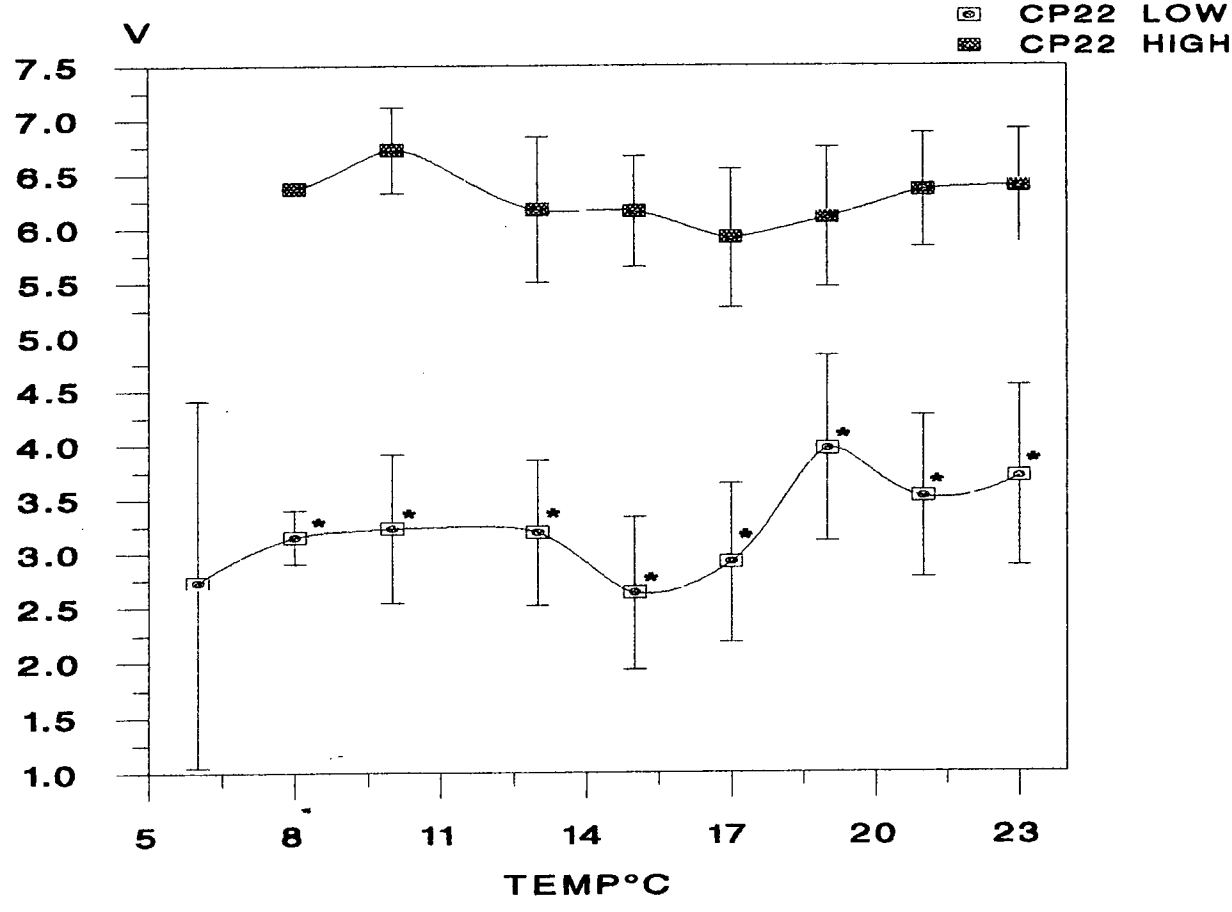


Figure 3.2B: Determination of voltage threshold in 22°C acclimated Cancer pagurus.



was seen that the warm acclimated low threshold axon required increased stimulation voltages with decreasing experimental temperature, which may indicate an acclimatory change. Generally no direct acclimatory changes were found on the thresholds of either motor axon.

Cancer pagurus.

Figures 3.2A and 3.2B show voltage thresholds for cold and warm acclimated *C.pagurus* respectively. Low and high voltage thresholds in both warm and cold acclimated crabs were significantly different (Paired t-test) over most of the experimental temperature range. Cold acclimated *C.pagurus* walking leg motor axon thresholds were similar to those of cold acclimated *C.maenas*, in that an increase in voltage threshold in both motor axons with increasing experimental temperature was seen, although the low threshold motor axon results were more variable than the high threshold axon.

Figure 3.2B shows warm acclimated *C.pagurus* motor axon voltage thresholds which were again more variable than those of cold acclimated crabs. There were significant differences (Paired t-test) between stimulation voltages of low and high threshold motor axons over the whole temperature range, except at 6°C, where for the high threshold axon no EJP could be initiated.

Other electrophysiological parameters were recorded coincidentally over the experimental temperature range whilst investigating the voltage thresholds (results not shown). Results were compared (Paired t-test) to determine any significant differences between the neuromuscular parameters generated through alternate low and high threshold motor axon stimulation within an acclimatory group. The neuromuscular parameters investigated were double pulse stimulated EJP amplitude, facilitation, single EJP amplitude and single EJP decay time constant. The resting potential was not investigated as low and high threshold results were recorded from the same muscle fibre.

No significant differences in neuromuscular parameters were found between any low and high threshold motor axon comparison, irrespective of acclimation temperature at the stimulation frequency of 0.5Hz. The neuromuscular results are not shown as they were similar to the results shown in Chapter Four, furthermore the comparisons were done to determine if axon type affected result variability.

Variation in closer muscle fibre electrophysiological characteristics.

A comparison of closer muscle properties from a single crab are shown summarised in Table 3.1. The data was taken from every visible surface muscle fibre observed after dissection. Rathmayer and Erxleben (1983) reported that identical muscle fibre types could be found in identical locations from one animal to the next, however, that could not be reproducibly achieved here. Therefore no statistical comparisons were attempted as the results would be different from one animal to the next. Table 3.1 shows large variations in EJP amplitude and EJP decay time constants.

Table 3.1: Variation in electrophysiological parameters recorded over 6-10°C from individual closer muscle fibres from a single *C.maenas* 8°C acclimated crab when tonically stimulated.

Muscle fibre no.	RP (mV).	Latency (msec).	2nd EJP amplitude (mV).	Tau (msec).
1	-75	14.5	5.5	61.9
2	-75	14.2	4.6	40.7
3	-85	13.7	5.8	82.6
4	-84	13.2	3.6	83.9
5	-95	13.3	3.7	47.6
6	-84	10.2	1.1	16.4
7	-85	11.2	4.8	28.1
8	-80	11.8	5.0	31.1
9	-80	13.5	2.6	67.2

Walking leg closer muscle force development.

Force was recorded at frequencies of 10, 20 and 50Hz from 8°C and 22°C acclimated *C.maenas* over the 6-26°C experimental temperature range using a 50g isometric force transducer. Force was measured in the closer muscle through selective stimulation of the low threshold motor axon.

Figure 3.3A shows the mean force generated by cold acclimated *C.maenas* walking leg closer muscles, it can be seen that the higher the frequency of stimulation the greater the force generated. All cold acclimated crabs irrespective of the stimulation frequency generated decreasing force with increasing experimental temperature. It was notable that no measurable force was generated at temperatures

Figure 3.3A: Tension produced in 8°C acclimated *Carcinus meanas* at different frequencies. Force production with different stimulation frequencies was determined in 8°C acclimated *C.maenas* over the 6-26°C experimental temperature range. Data was presented as mean ±S.E.mean; numbers of experiments were, 10Hz n=6, 20Hz n=6, 50Hz n=6. Students t-tests ($p<0.05$) were carried out on all data points, significant differences between groups are denoted by a>b>c on the figures.

Figure 3.3B: Tension produced in 22°C acclimated *Carcinus maenas* at different frequencies. The data is presented as mean ±S.E.mean; numbers of experiments were, 10Hz n=3, 20Hz n=6 50Hz n=5. Students t-tests were carried out on all data points, significant differences between the groups are denoted as a>b>c on the figures.

Figure 3.3A: Tension produced in 8°C acclimated Carcinus maenas at different frequencies.

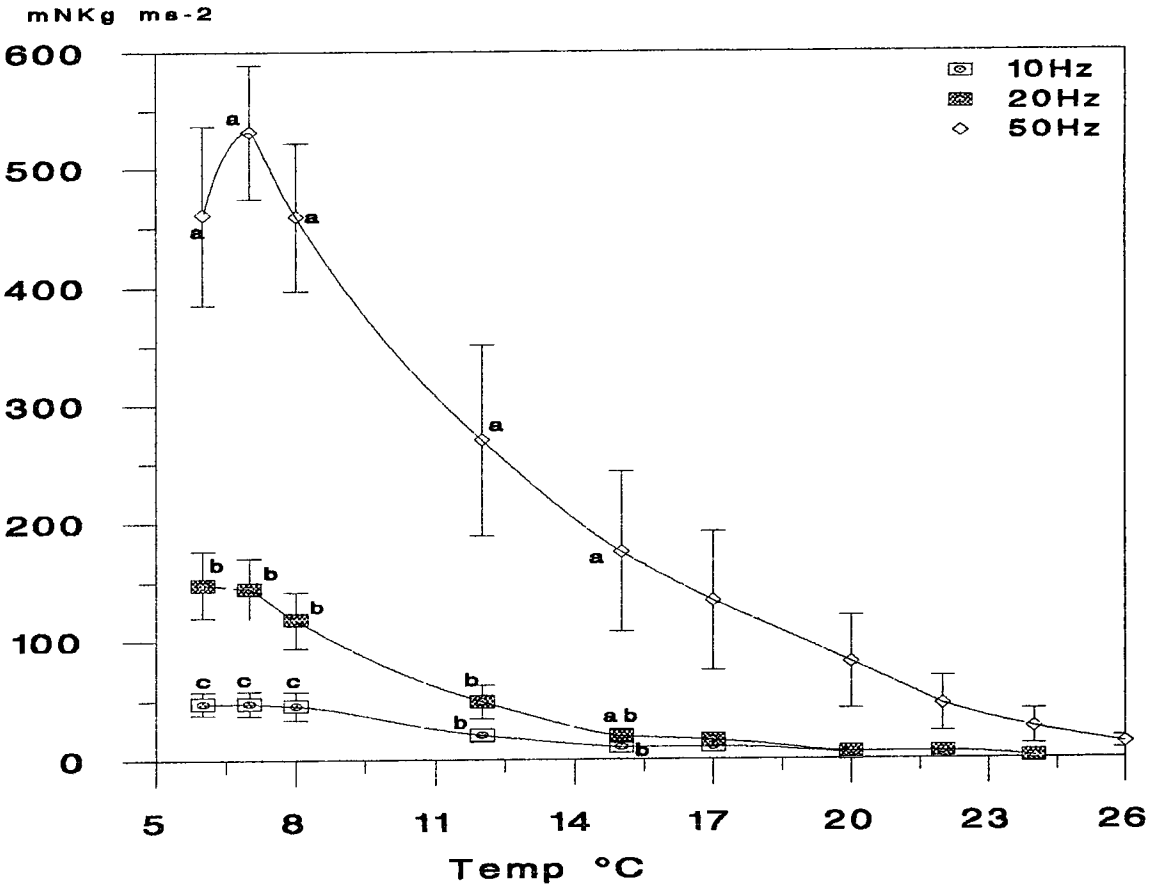
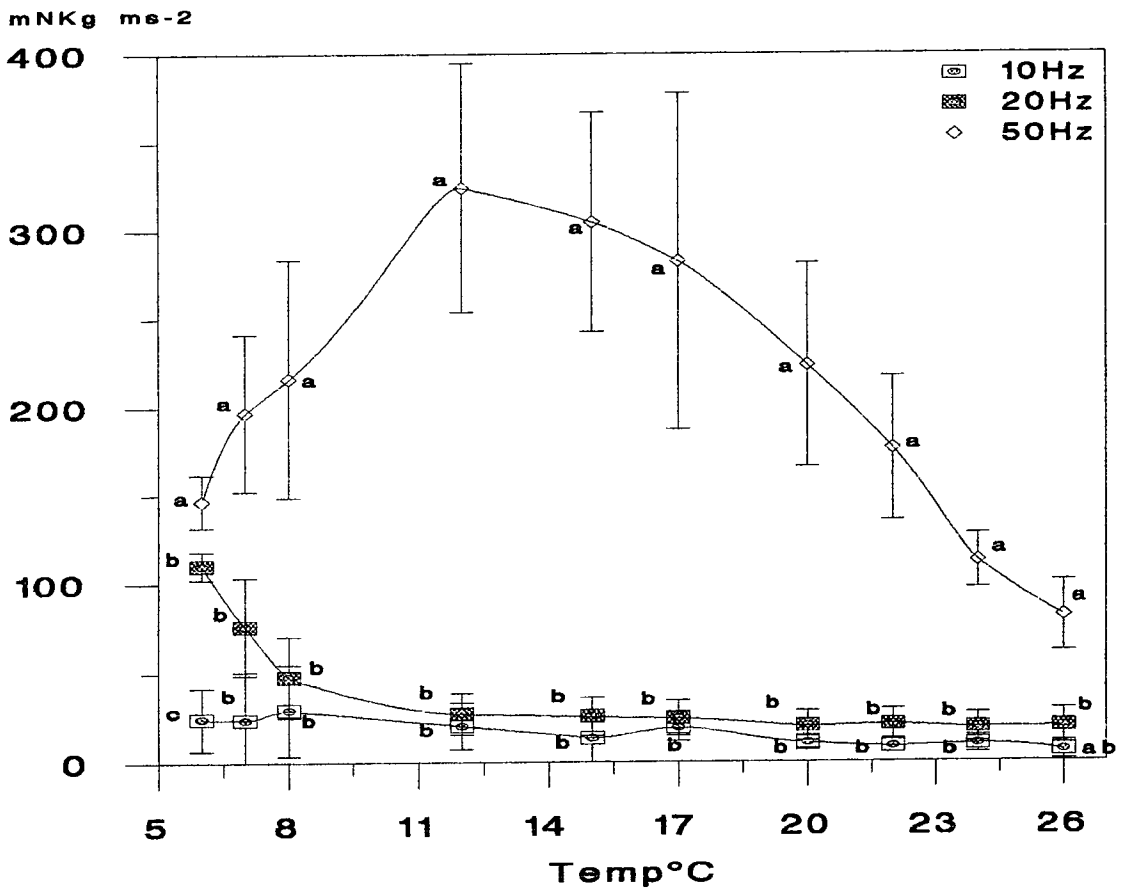


Figure 3.3B: Tension produced in 22°C acclimated Carcinus maenas at different frequencies.



warmer than 20°C and 24°C for the 10Hz and 20Hz stimulation frequency groups respectively. Statistically there were significant differences (letters on the figures show statistically significant different means; Student's t-test, a>b>c, $P<0.05$) between the different stimulation frequencies over 6-15°C of the experimental temperature range. At experimental temperatures warmer than 15°C no significant differences were found between the different stimulation frequency groups.

Figure 3.3B shows the force generated at different stimulation frequencies over 6-26°C in 22°C acclimated *C.maenas*. It was notable that all stimulation frequencies generated some measurable force over the whole experimental temperature range, unlike measurements from 8°C acclimated crabs at stimulation frequencies of 10Hz and 20Hz.

Increased force was generated with increasing stimulation frequency, which was similarly shown for cold acclimated crabs. It was apparent that the force generated at 10Hz over the experimental temperature range was comparatively constant, although it did decrease slightly with increasing temperature. Force generated at 20Hz decreased quickly over 6-9°C but was generally constant over the remainder of the experimental temperature range. Force generated at 50Hz revealed an n-shaped force versus temperature curve, maximal force was recorded at 11-15°C which, when compared to maximal force generated in cold acclimated animals (6-8°C) at 50Hz indicated an acclimatory shift of between 5-9°C along the temperature axis, indicating partial acclimation (type III acclimation, after Precht 1958).

Statistical analysis of the force results from warm acclimated crabs (Student's t-test) identified no significant differences between force generated at 10Hz and 20Hz except at 6°C, but significant differences between the force at 50Hz and both 10, 20Hz over the whole experimental temperature range.

It was noted that warm acclimated crab walking leg force was smaller than that generated by equivalently stimulated cold acclimated crabs when compared at the same temperatures, although with varying significance (Student's t-test). Statistical comparisons (Student's t-test) of the force generated at the different stimulation frequencies showed no significant differences between warm and cold acclimated crabs at 10Hz, and a significant difference at 8°C only for crab walking legs stimulated at 20Hz. Warm acclimated walking legs stimulated at 50Hz revealed significantly smaller force over 6-8°C and significantly larger force over 20-24°C when compared to cold acclimated walking legs stimulated at 50Hz. Acclimatory compensation was seen to a lesser extent in warm acclimated walking legs stimulated at 10Hz and 20Hz, warm acclimated walking legs maintained measurable force over the whole experimental temperature range, whereas cold acclimated walking legs stimulated at 10Hz and 20Hz were blocked at 20°C and 24°C respectively.

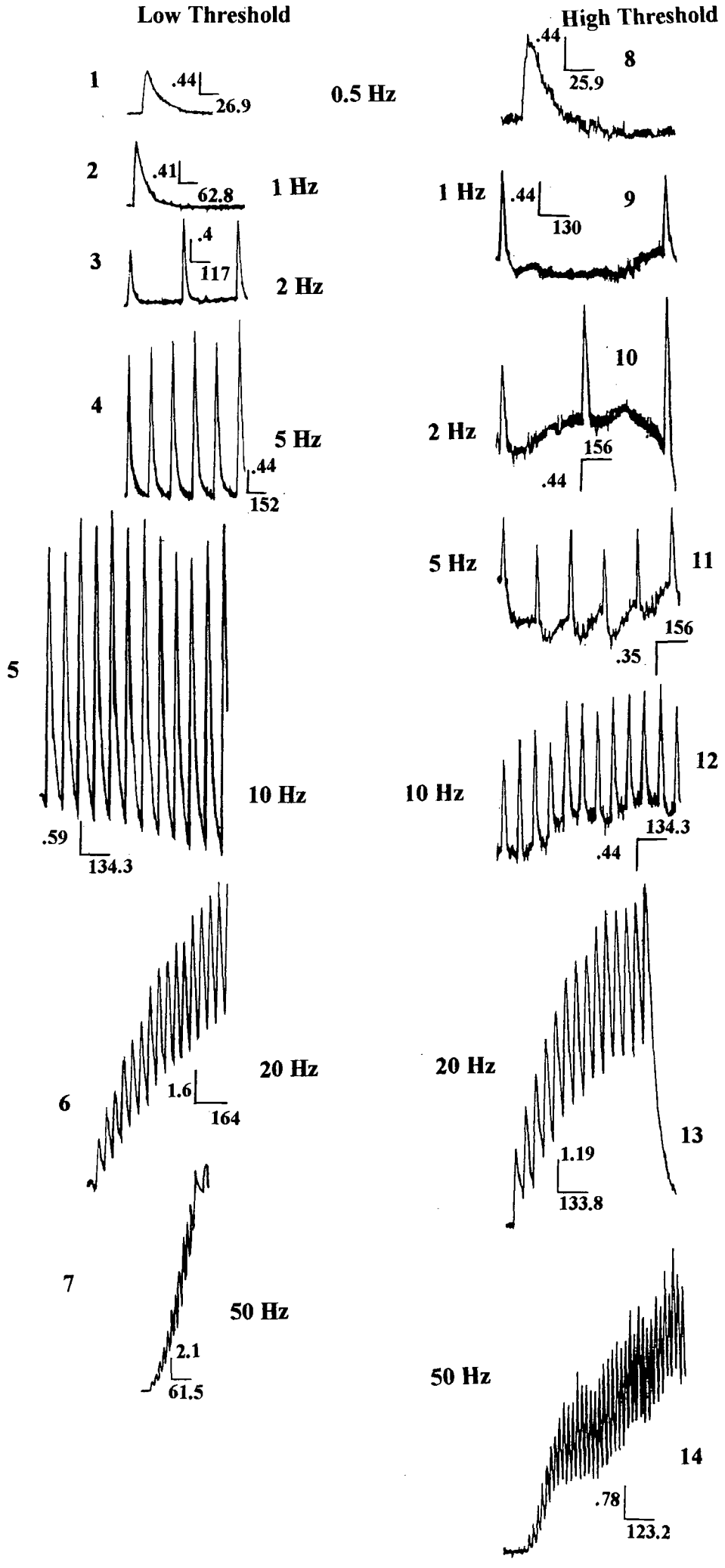
Figure 3.4: Change in EJP amplitude and facilitation with stimulation frequency in 8°C acclimated *Carcinus meanas* at 6-8°C. Results recorded from a muscle fibre stimulated by the low threshold motor axon are numbered 1-7; results numbered 8-14 were generated through stimulation of the high threshold motor axon. The low and high threshold axons were stimulated at 1.33 and 6.86 volts respectively. The microelectrode was maintained in a single closer muscle fibre to allow EJP amplitudes and facilitation comparisons between axon types.

Different amplitude (mV) and duration (msec) calibration bars are shown next to each trace;

Abscissa- msec.

Ordinate- mV.

Figure 3.4: Change in EJP amplitude and facilitation with stimulation frequency in 8°C acclimated *Carcinus maenas* at 6-8°C.



Change in EJP amplitude and facilitation with frequency of stimulation.

Figure 3.4 shows EJPs recorded at different stimulation frequencies at an experimental temperature of 7±1°C from a single 8°C acclimated *C.maenas*. No quantitative analysis was carried out between different walking legs results as different individual muscle fibres were used. It is known that the results shown are from muscle fibre types I or II only, it has been shown previously that fibre types I and II are dually innervated whereas types III and IV are phasically innervated only (Rathmayer and Maier 1987). There was little difference in the EJP amplitude of a muscle fibre stimulated alternately by both axons at frequencies of 2Hz or lower (note differences in calibration bars). The EJP amplitudes recorded from a low threshold axon stimulated muscle fibre were generally larger than the amplitudes generated by the high threshold motor axon when recorded from the same muscle fibre at stimulation frequencies of 5Hz and 10Hz. Differences in R_m could not account for the differences in EJP amplitude generated through the stimulation of the low and high threshold motor axons since recordings were taken from the same muscle fibre. Decreasing EJP amplitudes were not noted from either motor axon during sustained stimulation, but the high threshold motor axons EJPs ceased to increase in amplitude whereas the low threshold stimulated EJP amplitudes did continue to increase with sustained stimulation. Therefore it remained difficult to identify which axon was tonic or phasic.

Facilitation was calculated for each individual trace shown in Figure 3.4. At stimulation frequencies of 10Hz or lower the calculated facilitation was small i.e. less than 0.2 in almost all traces. An exception being at 2Hz where facilitation was greatest at 0.58 irrespective of the stimulating axon type. Axon stimulation at 20Hz and 50Hz generated EJP amplitudes whose facilitation was unexpectedly small, ranging from 0.4 to -0.01 from the high threshold motor axon, and 0.8 to -0.07 when stimulating the low threshold motor axon. It was not possible to identify the axon type from the facilitation results.

Inter-stimulus interval (I.S.I) changes on facilitation and double pulse EJP amplitude.

The affect of changing the I.S.I to 10, 20, 40, 70 and 100 msec on facilitation and EJP amplitude were investigated in 8°C and 22°C homothermally acclimated *C.maenas* and *C.pagurus* at experimental temperatures of 8, 15 and 22°C.

Figure 3.5A shows facilitation changes with temperature and I.S.I for cold acclimated *C.maenas*. Overall it was observed that facilitation decreased with

Figure 3.8A: Second EJP amplitude of a double pulse in 8°C acclimated Cancer pagurus at different experimental temperatures and varied I.S.I.

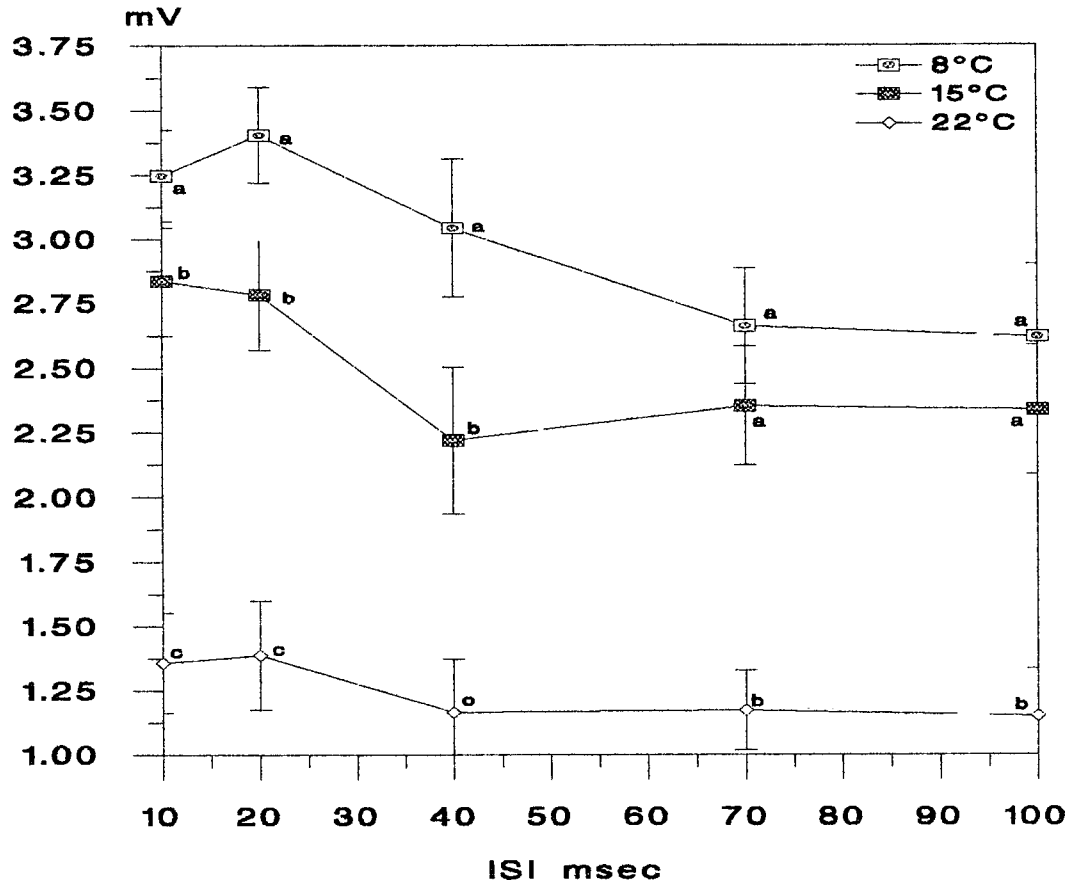


Figure 3.8B: Second EJP amplitude of a double pulse in 22°C acclimated Cancer pagurus at different experimental temperatures and varied I.S.I.

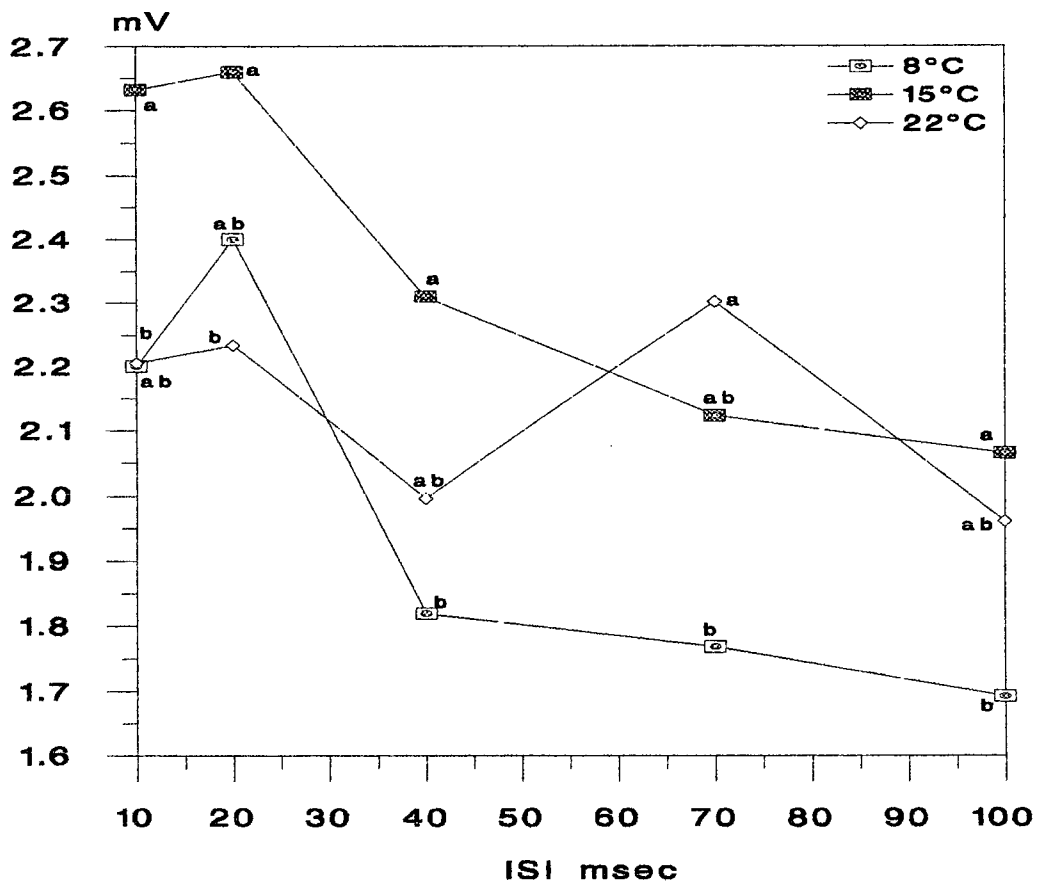


Figure 3.8A: Second EJP amplitude of a double pulse in 8°C acclimated *Cancer pagurus* at different experimental temperatures and varied I.S.I. Double pulse EJP amplitudes stimulated using the low threshold motor axon and corrected for non-linear summation are shown. All data is presented as mean ±S.E.mean; experimental numbers were, 8°C n=11, 15°C n=11, 22°C n=11. No curve fitting was attempted and data sets are linked with straight lines for identification purposes. Paired t-tests were carried out on all data points and significant differences between groups are denoted as a>b>c.

Figure 3.8B: Second EJP amplitude of a double pulse in 22°C acclimated *Cancer pagurus* at different experimental temperatures and varied I.S.I. Mean data is presented but no error bars are shown; experimental numbers were, 8°C n=9, 15°C n=9, 22°C n=9. No curve fitting was attempted and data sets are linked with straight lines for identification purposes. Paired t-tests were carried out on all data points and significant differences between groups are denoted as a>b.

Figure 3.7A: Second EJP amplitude of a double pulse in 8°C acclimated Carcinus maenas at different experimental temperatures and varied I.S.I.

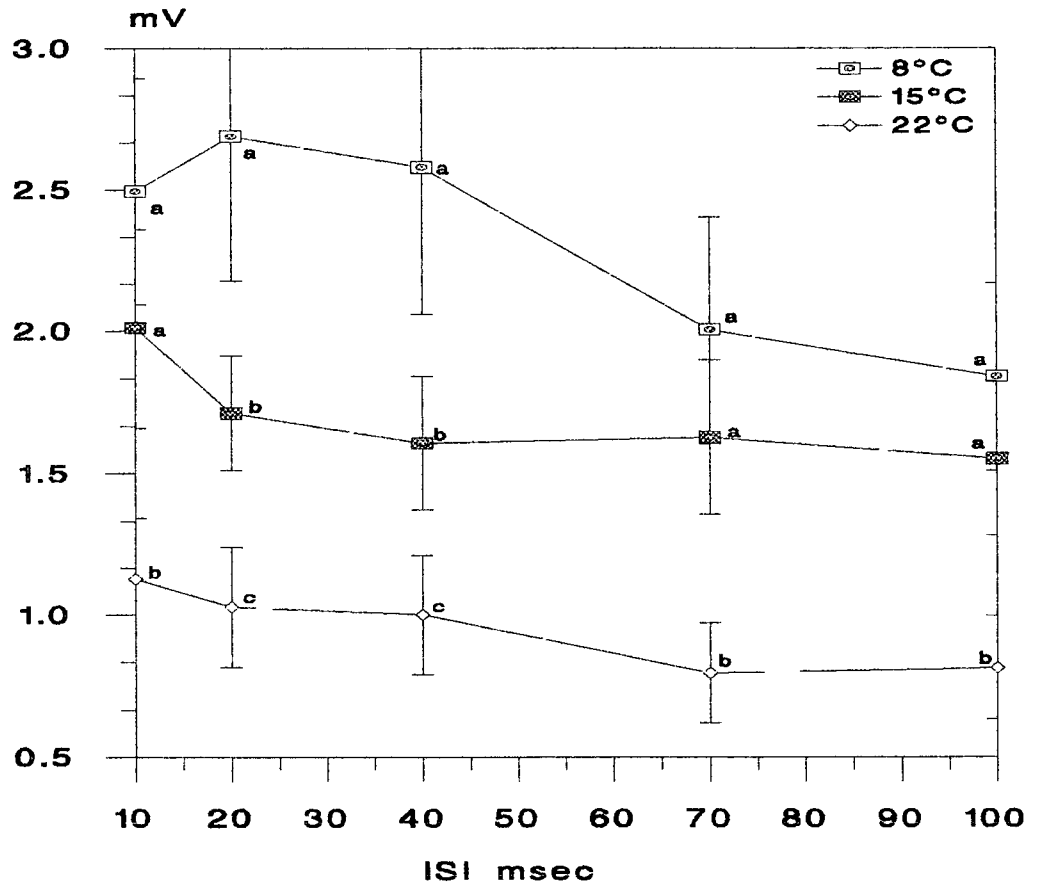


Figure 3.7B: Second EJP amplitude of a double pulse in 22°C acclimated Carcinus maenas at different experimental temperatures and varied I.S.I.

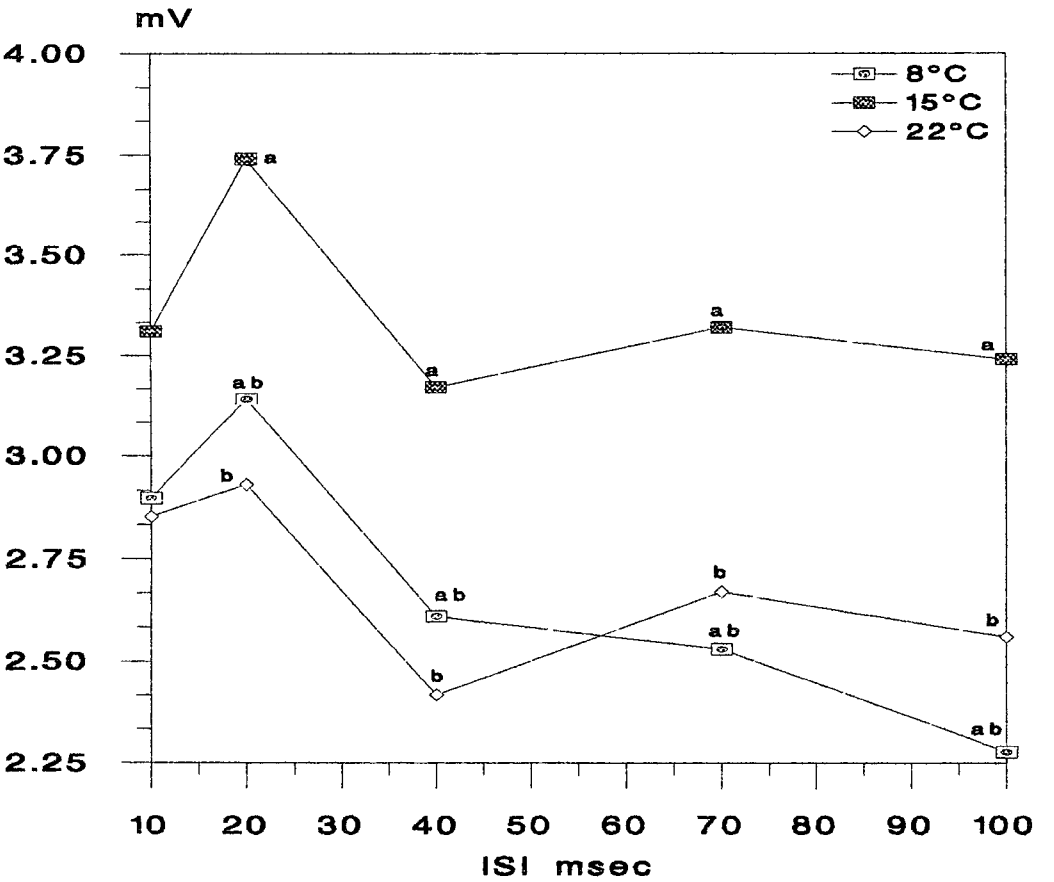


Figure 3.7A: Second EJP amplitude of a double pulse in 8°C acclimated *Carcinus maenas* at different experimental temperatures and varied I.S.I. Double pulse EJP amplitudes stimulated using the low threshold motor axon and corrected for non-linear summation are shown. All data is presented as mean ±S.E.mean; experimental numbers were, 8°C n=11, 15°C n=11, 22°C n=11. No curve fitting was attempted and data sets are linked with straight lines for identification purposes. Paired t-tests were done on all data points and significant differences between groups are denoted as a>b>c.

Figure 3.7B: Second EJP amplitude of a double pulse in 22°C acclimated *Carcinus meanas* at different experimental temperatures and varied I.S.I. Mean EJP amplitudes are presented, no error bars are shown; experimental numbers were, 8°C n=8, 15°C n=9, 22°C n=9. No curve fitting was attempted and data sets are linked with straight lines for identification purposes. Paired t-tests were carried out on all data points and significant differences between groups are denoted as a>b.

increasing I.S.I. Facilitation at 8°C and 22°C were not significantly different (Paired t-test) from each other, whereas facilitation at 8°C was significantly larger than facilitation at 15°C over 10-40 msec I.S.I. Facilitation at 22°C was not significantly different (Paired t-test) from facilitation at 15°C over all of the I.S.I range, it was generally noted that facilitation at 15°C was smaller than at any other experimental temperature.

Facilitation from warm acclimated *C.maenas* (Figure 3.5B) also decreased with increasing I.S.I. Facilitation from warm acclimated *C.maenas* was similar at experimental temperatures of 8, 15 and 22°C.

Warm acclimated *C.maenas* exhibited greater facilitation over most of the investigated I.S.I and temperature ranges when compared to cold acclimated crabs. Comparing facilitation from cold and warm acclimated groups (Student's t-test) at different temperatures and I.S.I's revealed some significant differences (separately graphed). Significant differences were found between facilitation from warm and cold acclimated *C.maenas* at an experimental temperature of 15°C over the whole I.S.I range. Furthermore at an experimental temperature of 22°C significant differences between warm and cold acclimated *C.maenas* were at I.S.Is greater than 40msec.

Facilitation changes with temperature and I.S.I for cold acclimated *C.pagurus* are shown in Figure 3.6A. It can be seen that facilitation decreased with increasing I.S.I, significant differences (Paired t-test) between facilitation at 8, 15 and 22°C were found over 10-40 msec I.S.I range only. Facilitation at 8°C was significantly greater than facilitation at 22°C over 10-40 msec I.S.I, no differences in facilitation were found at 15 and 22°C except at 20 msec I.S.I.

Figure 3.6B shows facilitation changes with temperature and I.S.I for warm acclimated *C.pagurus*. The warm acclimated crab facilitation data was quite variable, facilitation decreased with increasing I.S.I and facilitation at 8°C was significantly (Paired t-test) larger than facilitation at 15°C over 20-40 msec I.S.I. Generally there were almost no differences in facilitation generated at 8, 15 and 22°C over the whole I.S.I range. Furthermore, there were no significant (Student's t-test) differences between facilitation at experimental temperatures of 8, 15 and 22°C when comparing warm and cold acclimated *C.pagurus*.

It was noted that irrespective of warm and cold acclimation, facilitation decreased markedly with increasing I.S.I for both species, and the temperature affects on facilitation were most clearly seen in cold acclimated crabs.

Figure 3.6A: Facilitation in 8°C acclimated Cancer pagurus at different experimental temperatures and varied I.S.I

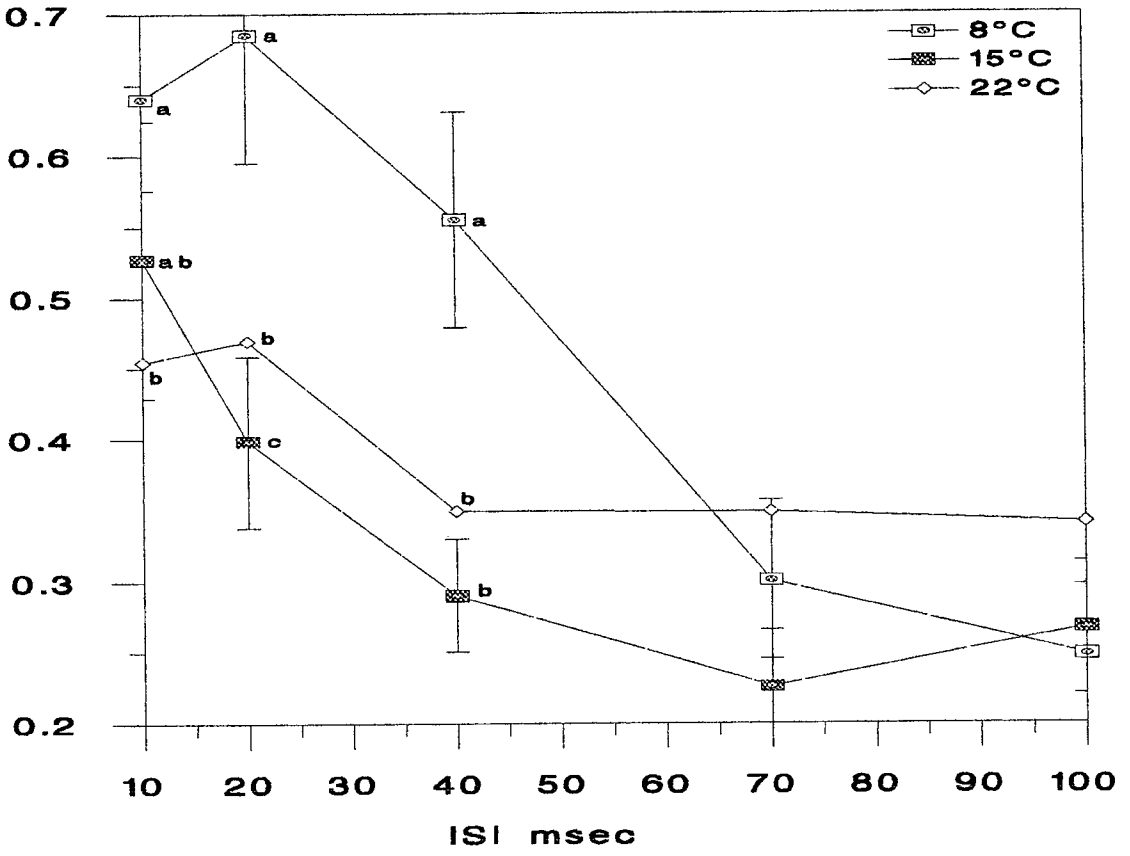


Figure 3.6B: Facilitation in 22°C acclimated Cancer pagurus at different experimental temperatures and varied I.S.I.

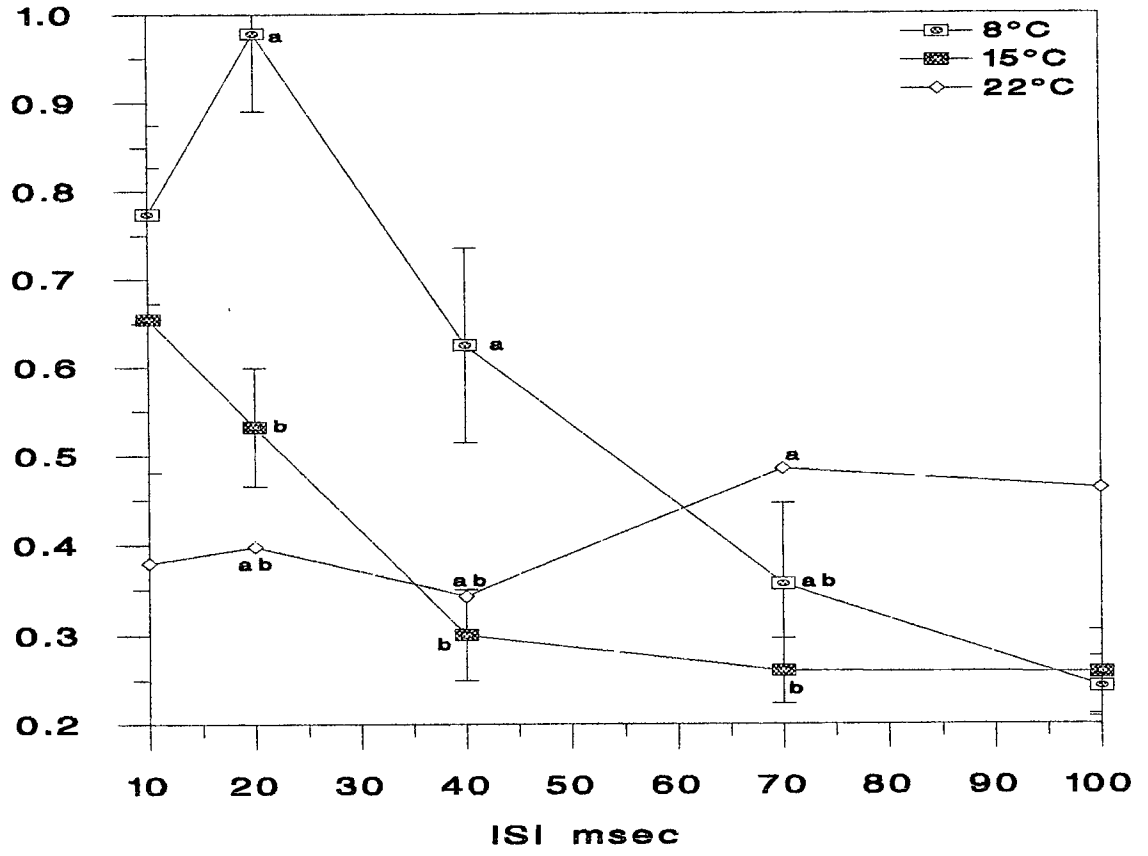


Figure 3.6A: Facilitation in 8°C acclimated *Cancer pagurus* at different experimental temperatures and varied I.S.I. Facilitation was determined through double pulse stimulation of the low threshold motor axon at different I.S.I. Mean data is presented where error bars are shown for 8°C and 15°C groups only; experimental numbers were, 8°C n=11, 15°C n=11, 22°C n=11. No curve fitting was attempted and data sets are linked with straight lines for identification purposes. Paired t-test were carried out on all data points, significant differences between groups are denoted as a>b>c.

Figure 3.6B: Facilitation in 22°C acclimated *Cancer pagurus* at different experimental temperatures and varied I.S.I. Mean data is presented, where error bars are shown for 8°C and 15°C groups only; experimental numbers were, 8°C n=9, 15°C n=9, 22°C n=9. No curve fitting was attempted and data sets are linked with straight lines for identification purposes. Paired t-tests were carried out on all data points, significant differences are between groups are denoted as a>b.

Figure 3.5A: Facilitation produced in 8°C acclimated Carcinus maenas at different experimental temperatures and varied I.S.I.

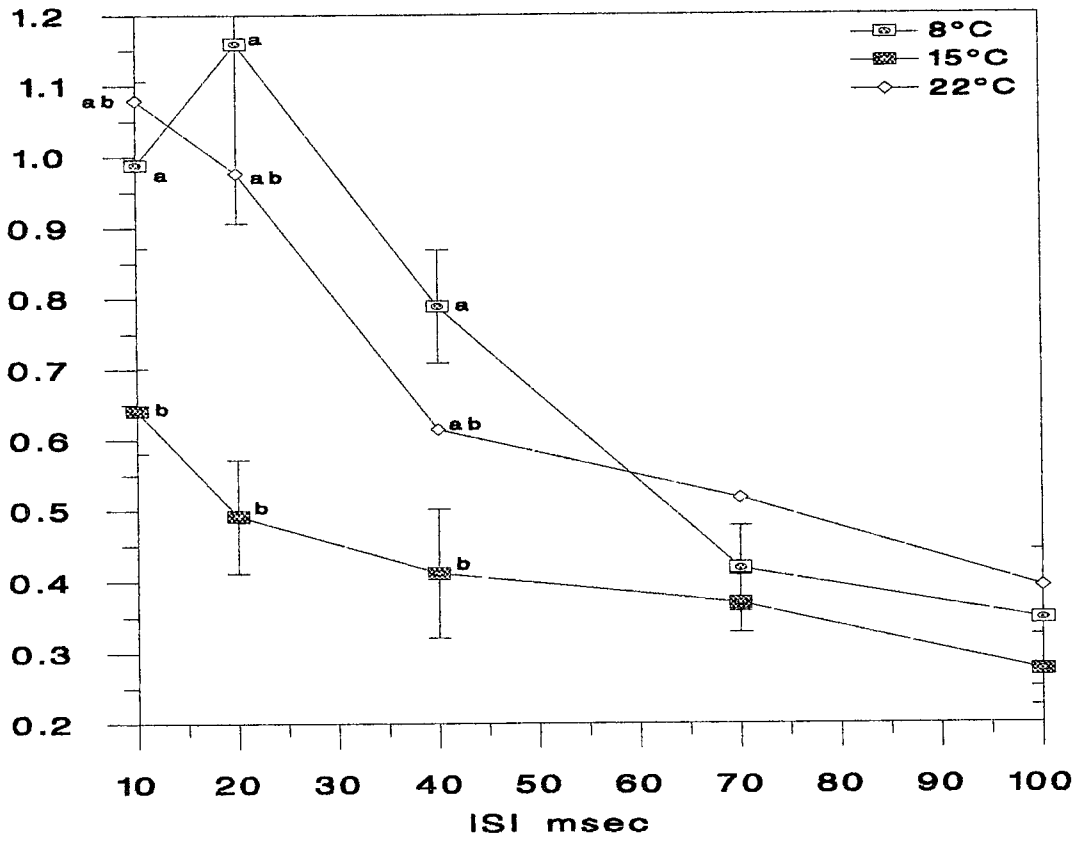


Figure 3.5B: Facilitation produced in 22°C acclimated Carcinus maenas at different experimental temperatures and varied I.S.I.

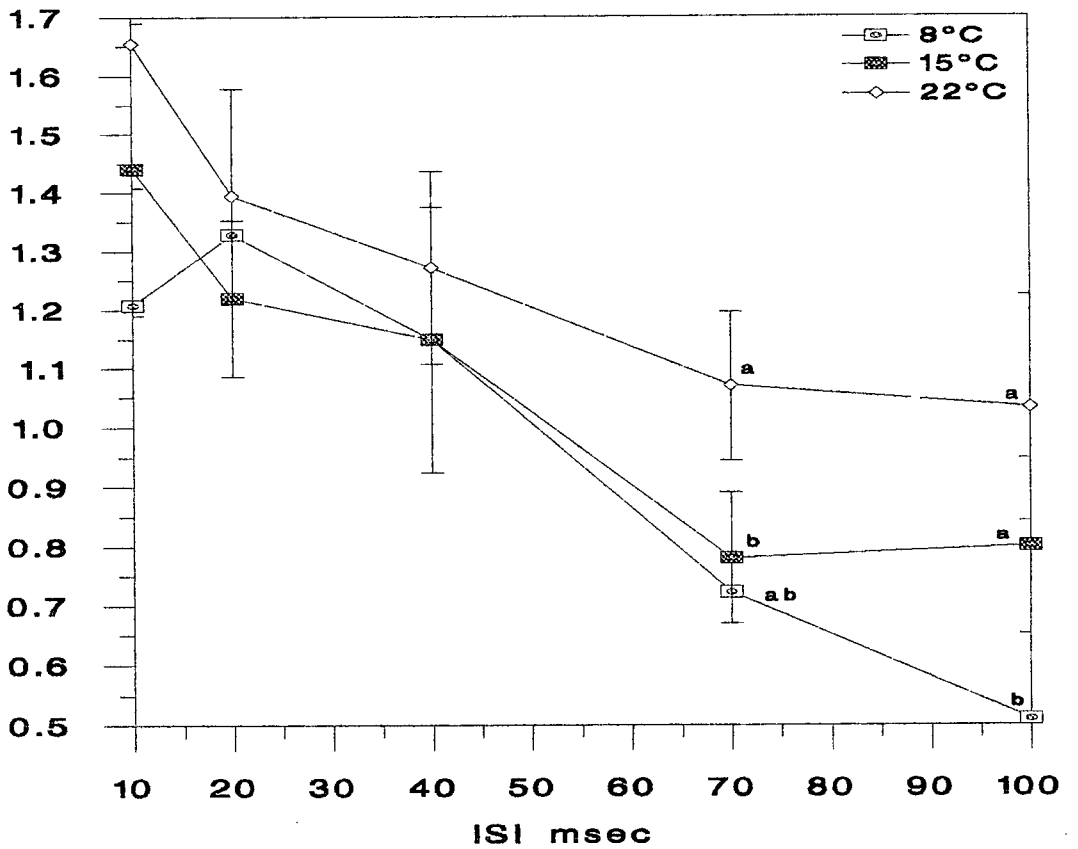


Figure 3.5A: Facilitation produced in 8°C acclimated *Carcinus meanas* at different experimental temperatures and varied I.S.I. Where I.S.I is the Inter-Stimulus-Interval. Double pulse EJP amplitudes were recorded in closer muscle fibres stimulated using the low threshold motor axon at different experimental temperatures and I.S.I. Mean data is presented, where error bars are shown for 8°C and 15°C groups only; experimental numbers were, 8°C n=11, 15°C n=11, 22°C n=11. No curve fitting was attempted and data sets are linked with straight lines for identification purposes. Paired t-tests were carried out on all data points, significant differences between groups are denoted as a>b.

Figure 3.5B: Facilitation produced in 22°C acclimated *Carcinus meanas* at different experimental temperatures with varied I.S.I. Mean data is presented where error bars are shown for 15°C and 22°C groups only; experimental numbers were, 8°C n=8, 15°C n=9, 22°C n=9. No curve fitting was attempted and data sets are linked with straight lines for identification purposes. Paired t-tests were carried out on all data points, significant differences are between groups are denoted as a>b.

Changes in double pulse EJP amplitude with temperature and I.S.I for cold and warm acclimated *C.maenas* are shown in Figure 3.7A and B respectively. It can be seen in Figure 3.7A that cold acclimated *C.maenas* EJP amplitudes decreased slightly with increasing I.S.I. In contrast the decrease in amplitude with increasing temperature was marked. The EJP amplitudes at 8°C were larger than the amplitudes at 15°C and 22°C over the whole I.S.I range, significantly (Paired t-test) so at 20-40 msec at 15°C and significantly so over the whole I.S.I range for 22°C. Amplitudes at 15°C were significantly larger than amplitudes at 22°C over the whole I.S.I range. The results show that temperature had more affect in changing EJP amplitudes than I.S.I.

Figure 3.7B shows EJP amplitude changes with temperature and I.S.I for warm acclimated *C.maenas*. Again there was a general decrease in amplitude with increasing I.S.I. However, it is important to note that the amplitudes generated at 15°C were larger than the amplitudes generated at 8°C and 15°C over the whole I.S.I range. Although the amplitudes generated at 15°C were not significantly (Paired t-test) larger than the amplitudes at 8°C, but were significantly larger than the amplitudes at 22°C over 20-100 msec I.S.I.

Cold acclimated *C.maenas* amplitudes were significantly (Student's t-test) smaller than warm acclimated *C.maenas* amplitudes at 22°C over 20-100 msec I.S.I, which indicates warm acclimated *C.maenas* maintained muscle depolarisation better than cold acclimated *C.maenas* at higher temperatures.

Figure 3.8A shows double EJP amplitude changes with I.S.I and temperature in cold acclimated *C.pagurus*. The EJP amplitudes decreased with increasing I.S.I and temperature. Amplitudes at 8°C were larger than amplitudes at 15°C over the whole I.S.I range, significantly (Paired t-test) so at 10-40 msec I.S.I. Amplitudes at 8°C and 15°C were significantly larger than amplitudes at 22°C over the whole I.S.I range, which indicated cold acclimated *C.pagurus* could not maintain their EJP amplitudes to higher experimental temperatures.

Figure 3.8B shows EJP amplitude changes with I.S.I and temperature in warm acclimated *C.pagurus*. The EJP amplitudes decreased with increasing I.S.I, except at 70 msec I.S.I when measured at 22°C. The EJP amplitudes of warm acclimated *C.pagurus* were quite variable, in general their EJP amplitudes at 15°C were larger than amplitudes generated at 8°C and 22°C over most of the I.S.I range, with varying degrees of significance (Paired t-test). Amplitudes at 15°C were significantly larger than amplitudes at 8°C at 40 and 100 msec I.S.I, and significantly larger than amplitudes at 22°C at 20 msec I.S.I only.

Cold acclimated *C.pagurus* generated significantly (Student's t-test) larger EJP amplitudes than warm acclimated *C.pagurus* at 8°C over the whole I.S.I range. Whereas warm acclimated *C.pagurus* generated EJP amplitudes which were significantly larger than amplitudes from cold acclimated *C.pagurus* at 22°C over the whole I.S.I range, which indicated warm acclimated *C.pagurus* maintained muscle depolarisation to warmer experimental temperatures equally as well as warm acclimated *C.maenas* which was unexpected. This may be linked with warm acclimated *C.pagurus*'s significantly (Student's t-test) larger EJP amplitudes at experimental temperatures of 22°C over 10-100 msec I.S.I when compared to cold acclimated *C.pagurus* (Figure 3.8A), which was also shown for warm acclimated *C.maenas*.

It was noted that temperature affected EJP amplitudes more than I.S.I in cold acclimated *C.maenas* and *C.pagurus*. Whereas warm acclimated walking leg EJP amplitude changes with I.S.I were increased slightly, the temperature affect being masked by acclimatory shifts in EJP amplitude.

Discussion.

Motor axon identification was achieved in all individual cases, the low threshold axon could always be successfully separated from the high threshold axon. Identification of different axon types by voltage threshold has been achieved in *Pachygrapsus crassipes* to identify the slow or tonic (Stephens 1985b) and fast or phasic axons (Stephens and Church 1988). It has also been shown in crayfish *Astacus leptodactylus* by Harri and Florey (1979) that the tonic axon had a lower voltage threshold than the phasic axon. Low threshold or tonic axons generate EJPs which continue to increase in amplitude with continued stimulation, indicating a more sustainable source of transmitter release (Shupliakov *et al.*, 1995). Stimulation of the low threshold axon here sometimes generated EJPs which hyperpolarised, the common inhibitor axon is known to be closely associated with the tonic axon (Günzel *et al.*, 1993), therefore it can be determined in this case that the low threshold axon was tonic in nature. Which further correlated with the general lack of increase in EJP amplitude recorded from muscle fibres during sustained stimulation of the phasic (or high threshold) axon at higher stimulation frequencies (see Figure 3.4), this is supported by King *et al.*, (1996) who reported phasic axons as being less able to maintain transmitter exocytosis.

The voltage threshold required by both motor axons to initiate an EJP increased with increasing experimental temperature (except for the low threshold axon of warm

acclimated *C.maenas*), this could be related to decreasing R_m with increasing experimental temperature (Fatt and Katz 1953). Larger diameter axons require lower stimulation voltages (Nicholls *et al.*, 1992) to initiate an EJP. Atwood *et al.*, (1994) reported phasic axon synaptic terminals as being particularly fine and therefore have increased R_m which may account for their higher voltage thresholds. Lnenicka *et al.*, (1986) reported that crayfish tonic axon terminals were of a larger diameter than phasic axon terminals, this accounts for the lower voltage thresholds of tonic motor axons and correlates with the findings of Atwood *et al.*, (1994), further supporting the conclusion that the low threshold axon was tonic. Little evidence for voltage threshold acclimation was found, in all cases warm acclimated crab axon voltage thresholds were higher, typically between 130-200% when both motor axon stimulation thresholds were compared at the warm and cold acclimation temperatures. General cell hypertrophy has been shown to occur in some warm acclimated organisms (Triestman and Grant 1993), hypertrophy may have been expected to reduce the voltage thresholds of both motor axons in warm acclimated crabs (Hille 1992; Nicholls *et al.*, 1992) although that was not seen here. Since there was no acclimatory compensation of axon thresholds it may be concluded that no axon hypertrophy occurred with warm acclimation. Comparison of the EJP characteristics from muscle fibres of cold and warm acclimated crabs when stimulated alternately by phasic and tonic axons determined no significant differences between any electrophysiological parameter. Because no differences in the EJP characteristics were found when stimulating the different motor axons at a frequency of 0.5Hz, it can be determined that stimulating at 0.5Hz did not contribute to result variability and thus will be the stimulation frequency used throughout this thesis. Additionally it indicated that the muscle fibres response to stimulation by both motor axons was similar, despite different synaptic characteristics.

Differences in the ranges of voltage threshold between warm and cold acclimated crabs may be attributable to changes in quantal content and release (White 1983), restructuring of axon terminal active zones (Van der Goor 1995) or short circuiting of stimulatory impulses (White 1983). The tonic axon was identified in all cases, and importantly identified all experimental results as being from muscle fibres of type I or II only, as it is known that muscle fibre types III and IV are innervated by the phasic axon only (Rathmayer and Erxleben 1983; Rathmayer and Maier 1987). Identification of muscle fibre types into groups I and II could not be done electrophysiologically.

Table 3.1 shows the variation in EJP characteristics recorded from different individual surface muscle fibres in the distal portion of a walking leg closer muscle

from a single cold acclimated *C.maenas*. No statistical comparisons were carried out, although the results are from muscle fibre types I and II only (Rathmayer and Erxleben 1983). Some of the result differences between fibres can be attributed to the temperature change which occurred during recording i.e. from 6-10°C; faster Na^+ channel function with increasing temperature (MacDonald 1990) accounts for the latency changes. Additionally thermal activation of Na^+/K^+ ATPase (Prosser and Nelson 1981) accounts for changes in RP, and the Tau differences are dependent on the number of open channels in the muscle fibre (White 1983; Fatt and Katz 1953). Atwood and Bittner (1971) examined individual muscle fibre EJP amplitudes in the stretcher muscle of the crab *Grapsus grapsus*, twelve fibres generated EJPs that ranged in amplitude from 3.3mV to 29mV. The results in Table 3.1 justify the assertion that muscle fibre results shown in this thesis are quite homogenous, as the EJP amplitude range is smaller and the axon type and muscle fibre types are known. It is not known if the EJP amplitude differences between muscle fibres shown in Table 3.1 are due to R_m, receptor density and sensitivity alone or a combination of effects.

The affect of acclimation temperature on closer muscle force generation, when tonically stimulated at different frequencies, revealed an acclimatory shift to warmer experimental temperatures in warm acclimated *C.maenas*. The tonic axon was selectively stimulated therefore all force measurements were determined from muscle fibre types I and II only, no force was generated by muscle fibre types III and IV as they were not stimulated. The acclimatory shift was most clearly seen for warm acclimated *C.maenas* stimulated at a frequency of 50Hz, and is best described as partial or type III acclimation after Precht (1958). The acclimatory shift was approximately 5-9°C, in that maximal force was recorded at mid experimental temperatures nearer to the warm acclimation temperature. Similar acclimatory shifts in closer muscle tension, at different stimulation frequencies, have been shown for *Astacus leptodactylus* acclimated to 12°C and 25°C when tonically and phasically stimulated (Harri and Florey 1979). Throughout the force measurement experiments, warm acclimated crabs generated decreased force at equivalent experimental temperatures (except at temperatures warmer than 20°C when stimulated at 50Hz) when compared to cold acclimated crabs, the decreased force being linked to increased lability of E-CC by Harri and Florey (1979). Fischer and Florey (1981) reported E-CC in *Astacus leptodactylus* 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).

Muscle contraction in intact crabs is through summation and facilitation of hundreds of individual EJPs, as such, muscle depolarisation or electromechanical coupling has a significant role in muscle force production and therefore crab movement. The force changes with experimental temperature in cold acclimated *C.maenas* were similar to their EJP amplitude changes with temperature, see Figure's 3.3A and 4.6A which illustrate the similarities and therefore electromechanical coupling between the force and EJP amplitude results. 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). It cannot be determined from the force data of cold acclimated crabs if any pharmacomechanical coupling was contributing to muscle contraction, although it seems probable. Increasing force with increasing stimulation frequency in warm and cold acclimated *C.maenas* 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 changes in EJP amplitude and force with experimental temperature were also similar in warm acclimated crabs, therefore indicating electromechanical coupling. Force was maintained over the whole experimental temperature range in warm acclimated *C.maenas* when stimulated at 10Hz and 20Hz, force generated at 50Hz showed a clear n-shaped force versus temperature curve, indicating an acclimatory shift. Similar results have been presented by Harri and Florey (1979) for warm and cold acclimated *Astacus leptodactylus*. The force generated at 20Hz by warm acclimated *C.maenas* over 6-9°C was larger compared to the force generated over 10-26°C which may indicate pharmacomechanical coupling as Ca^{2+} sensitivity is known to increase at colder temperatures (Stevens and Godt 1990). However, warm acclimated *C.maenas* EJP amplitudes were also larger over that same 6-9°C experimental temperature range (see Figure 4.6A). On balance the changes in force with experimental temperature, and to a lesser extent with stimulation frequency are directly related to muscle fibre depolarisation, or electromechanical coupling. The force developed by warm acclimated *C.maenas* was generally smaller than that of cold acclimated *C.maenas* when compared at the same temperatures and stimulation frequencies, this is linked to the larger EJP amplitudes recorded from cold acclimated crabs at colder experimental temperatures. The situation was reversed at temperatures warmer than 20°C when stimulated at 50Hz, furthermore, muscle depolarisation was larger in warm acclimated crabs or maintained to warmer experimental temperatures when stimulating at 10Hz and 20Hz than that found in

cold acclimated crabs, which indicates warm acclimated crabs maintain muscle depolarisation to warmer experimental temperatures irrespective of stimulation frequency.

The response of a muscle fibre stimulated by both motor axons at different frequencies is shown in Figure 3.4. Little difference was found in EJP amplitude when both axons were stimulated at 0.5-2Hz. However, at stimulation frequencies of 5-50Hz the low threshold (tonic) axon generated larger EJP amplitudes which generally continued to increase in amplitude with impulse number and stimulation frequency. Whereas stimulation of the high threshold (phasic) axon generated EJPs which were initially smaller than tonically generated amplitudes, but then increased in amplitude faster with increased impulse number until a plateau was reached. Atwood *et al.*, (1994) considered this may be due to decreasing transmitter release or recycling, typical of a phasic axon during sustained stimulation. Shupliakov *et al.*, (1995) determined that tonic axons in the crayfish *Procambarus clarkii* contained twice as much glutamate when labelled with immunogold than phasic axons. The high glutamate levels of tonic axons increased the efficiency of synaptic vesicle refilling during sustained transmitter exocytosis, whereas the phasic axon vesicle refilling was slower. It is known in vertebrate systems that glutamate uptake into synaptic vesicles is dependent on a high affinity Na^+ transporter in the axon terminal plasma membrane, and then by a low affinity Na^+ independent transport system which transports glutamate into the synaptic vesicles (Wolosker *et al.*, 1996a/b). It would be interesting to know if the efficacy of these systems was different in phasic and tonic axons, or if it changed through acclimation in tonic/phasic axons.

Closer muscle fibres are polyterminally innervated by phasic and tonic motor axons (see Figure 1.4 and Wiersma and Ripley 1952), the tonic axon is continuously active being responsible for the maintenance of animal posture and movement (Atwood and Nguyen 1995). The phasic axon is intermittently active and is responsible for initiation of movement and escape responses (Atwood and Nguyen 1995), due to these different stimulation characteristics on the closer muscle it seems probable that the motor axon which is continuously active (i.e. tonic axon) would affect the phenotypic characteristics of the muscle fibre (Rathmayer and Maier 1987). The affect of sustained tonic motor axon activity on the muscle fibre phenotype probably accounts for the lack of differences between neuromuscular parameters recorded from the same muscle fibre (Figure 3.4) when both tonic and phasic motor axons are alternately stimulated at different frequencies, although the axon types are different, the muscle fibre phenotype was principally tonic in nature.

Facilitation generated by the low (tonic) and high (phasic) threshold motor axons in the same muscle fibre were not different, both exhibited a degree of depression with sustained stimulation typical of a phasic axon (Atwood *et al.*, 1994). At higher stimulation frequencies (20Hz and 50Hz), facilitation was comparatively small which might be related to the equivalent I.S.Is of 50 and 20msec respectively, although, Van der Kloot (1994) reported I.S.Is shorter than 10msec only affected maximal facilitation.

No statistical comparisons of amplitudes and facilitation shown in Figure 3.4 was carried out. However, it was obvious that the low threshold (tonic) axon when stimulated at 10Hz or greater generated EJPs which continued to increase in amplitude with sustained stimulation more rapidly than that observed through stimulation of the high threshold (phasic) axon. Because muscle fibre R_m and receptor sensitivity/number cannot account for motor axon differences in evoked EJP amplitude, the differences can be attributed directly to transmitter availability and release, this being supported by the work of Harri and Florey (1979); Atwood and Nguyen (1995). The low and high threshold axons were identified as tonic and phasic respectively.

Facilitation decreased in both species with increasing I.S.I, the likely cause being the removal of transmitter, but principally Ca^{2+} buffering and a decrease in the probability of transmitter release, together with at longer I.S.I R_m having a profound affect on the decrease in muscle fibre depolarisation (Fatt and Katz 1953). Similar facilitation results have been shown for *Pachygrapsus crassipes* at temperatures lower than 12°C (Stephens 1985a). Facilitation at 10msec I.S.I was variable, however, previous workers determined that complete facilitation could not be generated at I.S.Is as short as 10msec (Van der Kloot 1994) due to incomplete transmitter interaction with post synaptic receptors. Increased facilitation at 20msec I.S.I is linked to increased EJP amplitude shown by almost all acclimatory groups and by both species, possibly through more complete utilisation of the exocytosed transmitter.

Facilitation in warm acclimated walking leg muscles, from both species, decreased with increasing I.S.I, except for warm acclimated *C.pagurus* at an experimental temperature of 22°C over 70-100msec I.S.I. Differences between facilitation from warm acclimated *C.maenas* at the different experimental temperatures were minor. Facilitation recorded from warm acclimated *C.maenas* revealed a comparatively flat facilitation versus temperature curve, due to facilitation at an experimental temperature of 15°C being comparatively large. This indicates that warm acclimated *C.maenas* maintained muscle fibre function through increased

facilitation. Increased facilitation at experimental temperature extremes correlates with increasing R_m at low temperatures, and increased quantal content and release or Ca^{2+} buffering failure at high temperatures.

Facilitation in cold acclimated *C.pagurus* was minimal at an experimental temperature of 15°C over almost all the I.S.I range, with varying degrees of significance. The results indicate facilitation was decreasingly sensitive to increasing I.S.I with increasing experimental temperature. Figure 3.6B shows facilitation from warm acclimated *C.pagurus*, facilitation at an experimental temperature of 8°C was significantly larger than facilitation at experimental temperatures of 15°C or 22°C over 20-40msec I.S.I, which may indicate a lack of acclimation, generally facilitation was again decreasingly sensitive to increasing I.S.I with increasing experimental temperature. Facilitation was similar from warm and cold acclimated *C.pagurus*, no significant differences (Student's t-test) were found between warm and cold acclimatory groups. This generally indicates decreased acclimation and decreased ability to maintain sustained muscle function at the warmer experimental temperatures.

EJP amplitudes from cold acclimated *C.maenas* (Figure 3.7A) and *C.pagurus* (Figure 3.8A) were similar, showing decreased EJP amplitude with increases in I.S.I and experimental temperature. Fischer and Florey (1981) reported cold acclimated crayfish at lower experimental temperatures required lower stimulation frequencies to produce tension, than the stimulation frequency required to produce identical tension at warmer experimental temperatures. The decrease in EJP amplitude with increasing experimental temperature indicates increased thermal sensitivity and correlates with decreasing R_m and Ca^{2+} sensitivity (Fatt and Katz 1953; White 1983; MacDonald 1990). Wolosker and Meis (1994) considered that a decrease in EJP amplitude with increasing I.S.I is due to removal of Ca^{2+} and transmitter. The increase in EJP amplitude from 10-20msec I.S.I seen in cold acclimated crabs at an experimental temperature of 8°C, but recorded in all warm acclimated crabs irrespective of experimental temperatures and species, was similarly reported by Van der Kloot (1994) who reported I.S.I.s shorter than 10msec did not allow maximal facilitation to be generated.

EJP amplitudes from warm acclimated *C.maenas* were more variable, but clearly show an acclimatory shift in peak EJP amplitude to warmer experimental temperatures nearer to the acclimation temperature. Warm acclimated *C.maenas* generated significantly larger EJP amplitudes than cold acclimated *C.maenas* at warmer experimental temperatures. EJP amplitudes generated by warm acclimated *C.maenas* at experimental temperatures of 8°C and 22°C exhibited no significant

differences from each other indicating similar levels of muscle function at those temperatures, whereas muscle depolarisation at 15°C was increased.

EJP amplitudes from warm acclimated *C.pagurus* were more variable than amplitudes from cold acclimated *C.pagurus*. Amplitudes generated at an experimental temperature of 15°C by warm acclimated *C.pagurus* were larger than those from cold acclimated *C.pagurus,* and therefore exhibited an acclimatory shift. There were no significant differences between EJP amplitudes from warm acclimated crabs at experimental temperatures of 8°C and 22°C indicating similar levels of muscle function at those temperatures. Warm acclimated *C.pagurus* exhibited significantly decreased muscle depolarisation at 8°C but significantly increased muscle depolarisation at 22°C when compared to cold acclimated *C.pagurus*, indicating an acclimatory shift similar to that reported for *C.maenas*.

The I.S.I results indicate that maximal facilitation in most cases and maximal EJP amplitudes in all warm and most cold acclimated crab experiments, irrespective of species were recorded at 20msec I.S.I, which is equivalent to a stimulation frequency of 50Hz. This correlates perfectly to maximal force recorded from warm and cold acclimated *C.maenas* (Figure's 3.3A and 3.3B). Similar force results have been reported by Stephens (1985a) for a single muscle fibre from the stretcher muscle of *Pachygrapsus crassipes*. The data here indicates facilitation was decreasingly sensitive to increasing I.S.I with increasing experimental temperature, the experimental temperature determined the relative extent of muscle facilitation through changes in Tau. Warm acclimated crabs facilitated more over the whole I.S.I range irrespective of the experimental temperature and may be the method used by warm acclimated crabs to maintain muscle function at temperature extremes. It was clear that the I.S.I did not significantly affect the EJP amplitude as much as temperature, where facilitation was affected by both.

Chapter Four

Homothermal acclimation of *Carcinus maenas* and *Cancer pagurus* to 8°C and 22°C when free and immobilised.

Introduction.

The body temperatures of ectothermal animals will be subject to fluctuation as a consequence of the natural seasonal and daily temperature changes. Through the processes of acclimatization such animals are able to make physiological and biochemical adjustments, that compensate for the seasonal temperature changes, that contribute to the maintenance of fitness over the range of temperatures experienced (Cossins and Bowler 1987). An important point to emphasise is that, for ectothermal animals, temperature is an all pervasive parameter. In consequence a change in environmental temperature will be experienced by all tissues. A vast literature exists, from studies at all levels of biological organisation, that confirms the adaptive nature of acclimatization responses (which has included goldfish (Sidell *et al.*, 1973), crab closer muscle (Rathmayer and Maier 1987), *Aplysia* (Triestman and Grant 1993), seasonal changes in axon terminal morphology (Lnenicka and Zhao 1991), muscle biochemistry (Silverman *et al.*, 1987; Sakurai *et al.*, 1996), channel and pump function (Hochachka 1988a/b)). It is pertinent to this study that many such studies have been carried out on crustaceans (see White (1983); Atwood and Nguyen (1995); Dudel *et al.*, (1990); Kivivuori *et al.*, (1990); King *et al.*, (1996); Lnenicka (1993); Layne *et al.*, (1985); Harri and Florey (1979); Lazarus *et al.*, (1982); Aagaard (1996); Ahsanullah and Newell (1971); Atwood and Bittner (1971); Atwood (1976); Blundon (1989) etc).

In contrast, relatively few studies exist that concern the control of acclimatization (acclimation). There is uncertainty whether the acclimation responses of an ectotherm are the sum of the individual tissue and cellular changes or whether acclimation is driven by central controlling mechanisms of the CNS and endocrine system. This issue was identified by Prosser *et al.*, (1965) and Roberts (1966) but has received scant attention since, although Prosser and Nelson (1981) provided evidence of a function for the CNS in acclimatory responses in fish, a view supported by Lagerspetz (1974).

The experimental protocols developed in the present study were designed to address this specific question. The essence of the experimental design was to compare the acclimation responses in peripheral tissues from animals homothermally acclimated (the whole animal at one temperature, either warm or cold), with the responses from tissues from heterothermally acclimated animals (the animal with one part of its body warm whilst the other part was kept cold; see Chapter Six). In heterothermally acclimated

animals the partition was placed so that the CNS was either warm or cold (see Figure 6.0) and the peripheral tissue chosen for study, the neuromuscular system of the walking legs was at a different temperature. Thus, it was possible to determine the extent to which the temperature of the CNS influenced the acclimatory responses of peripheral tissues, and how these compared with cold and warm acclimation in homothermally acclimated control animals.

This approach also permits extensive comparisons to be made between a relatively stenothermal, *Carcinus maenas* and eurythermal *Cancer pagurus*, crab. Such comparison may enable a better insight into the physiological basis of steno- and eurythermy.

The employment of heterothermal protocols have been used infrequently in studies on the acute effects of temperature on individuals. Bowler (1960) studied the lethal death points in crayfish, Prosser *et al.*, (1965) studied the effect on metabolism in the eel, and Fahmy (1972 and 1973) studied critical thermal maxima in trout. The present study, however, is novel in that it concerns chronic exposure of an animal to heterothermal conditions and allows an assessment of control of the acclimation process to be made.

In this chapter the decapod crustacea *Carcinus maenas* and *Cancer pagurus* were both homothermally acclimated to 8°C or 22°C. This was carried out in free moving and immobilised crabs to determine warm and cold acclimation temperature differences, and to compare species differences. But also to determine if animal immobilisation an essential condition for heterothermal acclimation, affects the measured electrophysiological parameters.

The nomenclature used throughout the figures was; free *Carcinus maenas* homothermally acclimated to 8°C is abbreviated to free *C.maenas* 8, whereas free *Cancer pagurus* acclimated to the same temperature would be free *C.pagurus* 8. Immobilised animals acclimated to the same temperature would be IM *C.maenas* 8 and IM *C.pagurus* 8 respectively, if the acclimation temperature was warm it would appear as IM *C.maenas* 22 and IM *C.pagurus* 22 respectively.

Methods.

It was necessary to identify the low threshold or tonic motor axon innervating the closer muscle so as to reduce muscle fibre type variation, which made the data collected more homogeneous. This was carried out essentially using the procedures as described in Chapter Three.

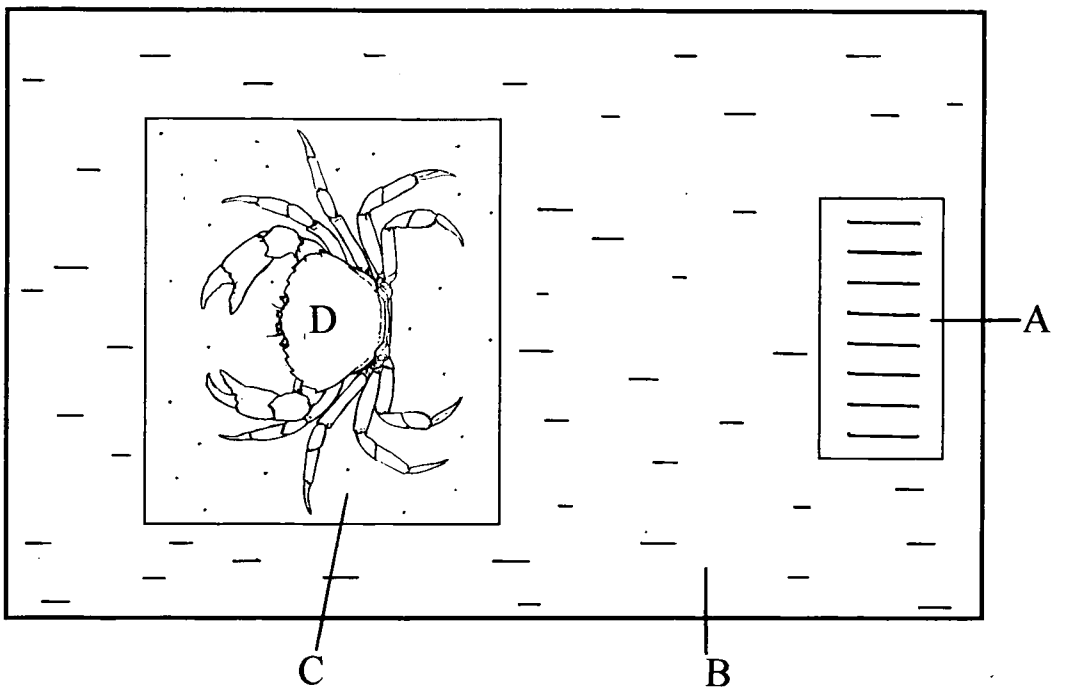


Fig 4.0

A= Water heater,
B= Heated fresh water,
C= Sea water,
D= Acclimating crab.

Pictured is a control free homothermally acclimating crab (Carcinus maenas). The crab was either warmed to 22°C or maintained at 8°C.

Homothermal acclimation.

Crabs were immediately put into plastic tanks (35 × 22cm) with aerated fresh sea water in an environmentally controlled room, set to 8°C (12 hour light/dark cycle). The crabs were fed a combination of mussel and frozen fish once a week and transferred to a fresh tank within 24 hours containing clean aerated sea water to prevent animal death due to fouled water. This protocol was maintained throughout the acclimation period.

Crabs were kept at 8°C for a minimum acclimation period of fourteen days, the crabs were considered to be acclimated after that period of time, proven by time course experiments done by Cuculescu (1996) on warm and cold acclimated *C.maenas* and *C.pagurus*.

Crabs to be acclimated to 22°C were initially maintained in the 8°C environmental room, after three days they were transferred to a different environmental room set to 12°C (12 hour light/dark cycle), the tanks were then put into a temperature controlled water bath, see Figure 4.0. The crabs were maintained in the room at 12°C for a further three days, thereafter the water bath temperature was increased to 15°C. The crabs were thereafter every three days subjected to temperature increases to 18°C and finally 22°C. The time taken to raise the sea water temperature up to the required acclimation temperature of 22°C typically took sixteen days, inclusive of feeding and fresh sea water changes. The fresh sea water when changed was at 8°C, the new water temperature increased up to the required temperature quickly i.e.<12 minutes. Upon reaching the final acclimation temperature the crabs were left for a period of fourteen days to acclimate fully to the new thermal environment (Prosser & Nelson 1981; Ahsanullah and Newell 1971). From the point of capture through to a fully acclimated condition typically took 28-30 days.

Electrophysiological measurements were recorded from both species acclimated to 8°C and 22°C (free and immobilised) as per the protocol in general methods; Chapter Two, and analysed for significance using one way analysis of variance (one way ANOVA).

To determine the Na^+/K^+ATPase contribution to the resting potential hyperpolarisation with increasing temperature, a group of free cold acclimated *C.maenas* crabs were dissected and walking leg muscle incubated in crab saline containing 1mM ouabain for 25 minutes to abolish the ATPase contribution to resting potential. The incubated preparation was then impaled with a microelectrode and resting potential data was taken over the experimental temperature range (6-26°C). Resting potential values from ouabain treated free cold homothermally acclimated *C.maenas* were averaged, the resting potential change with temperature was compared to that of other acclimatory groups using one way analysis of variance (ANOVA).

Immobilised acclimation.

Immobilised acclimation was carried out as a control to heterothermal acclimation (see Chapter Six), specifically to determine if clamping the crabs into the heterothermal apparatus had any quantifiable effect on the neuromuscular parameters investigated. It was important to determine if clamping the crabs within the apparatus affected acclimation, the crabs were restrained through clamps on their carapace, their legs however, were still free to move.

The thermal acclimation protocol used during immobilised acclimation was exactly the same as heterothermal acclimation (Table 6.1) except that the apparatus was homothermally maintained at 8°C or heated to 22°C. The immobilised crabs were not fed during acclimation.

Muscle fibre diameter.

After each electrophysiological experiment the exposed muscle fibres in the propodite article were measured transversely using an eye-piece graticule in a Zeiss dissection microscope. The muscle fibre diameters were measured (n=9-14 per leg) and averaged from a single crab, these mean diameter measurements were then pooled with results from like acclimated crabs (n=6-16) to provide an overall mean value. These average pooled diameters from different acclimatory groups were compared (one way ANOVA), no data was available for immobilised *Carcinus maenas* acclimated to 8°C.

Results.

Axon identification.

The voltage threshold required to initiate an EJP was determined for both motor axons innervating the closer muscle (see Table 4.0). It can be seen from Table 4.0 that the phasic (high threshold) axon had a higher voltage threshold than the tonic axon, thus identification of the tonic axon could be readily achieved in all experimental preparations.

Cold acclimated walking leg motor axon voltage thresholds were determined in the range 6-8°C, whereas warm acclimated walking leg voltage thresholds were determined in the range 20-26°C. Statistical comparisons (one way ANOVA) showed there were no significant differences between any phasic axon thresholds irrespective of acclimation condition. The low (tonic) threshold axon of free cold acclimated *C.maenas* was

significantly lower than the voltage threshold of immobilised cold acclimated *C.maenas*. There were significant differences between all low and high (Paired t-test $P<0.05$) threshold motor axon comparisons.

Table 4.0: Voltage threshold (mean±S.E. mean) required to initiate an EJP in all acclimatory groups.

Species and type of Acclimation.	High threshold axon voltage ±S.E (V).	Low threshold axon voltage ±S.E (V).	n
Free *C.maenas* 8°C	6.17±0.64	1.56±0.20	5
Immobilised *C.maenas* 8°C	6.63±0.14	2.85±0.32	15
Free *C.maenas* 22°C	6.69±0.20	2.20±0.20	18
Immobilised *C.maenas* 22°C	6.52±0.20	1.72±0.49	9
Free *C.pagurus* 8°C	6.41±0.28	2.71±0.54	6
Immobilised *C.pagurus* 8°C	6.24±0.23	2.35±0.28	13
Free *C.pagurus* 22°C	6.52±0.19	1.97±0.23	12
Immobilised *C.pagurus* 22°C	6.47±0.23	1.73±0.22	11

Resting membrane potential.

Resting potential (RP) data from homothermal warm and cold acclimated free and immobilised *C.maenas* are presented on Figure 4.1A. RP's hyperpolarised with increasing experimental temperatures in all acclimatory groups, the RP change with temperature was 1.34mV/°C for free cold acclimated *C.maenas* which was significantly different from (one way ANOVA) 1.11mV/°C for free warm acclimated *C.maenas* (see Table 4.2). The RP change with temperature for both free warm and cold acclimated *C.maenas* were significantly greater than that predicted by Nernst (0.3163 mV/°C). It can be seen from Figure 4.1A that the RPs of free cold acclimated crabs were hyperpolarised over the whole experimental temperature range when compared at the same experimental temperatures to RPs of free warm acclimated crabs. Statistical analysis (one way ANOVA) showed that RP of free cold acclimated crabs were significantly different from those of free warm acclimated crabs over almost all of the experimental temperature range i.e. 8-24°C. The differences between free warm and cold acclimated *C.maenas* walking leg RP values indicated an acclimatory shift (Cossins and Bowler 1987). The acclimatory shift (Precht 1958) was estimated by determining the RP of free cold acclimated *C.maenas* at 8°C, then traversing along the temperature axis at that RP until the free warm acclimated *C.maenas* RP curve was intercepted. The acclimatory shift was calculated as the percentage temperature difference as a function of

Figure 4.1A: Resting potential changes in *Carcinus maenas* homothermal free and immobilised controls. Numbers of experiments were; free *C.maenas* 8 (n= 20), free *C.maenas* 22 (n= 24), IM *C.maenas* 8 (n= 15) and IM *C.maenas* 22 (n= 24). Mean data is plotted and standard errors of mean are shown for free *C.maenas* 8 and IM *C.maenas* 22 only, not all error bars are shown so as to keep the figure un-cluttered. Best fit lines were drawn through the data using a least squares regression, equations for the lines are shown below.

Free *C.maenas* 8= -71.72-0.909x R=0.9745
Free *C.maenas* 22= -65.42-0.90153x R=0.899
IM *C.maenas* 8= -72.53-0.872x R=0.99
IM *C.maenas* 22= -68.66-0.56003x R=0.982

Figure 4.1B: Resting potential changes in *Cancer pagurus* homothermal free and immobilised controls. Numbers of experiments were; free *C.pagurus* 8 (n= 17), free *C.pagurus* 22 (n= 19), IM *C.pagurus* 8 (n= 16) and IM *C.pagurus* 22 (n= 14). Mean data is plotted and standard errors of mean are shown for free *C.pagurus* 22 and free *C.pagurus* 8, not all error bars are shown so as to keep the figure un-cluttered. Equations for best fit lines using a least squares regression are shown below.

Free *C.pagurus* 8= -71.71-1.184x R=0.986
Free *C.pagurus* 22= -65.34-0.924x R=0.966
IM *C.pagurus* 8= -72.434-1.0744x R=0.9831
IM *C.pagurus* 22= -68.3-0.56x R=0.9725

Figure 4.1A: Resting membrane potential in Carcinus meanas homothermal free and immobilised controls.

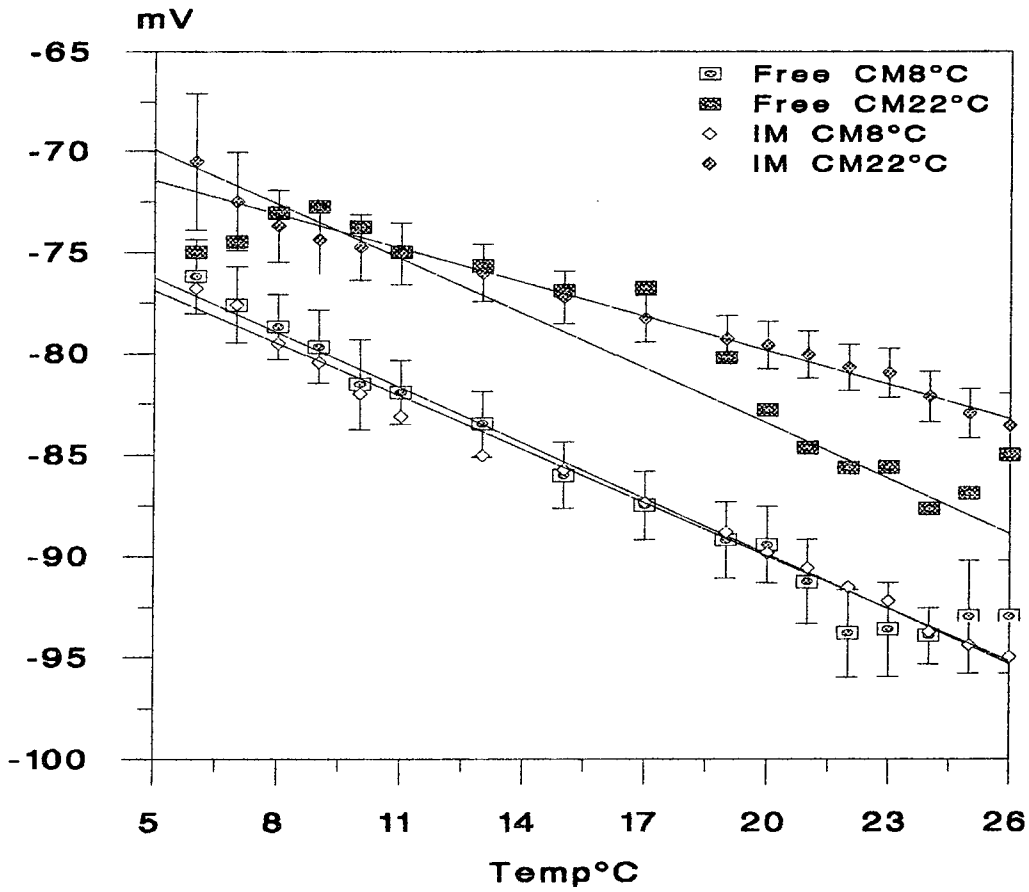
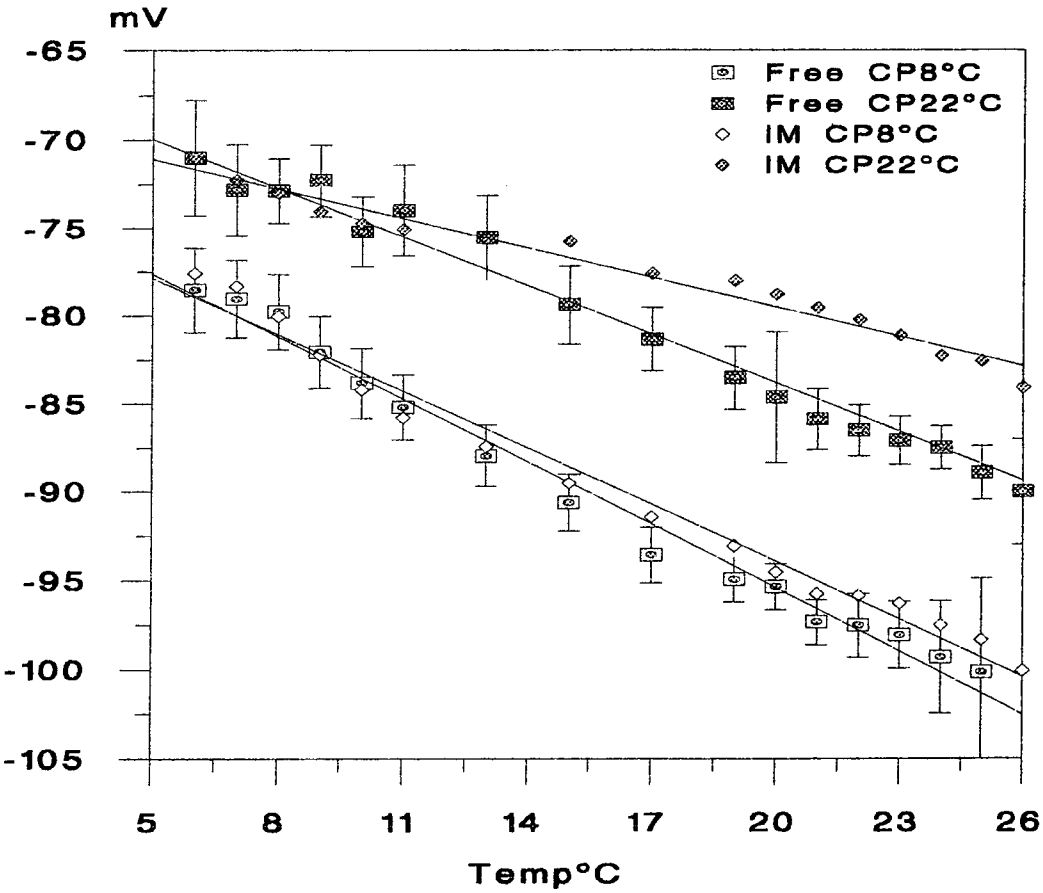


Figure 4.1B: Resting membrane potential in Cancer pagurus homothermal free and immobilised controls.



the differences between the acclimation temperature (8-22°C), See Table 4.1 for all acclimatory groups estimated acclimatory shifts.

Table 4.1: Estimated acclimatory shifts of muscle membrane RPs from walking legs of *C.maenas* and *C.pagurus* free and immobilised acclimatory groups.

Acclimation Type.	Acclimatory shift (%).
Free *C.maenas* 8°C→ Free *C.maenas* 22°C	53.6
Immobilised *C.maenas* 8°C→ Immobilised *C.maenas* 22°C	82.14
Free *C.pagurus* 8°C→ Free *C.pagurus* 22°C	64.3
Immobilised *C.pagurus* 8°C→ Immobilised *C.pagurus* 22°C	104.3

It was estimated that free warm acclimated *C.maenas* RP acclimatory shift was 53.6%, the RP shift exhibited partial acclimation (or Type III after Precht 1958).

Figure 4.1A also shows homothermal cold and warm acclimated immobilised *C.maenas* RP changes over the experimental temperature range. Both immobilised warm and cold acclimated experimental groups exhibited hyperpolarising RPs with increasing experimental temperatures, the RP change with temperature was also greater than the 0.3163mV/°C change predicted by Nernst (see Table 4.2). It was calculated that immobilised cold acclimated RP change with temperature at 0.878mV/°C was significantly different from immobilised warm acclimated RP change at 0.596mV/°C. Immobilised cold acclimated *C.maenas* walking leg RP values again hyperpolarised with increasing experimental temperature, being more hyperpolarised than immobilised warm acclimated RP values when compared at the same experimental temperatures. Immobilised cold acclimated RP values were significantly (one way ANOVA) hyperpolarised over almost all the experimental temperature range i.e. 6-25°C.

There was a clear difference in RP between the cold and warm acclimated groups, irrespective of their free or immobilised state, however, comparisons were then undertaken to reveal any immobilisation affect. When the dependence of RP on experimental temperature is determined it is clear that consistently cold acclimation results in RPs being hyperpolarised as compared with warm acclimation. This is the case for both free and immobilised crabs. The relationship between RP and temperature

(slope) was then investigated for every individual experiment for warm and cold acclimated free and immobilised animals (Table 4.2). It was found that immobilised warm and cold acclimated walking legs RP change with temperature was significantly smaller (one way ANOVA) than equivalent free warm and cold acclimated *C.maenas* walking leg RP changes with temperature, a situation which is not clearly expressed for immobilised cold acclimated *C.maenas* on Figure 4.1A. This may indicate immobilisation affected the sensitivity RP changes with experimental temperature. However, statistical analysis (one way ANOVA) of the individual RP values recorded over the experimental temperature range identified no significant differences between free or immobilised cold acclimated RP values. The cold acclimated free and immobilised crab walking legs exhibited qualitatively identical individual RP changes with temperature. On the other hand warm acclimated free and immobilised acclimatory group muscle RPs were significantly hyperpolarised over the 21-24°C temperature range. The estimated acclimatory shifts revealed immobilised acclimated walking legs exhibited more complete acclimation at 82.14% than free acclimated *C.maenas* walking legs acclimatory shift at 53.6%. The absolute differences between the RPs of warm acclimated free and immobilised crabs were minor, and no differences were found between the RPs of cold acclimated free and immobilised crabs.

C.pagurus.

RP data from cold and warm acclimated free and immobilised *C.pagurus* are shown on Figure 4.1B. Free warm and cold acclimated *C.pagurus* RP's hyperpolarised with increasing experimental temperature, the RP change with temperature (see Table 4.2) was significantly greater than that predicted by Nernst.

There was a significant difference between the walking leg RP change with temperature when comparing free warm and cold acclimated experimental groups (Table 4.2), warm acclimation reduced the electrogenic component slightly. Free cold acclimated *C.pagurus* RP values were significantly hyperpolarised (one way ANOVA) when compared to free warm acclimated *C.pagurus* RP values over all of the experimental temperature range i.e. significantly different over 6-25°C. The estimated acclimatory shift for free warm acclimated *C.pagurus* with respect to free cold acclimated walking leg RP was 64.3% (see Table 4.1), which again indicated partial acclimation.

The mean correlation coefficient shown is for data sets used to determine the mean RP change with temperature. All acclimatory group RP changes with temperature were significantly greater than the RP change determined in the presence of 1mM ouabain, except for immobilised warm acclimated *C.pagurus/C.maenas.*

Immobilised warm and cold acclimated *C.pagurus* (see Figure 4.1B) RP also hyperpolarised with increasing experimental temperatures. The hyperpolarisation was again significantly greater than that predicted by Nernst (see Table 4.2). The RP change with temperature of immobilised warm acclimated *C.pagurus* was significantly smaller at 0.763±0.08mV/°C than that of immobilised cold acclimated *C.pagurus* at 1.08±0.06mV/°C. Throughout it has been calculated that warm acclimated walking legs (both free and immobilised) exhibited smaller RP changes with experimental temperature, which may indicate a degree of thermal insensitivity for warm acclimated walking legs i.e. a reduction in the electrogenic component.

Table 4.2: Change in resting potential with experimental temperature. The mean RP changes were then compared to that of free cold acclimated *C.maenas* incubated with 1mM ouabain* (expressed as a percentage) to determine the contribution of the Na^+/K^+ATPase to the resting potential change with temperature.

Species and type of Acclimation.	Mean RP change with temperature mV/°C.	Mean correlation coefficient.	%* difference.	n
Free *C.maenas* 8 +Ouabain(1mM)	-0.4065±0.078	0.996	0	5
Free *C.maenas* 8°C	-1.34±0.078	0.975	230.6	23
Immobilised *C.maenas* 8°C	-0.878±0.056	0.990	116	14
Free *C.maenas* 22°C	-1.106±0.083	0.90	172	16
Immobilised *C.maenas* 22°C	-0.595±0.062	0.982	46.5	17
Free *C.pagurus* 8°C	-1.29±0.1619	0.986	218.1	14
Immobilised *C.pagurus* 8°C	-1.079±0.064	0.983	165.4	16
Free *C.pagurus* 22°C	-1.052±0.061	0.966	158	16
Immobilised *C.pagurus* 22°C	-0.763±0.078	0.973	87.7	10
Nernst prediction♣	-0.3163	0.999	-22.2	-

* % change in resting potential mV/°C.

♣ Assumes using Nernst (equation 1.2) that $[K^+]_o$=10mM and $[K^+]_i$=400mM.

Immobilised cold acclimated *C.pagurus* walking leg RP values were significantly hyperpolarised over most of the experimental temperature range when compared to immobilised warm acclimated walking leg RP values i.e. significantly different over 7-26°C (one way ANOVA). The estimated acclimatory shift of immobilised warm and cold acclimated *C.pagurus* was 104.3% (see Table 4.1), indicating perfect acclimation (or Type II after Precht 1958).

Comparison of free to immobilised acclimatory group RP results revealed no significant differences between cold acclimated *C.pagurus*. A significant difference was found between the free and immobilised warm acclimated crab RP change with temperature (Table 4.2), significant differences were found at 19-25°C between warm acclimated free and immobilised *C.pagurus* walking leg RPs. The immobilised warm acclimated *C.pagurus* walking leg RPs were significantly depolarised over the 19-25°C temperature range.

The observed individual RP changes with temperature for immobilised compared to free acclimated walking legs were similar for both warm and cold acclimatory groups (8°C and 22°C) irrespective of species. There were no significant differences between cold acclimated free or immobilised walking leg RPs of *C.maenas* or *C.pagurus*, illustrating that immobilisation did not affect the acclimation process in either species. Warm (22°C) acclimated crabs revealed minor but significant differences between free and immobilised acclimated preparations. Specifically, *C.maenas* immobilised compared to free *C.maenas*, significant differences were observed at 21-24°C, and for identically acclimated *C.pagurus* significant differences were observed at 19-25°C (one way ANOVA). Therefore only at higher experimental temperatures was there any immobilisation affect on warm acclimated walking leg RPs. It is unclear if this effect is due to the immobilisation *per se* or if there is increased variation between preparations at the warmer acclimation temperature due to other factors. Both *C.maenas* and *C.pagurus* immobilised warm acclimated walking legs shifted their RPs with acclimation temperature in an appropriate manner, whereas over the same temperature range free warm acclimated crabs shifted their RPs less which was unexpected. All free warm compared to free cold acclimatory groups exhibited partial acclimation, whereas the acclimatory shift estimated for immobilised acclimated walking legs (both *C.maenas* and *C.pagurus*) was always more complete (Table 4.1) than that of free walking legs.

Other groups have investigated RP changes with experimental temperature using Arrhenius plots to identify break points or discontinuities (Kivivuori *et al.*, 1990). Discontinuities identified on Arrhenius plots have been linked to changes in reaction rate limiting steps or membrane phase transitions (Cossins and Bowler 1987). Attempts to identify significant RP discontinuities were also attempted here for warm and cold acclimated free and immobilised walking legs of both species. Individual experiment RP values were firstly fitted with a single straight line (monophasic plot), noting the correlation coefficient of the line. It was note worthy that all monophasic single line plots had correlation coefficients greater than 0.95 which indicated no bi-phasic plots could be identified.

Figure 4.2A: Latent period to first EJP in *Carcinus maenas* homothermal free and immobilised controls. Numbers of experiments were; free *C.maenas* 8 (n= 20), free *C.maenas* 22 (n= 24), IM *C.maenas* 8 (n= 15) and IM *C.maenas* 22(n= 25). Exponential curves were fitted to all experimental data using a least squares fit, the data was plotted on a log/linear scale, equations for the lines are shown below.

Free *C.maenas* 8= 26.89*10(-0.0287x) R=0.9968
Free *C.maenas* 22= 26.06*10(-0.0323x) R=0.968
IM *C.maenas* 8= 26.14*10(-0.02172x) R=0.992
IM *C.maenas* 22= 15.936*10(-0.0269x) R=0.9976

Figure 4.2B: Latent period to first EJP in *Cancer pagurus* homothermal free and immobilised controls. Numbers of experiments were; free *C.pagurus* 8 (n= 17), free *C.pagurus* 22 (n= 19), IM *C.pagurus* 8 (n= 16) and IM *C.pagurus* 22 (n= 14). Mean data is plotted and standard error of mean are shown for free *C.pagurus* 8 and IM *C.pagurus* 8 only, certain error bars only are shown so as to keep the figure un-cluttered. Exponential curves were fitted to the data using a least squares fit and plotted on a log/linear scale, equations for the exponential plots are shown below.

Free *C.pagurus* 8= 21.37*10(-0.0302x) R=0.985
Free *C.pagurus* 22= 27.9*10(-0.0379x) R=0.978
IM *C.pagurus* 8= 14.112*10(-0.032x) R=0.993
IM *C.pagurus* 22= 12.974*10(-0.0309x) R=0.9985

Figure 4.2A: Latent period to first EJP in Carcinus maenas homothermal free and immobilised controls.

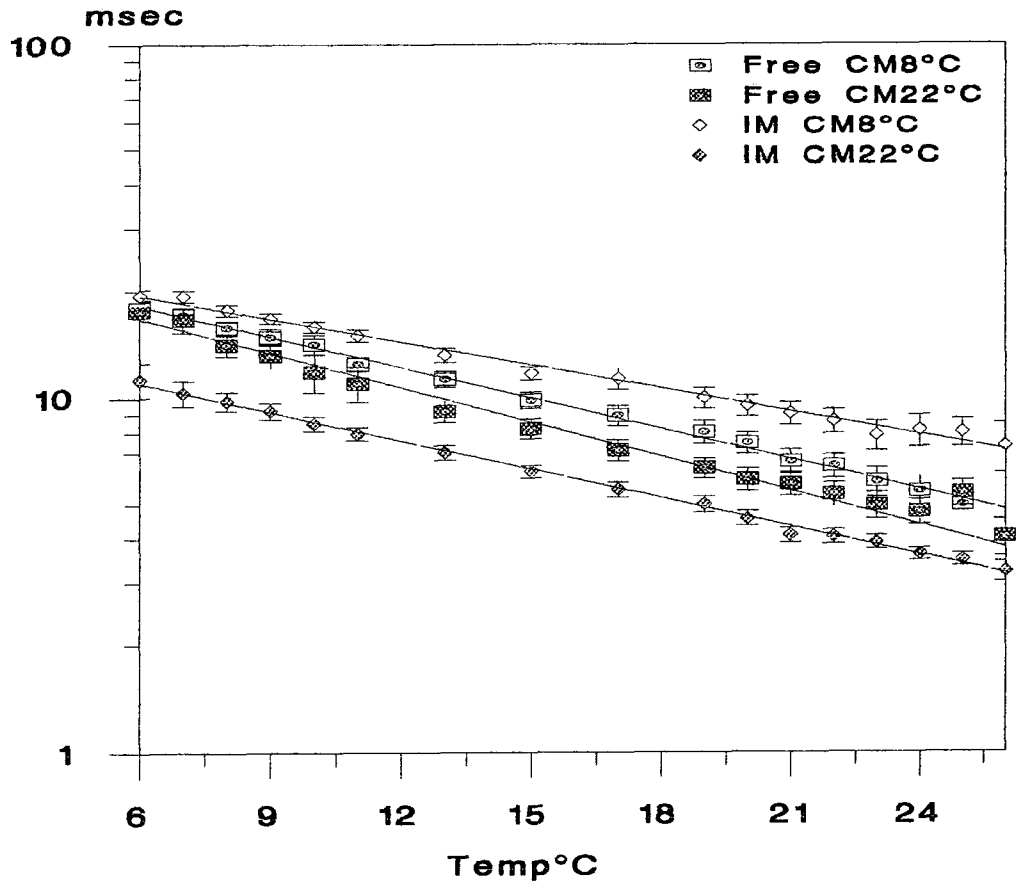
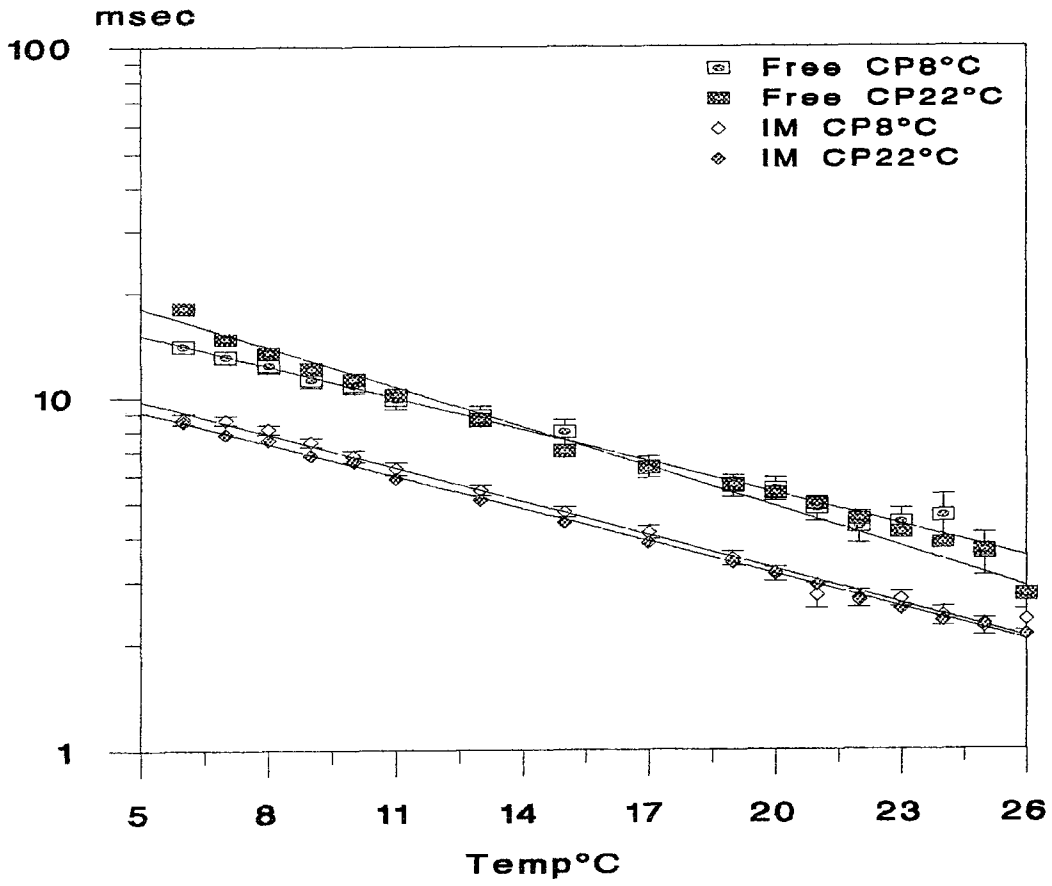


Figure 4.2B: Latent period to first EJP in Cancer pagurus homothermal free and immobilised controls.



Latency.

C.maenas.

Latent periods recorded from free and immobilised homothermal warm and cold acclimated *C.maenas* are shown in Figure 4.2A. The free cold and warm acclimated walking leg latent periods decreased with increasing experimental temperature, the decrease over the whole experimental temperature range being approximately 72% and 76% respectively. Statistical analysis (one way ANOVA) revealed a number of significant differences between the free warm and cold acclimated walking leg latent periods. Free cold acclimated walking leg latent periods were generally longer over the whole temperature range, but were significantly longer over 10, 13-20°C than free warm acclimated walking leg latent periods.

Immobilised warm and cold acclimated crab muscle latent periods also decreased with increasing experimental temperature, the immobilised warm acclimated crab muscle latent periods were noticeably shorter than those from immobilised cold acclimated crabs over the whole experimental temperature range. The decrease in latency calculated for immobilised warm and cold acclimated walking leg latent periods over the experimental temperature range was 72% and 65% respectively. Statistical analysis revealed a high number of significant differences (one way ANOVA) between the immobilised warm and cold acclimated walking leg latent periods i.e. significantly different over 6-26°C. The results also show that immobilised cold acclimated crab muscle latent periods were longer than all other acclimatory groups, including free acclimated crab walking legs, whereas immobilised warm acclimated crab walking leg latent periods were shorter than all other acclimatory groups.

Comparison of free and immobilised cold acclimated crab muscle latent periods revealed significant differences over 11-23, 25°C, immobilised animals having longer latent periods. Furthermore, warm acclimated free and immobilised crab muscle latent periods were significantly different over 6-15, 20-22, 25°C, the warm acclimated immobilised walking leg latent periods being shorter. The results indicate immobilisation does have an affect on EJP latency acclimation, immobilised cold acclimation lengthens the latent period, whereas immobilised warm acclimation shortens the latent period.

C.pagurus.

Latent periods from free and immobilised warm and cold acclimated *C.pagurus* are shown in Figure 4.2B. Free cold and warm acclimated walking leg latent periods

decreased with increasing experimental temperature, the decrease in latency over the experimental temperature range was calculated as being 75% and 81.8% respectively. The latent period versus temperature curves for free warm and cold acclimated *C.pagurus* were qualitatively and quantitatively identical, individual latency comparisons revealed no significant differences (one way ANOVA) between warm and cold acclimated walking leg latent periods.

Immobilised warm and cold acclimated latent periods also decreased with increasing experimental temperature, the decrease being approximately 77% for both immobilised acclimated conditions. Immobilised warm and cold acclimated walking leg latent periods were not significantly (one way ANOVA) different from each other over all the experimental temperature range, their latent periods were effectively identical.

Comparison of free to immobilised acclimated walking legs revealed two populations of results, no significant differences were found with respect to the acclimation temperature. Cold acclimated free latent periods were significantly longer than cold acclimated immobilised walking leg latent periods over 6-25°C, and warm acclimated free and immobilised latent periods were significantly different over 7-25°C. Latency acclimation was apparently independent of the acclimation temperature, where latency differences were clearly separated into free or immobilised populations, indicating the significantly shorter immobilised latent periods were dependent on the type of acclimation and not the acclimation temperature.

Single pulse stimulated EJP amplitude.

C.maenas.

Figure 4.3A shows free and immobilised homothermal warm and cold acclimated *C.maenas* single EJP amplitudes over the experimental temperature range. Single EJP amplitudes do not incorporate any facilitative component as the experimental stimulation frequency at 0.5Hz was too low (see Chapter Three).

Free cold acclimated *C.maenas* EJP amplitudes were generally constant over 6-17°C but then decreased rapidly with increasing experimental temperature, the decrease in amplitude over the whole experimental temperature range was approximately 70%. In comparison free warm acclimated walking leg EJP amplitudes exhibited fairly constant EJP amplitudes over most of the experimental temperature range (i.e. constant over 8-23°C). Free warm acclimated *C.maenas* EJP amplitudes decreased at temperatures colder than 9°C, but decreased only slightly at experimental temperatures warmer than 22°C. Statistical analysis (one way ANOVA) revealed no significant differences

Figure 4.3A: Single EJP amplitude from *Carcinus maenas* homothermal free and immobilised controls. Numbers of experiments were; free *C.maenas* 8 n= 20, IM *C.maenas* 8 n= 15, free *C.maenas* 22 n= 18 and IM *C.maenas* 22 n= 24). Mean data was plotted and connected using straight lines, no error bars are shown.

Figure 4.3B: Single EJP amplitude from *Cancer pagurus* homothermal free and immobilised controls. Numbers of experiments were; free *C.pagurus* 8 n= 16, IM *C.pagurus* 8 n= 13), free *C.pagurus* 22 n= 17 and IM *C.pagurus* 22 n= 16. Mean data was plotted and connected using straight lines, no error bars are shown.

Figure 4.3A: Single pulse EJP amplitudes from Carcinus maenas homothermal free and immobilised controls.

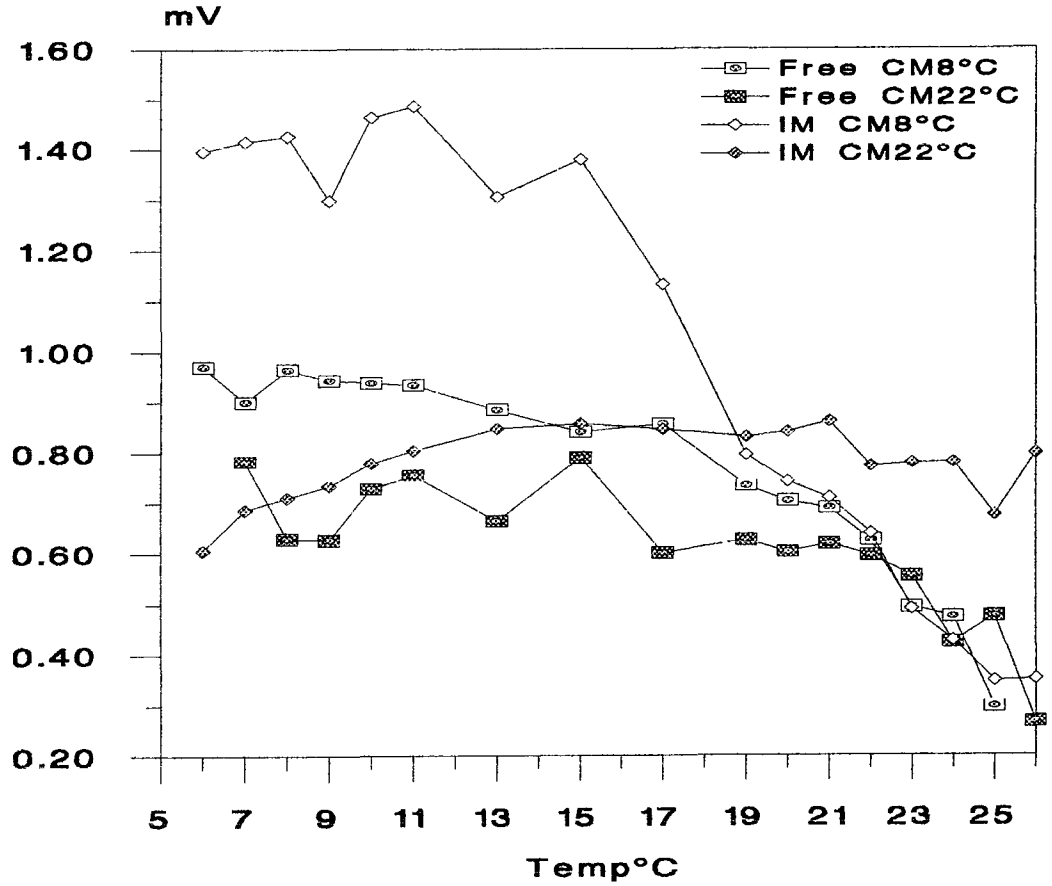
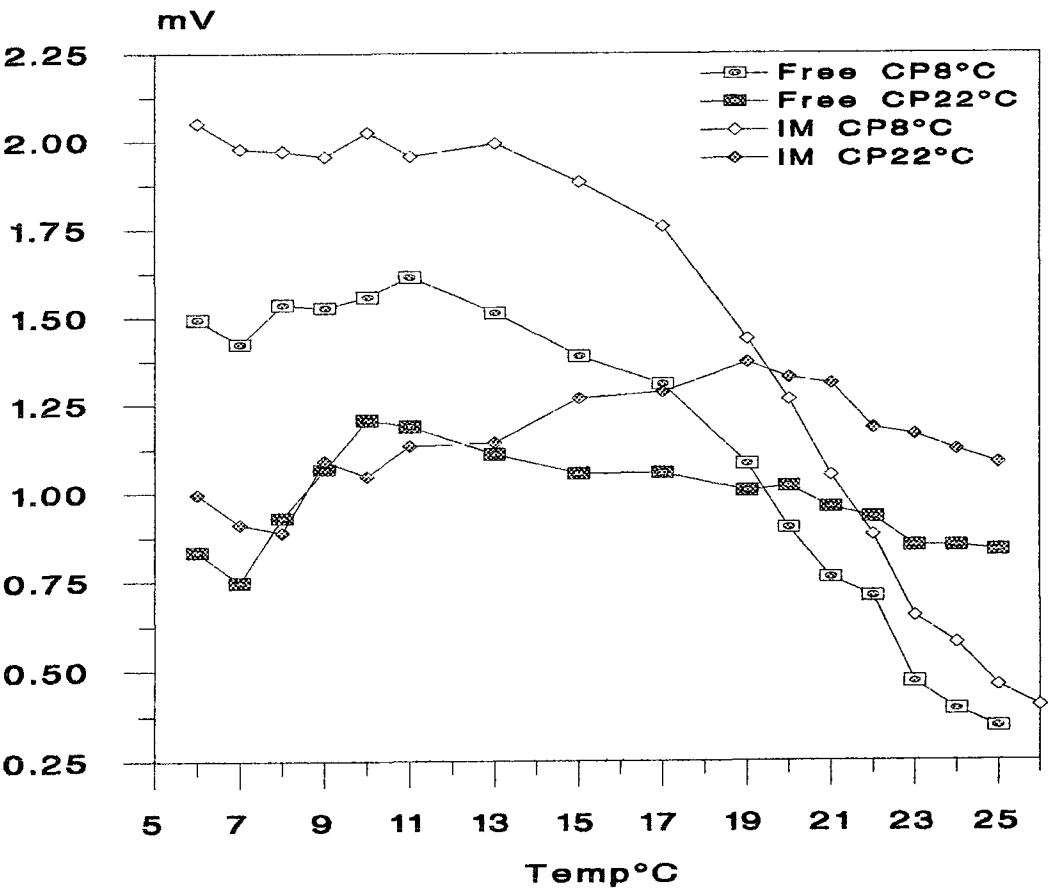


Figure 4.3B: Single pulse EJP amplitudes from Cancer pagurus homothermal free and immobilised controls.



between the free warm and cold acclimated walking leg EJP amplitudes over the whole experimental temperature range. The important difference between the free warm and cold acclimated EJP amplitude versus temperature curve was qualitative, in that warm acclimated walking legs maintained their EJP amplitudes over most of the temperature range up to 22°C, whereas cold acclimated walking leg amplitudes were maintained up to 15-17°C and then decreased significantly.

Immobilised warm and cold acclimated *C.maenas* single EJP amplitudes are shown on Figure 4.3A. Immobilised cold acclimated crab walking leg EJP amplitudes decreased with increasing experimental temperature, rapidly so at experimental temperatures warmer than 15°C, the decrease over the whole experimental temperature range was approximately 75%. Whereas immobilised warm acclimated crab walking leg EJP amplitudes exhibited an n-shaped EJP amplitude versus temperature curve. Maximal EJP amplitudes were recorded over 13-21°C, the single EJP amplitudes generally decreased at experimental temperatures colder than 13°C or warmer than 21°C. Statistical analysis (one way ANOVA) determined immobilised warm acclimated *C.maenas* walking leg EJP amplitudes were significantly smaller than immobilised cold acclimated walking leg amplitudes over 6-15°C, but significantly larger over 23-26°C. This difference between immobilised warm and cold acclimated walking leg EJP amplitudes indicated immobilised warm acclimated crabs maintained their EJP amplitudes over most of the experimental temperature range, whereas immobilised cold acclimated crab EJP amplitudes decreased significantly with increasing experimental temperatures.

Comparison of cold acclimated free and immobilised walking leg EJP amplitudes revealed a number of significant differences, immobilised cold acclimated walking leg EJP amplitudes were significantly larger than free cold acclimated walking leg amplitudes over 6-15°C. However, it is important to note that both cold acclimated free and immobilised walking leg EJP amplitudes decreased significantly with increasing experimental temperature in a qualitatively similar manner. In comparison significant differences between warm acclimated free and immobilised walking leg EJP amplitudes were very small at 24°C and 26°C. These results for EJP amplitude indicate immobilisation had no affect on warm acclimated walking legs, but may indicate an immobilisation affect on cold acclimated walking legs at colder experimental temperatures.

To identify more clearly the differences between warm and cold acclimated free or immobilised walking legs, the EJP amplitudes generated within an acclimatory group at low (8±1°C) and high (22±1°C) temperature ranges were compared (Student's t-test). Figure 4.4A shows *C.maenas* EJP amplitudes of free and immobilised warm and cold acclimated crab walking legs determined at the acclimation temperatures. It was found

Figure 4.4A: Single EJP amplitudes in homothermal free and immobilised *Carcinus maenas* at low (8±1°C) and high (22±1°C) temperatures. Mean EJP amplitudes were plotted and analysed for significant differences (Student's t-test). Numbers of experiments are shown below each result column. The relative change in actual EJP amplitude at low to high temperatures was; low to high free *C.maenas* 8= 35%, low to high free *C.maenas* 22= 10.7%, low to high IM *C.maenas* 8= 55.4% and low to high IM *C.maenas* 22= 12.7%. Mean data was plotted with standard error of means. Significant differences in amplitude at low and high temperature ranges within an acclimatory group are denoted on the figure by an asterisk.

Figure 4.4B: Single EJP amplitude in homothermal free and immobilised *Cancer pagurus* at low (8±1°C) and high (22±1°C) temperatures. Mean EJP amplitudes for *C.pagurus* were analysed using student t-test (numbers of experiments are shown below each result column). The relative change in EJP amplitude at low and high temperatures was; low to high free *C.pagurus* 8= 56.8%, low to high free *C.pagurus* 22= 0.44%, low to high IM *C.pagurus* 8= 56.3% and low to high IM *C.pagurus* 22= 26%. Mean data was plotted with standard error of mean. Significant differences in amplitude at low and high temperature ranges within an acclimatory group are denoted on the figure by an asterisk.

Figure 4.4A: Single EJP amplitude in homothermal free and immobilised Carcinus maenas at low (8±1°C) and high (22±1°C) temperatures.

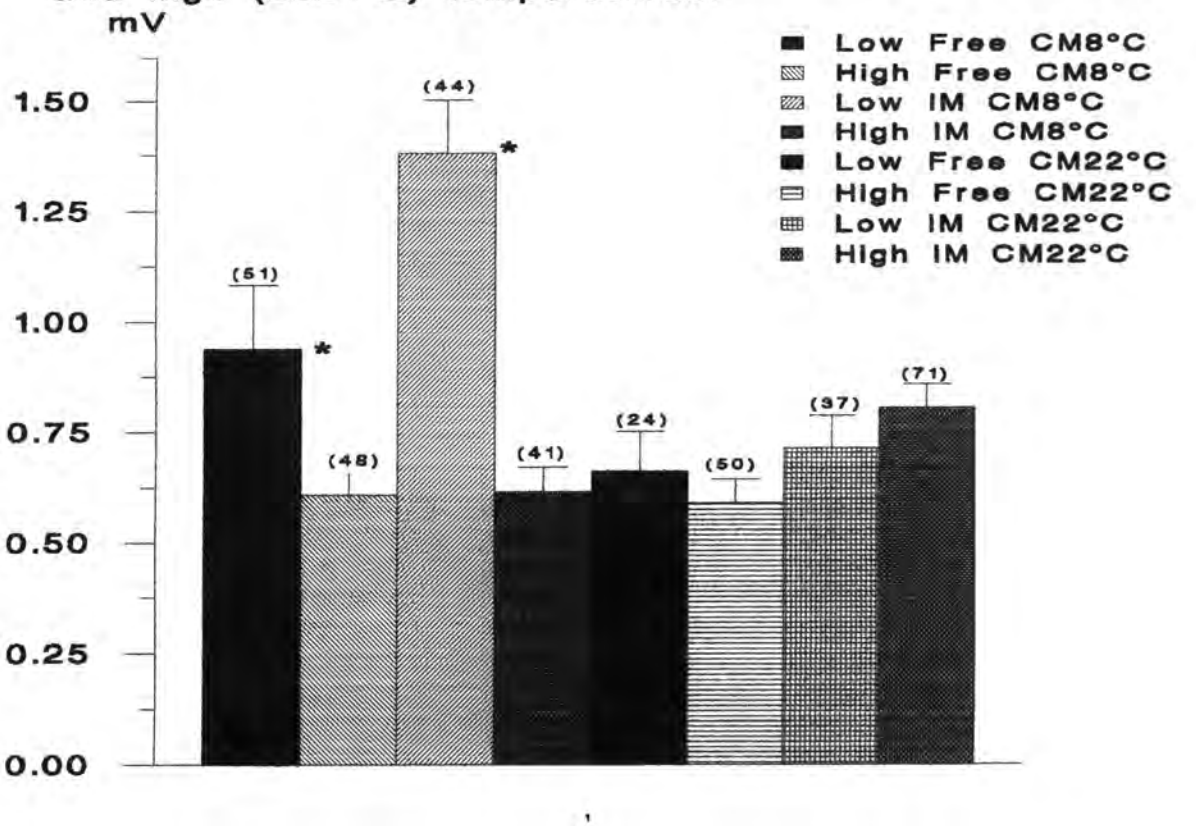
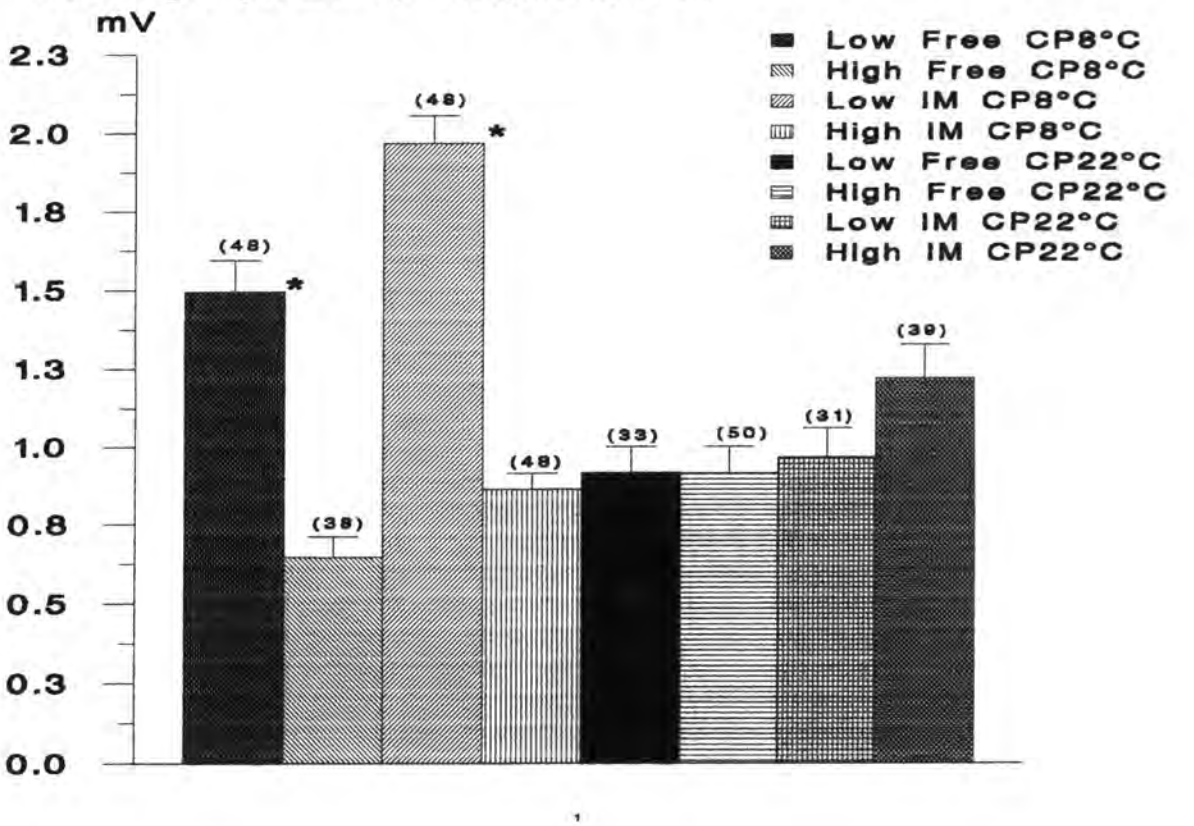


Figure 4.4B: Single EJP amplitude in homothermal free and immobilised Cancer pagurus at low (8±1°C) and high (22±1°C) temperatures.



that cold acclimated walking leg EJP amplitudes (whether free or immobilised) generated significantly smaller EJP amplitudes at the high (22±1°C) experimental temperature range than the EJP amplitudes generated in the same (crab) acclimatory group at the low (8±1°C) experimental temperature range. In comparison the warm acclimated walking legs (whether free or immobilised) revealed no significant differences in EJP amplitude at the low and high temperature ranges. The results indicate no immobilisation affect on single EJP amplitude acclimation.

C.pagurus.

Figure 4.3B shows free and immobilised warm and cold acclimated *C.pagurus* walking leg single EJP amplitude changes over the experimental temperature range. Free cold acclimated crab walking leg EJP amplitudes were generally maintained over 6-13°C but then decreased with increasing experimental temperatures, the decrease in amplitude over the whole experimental temperature range was approximately 77%. In contrast, free warm acclimated crab walking leg EJP amplitudes revealed a generally n-shaped EJP amplitude versus temperature curve. Maximal EJP amplitudes were recorded over 9-15°C, at experimental temperatures warmer than 17°C or colder than 9°C the EJP amplitudes decreased, the value at 26°C was omitted as only five of the seventeen preparations were responsive. Statistical analysis (one way ANOVA) revealed a small number of significant differences between free warm and cold acclimated *C.pagurus* walking leg EJP amplitudes, free cold acclimated walking legs generated significantly larger EJP amplitudes over 7-9°C, but significantly smaller EJP amplitudes over 23-24°C than EJP amplitudes generated by free warm acclimated *C.pagurus* walking legs. The qualitative differences between free warm and cold acclimated *C.pagurus* walking legs were that warm acclimated crabs maintained muscle depolarisation over most of the experimental temperature range, whereas cold acclimated crab EJPs decreased significantly with increasing experimental temperature.

Immobilised warm and cold acclimated *C.pagurus* walking leg EJP amplitudes were qualitatively identical to those already shown for free warm and cold acclimated *C.pagurus* respectively. Immobilised cold acclimated walking leg EJP amplitudes were generally stable over 6-13°C but then decreased with increasing experimental temperature, the decrease over the whole experimental temperature range was approximately 82%. In contrast immobilised warm acclimated walking legs revealed an n-shaped EJP amplitude versus temperature curve, maximal amplitudes were recorded over 15-22°C. Decreasing EJP amplitudes were recorded at experimental temperatures colder than 15°C or warmer than 22°C for immobilised warm acclimated walking legs. In this respect immobilised walking leg EJP amplitudes were qualitatively identical to

those of free warm acclimated walking legs, as both maintained relatively constant EJP amplitudes over the experimental temperature range, especially at higher temperatures. Statistically (one way ANOVA) immobilised cold acclimated *C.pagurus* generated significantly larger EJP amplitudes over 6-17°C, but then significantly smaller EJP amplitudes over 23-26°C than amplitudes generated by immobilised warm acclimated walking legs. These results were again qualitatively identical to those reported for free warm and cold acclimated *C.pagurus* walking legs, revealing a clear warm to cold acclimation temperature difference.

Comparison of cold acclimated immobilised to cold acclimated free crab walking leg EJP amplitudes revealed immobilised crab walking legs generated significantly larger EJP amplitudes over 6-10, 13-17°C than amplitudes generated by cold acclimated free crab walking legs, whereas no significant differences were found between free and immobilised warm acclimated *C.pagurus* walking leg EJP amplitudes. The results indicate immobilisation had no affect on warm acclimated walking legs, but did reveal a potential for increased sensitivity to released neurotransmitter for immobilised cold acclimated walking legs at colder experimental temperatures.

The EJP amplitudes at low (8±1°C) and high (22±1°C) temperature ranges were again compared, and are shown in Figure 4.4B. The results show cold acclimated walking legs (whether free or immobilised) generated significantly larger (Student's t-test) EJP amplitudes at colder experimental temperatures than amplitudes generated in the same animal at warm experimental temperatures, whereas warm acclimated crab walking legs (whether free or immobilised) revealed almost identical EJP amplitudes at the low and high temperature ranges. This indicated warm acclimated walking legs maintained their EJP amplitudes to warmer experimental temperatures nearer to their warm acclimation temperature, whereas cold acclimated walking legs EJP amplitudes decreased significantly with increasing experimental temperature.

The cold acclimated (whether free or immobilised) crab EJP amplitudes were qualitatively similar, as were the warm acclimated (whether free or immobilised) crab EJP amplitude results.

Facilitation.

C.maenas.

Facilitation was calculated as a ratio of the double pulse EJP amplitudes (see Figure 2.4).

Figure 4.5A shows free and immobilised warm and cold acclimated *C.maenas* walking leg facilitation changes over the 6-26°C experimental temperature range. These

Figure 4.5A: Facilitation in *Carcinus maenas* homothermal free and immobilised controls. Numbers of experiments were; free *C.maenas* 22 (n= 18), free *C.maenas* 8 (n= 20), IM *C.maenas* 8 (n= 15) and IM *C.maenas* 22 (n= 24). Mean data is plotted using interpolation and no error bars are shown so as to keep the figure un-cluttered.

Figure 4.5B: Facilitation in *Cancer pagurus* homothermal free and immobilised controls. Numbers of experiments were; free *C.pagurus* 8 (n= 16), free *C.pagurus* 22 (n= 17), IM *C.pagurus* 8 (n= 16) and IM *C.pagurus* 22(n= 14). Mean data is plotted and joined together using interpolated, no error bars are shown so as to keep the figure uncluttered.

Figure 4.5A: Facilitation in Carcinus meanas homothermal free and immobilised controls.

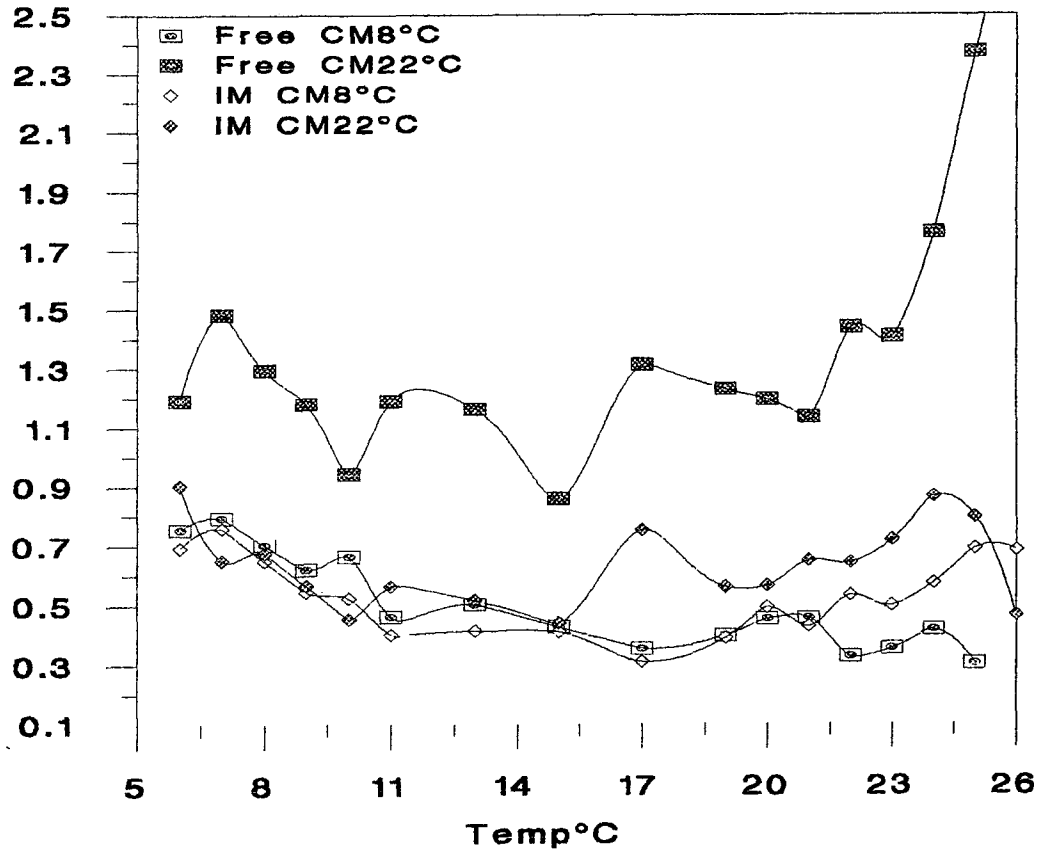
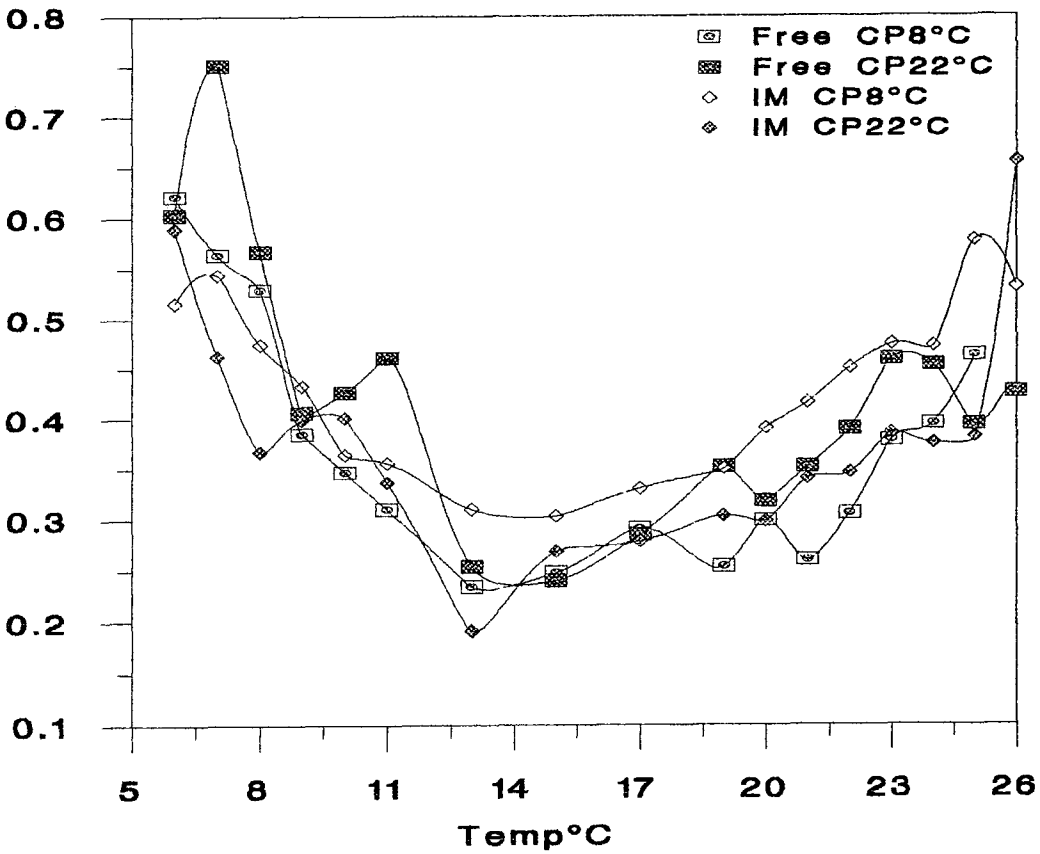


Figure 4.5B: Facilitation in Cancer pagurus homothermal free and immobilised controls.



were generally a shallow u-shape, exhibiting minimal facilitation at 14-17°C. Free cold acclimated *C.maenas* walking leg facilitation versus temperature curves showed a steady decline with increasing experimental temperature, no increase in facilitation was seen at temperatures warmer than 19°C which was unexpected. Free warm acclimated *C.maenas* walking leg facilitation values were significantly (one way ANOVA) larger than free cold acclimated walking leg facilitation over 7-25°C.

Immobilised warm and cold acclimated facilitation versus temperature curves also had a shallow u-shape, showing minimal facilitation over 14-15°C. There were no significant differences (one way ANOVA) between immobilised warm and cold acclimated walking leg facilitation values except at 17°C only. Facilitation was slightly increased at the warm and cold walking leg acclimation temperatures for both free and immobilised crabs, and increased at experimental temperatures extreme to that (except for free cold acclimated *C.maenas*) although these slight increases in facilitation were not significant.

Comparison of immobilised to free cold acclimated crab facilitation revealed no significant differences (one way ANOVA) over the whole experimental temperature range. Whereas comparison of free to immobilised warm acclimated crab facilitation identified significant differences over 7-26°C. Free warm acclimated *C.maenas* walking leg facilitation was significantly larger than all other acclimatory groups, it is not clear if this quantitative difference between warm acclimated free and immobilised crabs was anomalous, as the u-shaped facilitation versus temperature curve appeared to be reproducible for all acclimatory groups except free cold acclimated *C.maenas*.

C.pagurus.

Free and immobilised homothermal warm and cold acclimated *C.pagurus* walking leg facilitation changes with experimental temperature are shown in Figure 4.5B. Free warm and cold acclimated facilitation versus temperature curves were clearly u-shaped, showing minimal facilitation over 13-15°C, with increased (not significant) facilitation being recorded at temperatures lower than 8°C or warmer than 22°C. Statistically there were no significant differences (one way ANOVA) between the free warm and cold acclimated facilitation values, the response of free warm and cold acclimated crab walking leg facilitation to changing experimental temperature were qualitatively identical.

Immobilised warm and cold acclimated crab leg muscle facilitation versus temperature curves were qualitatively identical to the free warm and cold acclimated facilitation versus temperature curves, being u-shaped. Minimal facilitation was recorded from immobilised walking legs over 13-15°C, increased facilitation was

recorded at temperatures colder than 8°C or warmer than 22°C. Statistical analysis (one way ANOVA) revealed no significant differences between immobilised warm and cold acclimated facilitation values, their results were quantitatively and qualitatively identical.

Comparison of free and immobilised cold acclimated walking leg facilitation values revealed minor significant differences (one way ANOVA) at 7-8°C. Additionally, free and immobilised warm acclimated facilitation comparisons revealed no significant differences, the results were qualitatively and quantitatively identical, indicating immobilisation had no affect on *C.pagurus* walking leg facilitation irrespective of the acclimation temperature.

Double pulse stimulated EJP amplitude.

The amplitudes of double pulse EJPs evoked in the closer muscle fibres by the electrical stimulation of the excitatory tonic motor axon were recorded for both species from warm and cold homothermally acclimated free and immobilised crab walking legs. The double pulse stimulated EJP amplitudes contain a facilitative component, which contributes to EJP amplitude.

C.maenas.

Figure 4.6A shows double pulse stimulated EJP amplitudes from warm and cold acclimated free and immobilised *C.maenas* walking legs. Free cold acclimated *C.maenas* walking leg EJP amplitudes decreased steadily with increasing experimental temperature, the decrease in amplitude over the whole experimental temperature range was approximately 81%. In contrast, free warm acclimated *C.maenas* walking leg EJP amplitudes were almost constant over the whole experimental temperature range, but exhibited increased variability of EJP amplitudes at experimental temperatures colder than 8°C. The amplitude at 6°C was omitted as only three out of eighteen animals were responsive. Near maximal EJP amplitudes were recorded over 11-15°C from free warm acclimated walking legs, their EJP amplitudes were generally maintained up to 26°C. Statistical analysis (one way ANOVA) revealed a small number of significant differences between free warm and cold acclimated crab walking leg EJP amplitudes; free warm acclimated crab amplitudes were significantly larger than free cold acclimated crab amplitudes over 22-25°C. Qualitative differences between free warm and cold acclimated walking leg EJP amplitudes were evident, the free warm acclimated crab walking legs maintained their EJP amplitudes to warmer experimental temperatures than that shown by free cold acclimated crab walking legs (see Figure 4.6A).

Figure 4.6A: Double pulse EJP amplitudes from *Carcinus maenas* homothermal free and immobilised controls. Numbers of experiments were; free *C.maenas* 8 (n= 25), IM *C.maenas* 8 (n= 15), free *C.maenas* 22 (n= 22) and IM *C.maenas* 22 (n= 25). The data points were connected using straight lines. No error bars are shown to keep the figure un-cluttered.

Figure 4.6B: Double pulse EJP amplitudes from *Cancer pagurus* homothermal free and immobilised controls. Numbers of experiments were; free *C.pagurus* 8 n= 17, IM *C.pagurus* 8 n= 16, free *C.pagurus* 22 n= 19 and IM *C.pagurus* 22 n= 13. The data points were connected using straight lines, no error bars are shown to keep the figure un-cluttered.

Figure 4.6A: Double pulse EJP amplitude from Carcinus maenas homothermal free and immobilised controls.

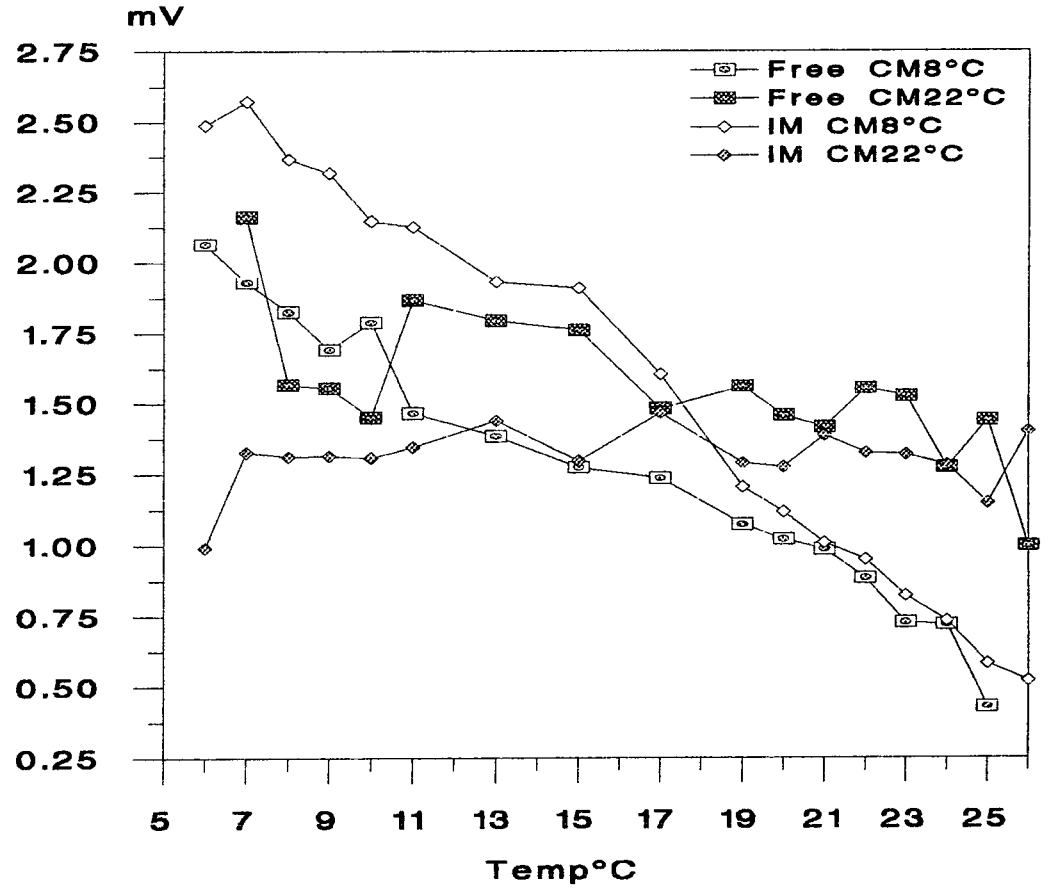
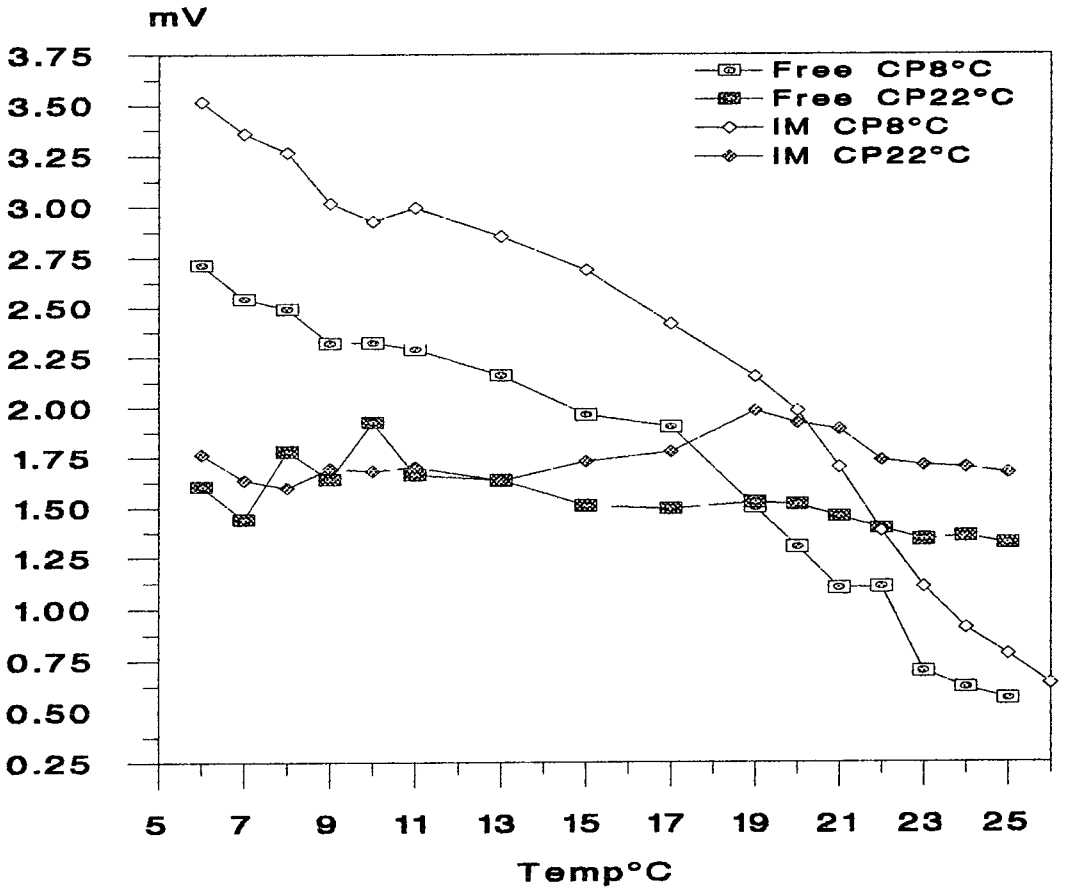


Figure 4.6B: Double pulse EJP amplitudes from Cancer pagurus homothermal free and immobilised controls.



Immobilised cold acclimated walking leg EJP amplitudes also decreased steadily with increasing experimental temperatures, their decrease in amplitude was approximately 80% over the whole experimental temperature range, which was similar to that reported for free cold acclimated walking legs. Immobilised warm acclimated *C.maenas* walking leg EJP amplitudes were almost constant over the whole temperature range, but, they decreased slightly at experimental temperatures colder than 9°C or warmer than 22°C. Statistical analysis (one way ANOVA) revealed immobilised cold acclimated walking leg EJP amplitudes were significantly larger than immobilised warm acclimated walking leg amplitudes over 6-11°C.

Comparison of free and immobilised cold acclimated crab EJP amplitudes revealed no significant differences (one way ANOVA), their EJP amplitudes were quantitatively identical, irrespective of restraint. Free warm acclimated EJP amplitudes compared to immobilised warm acclimated EJP amplitudes revealed no significant differences, which again indicated immobilisation had no affect on double pulse EJP amplitude changes with direct temperature.

To identify more clearly the differences between warm and cold acclimated free and immobilised *C.maenas* walking leg EJP amplitudes, the amplitudes generated at low (8±1°C) and high (22±1°C) experimental temperature ranges within an acclimatory group were compared (Student's t-test; see Figure 4.7A). It can be seen immediately that cold acclimated crab walking legs (whether free or immobilised) generated significantly larger EJP amplitudes at the low (8±1°C) temperature range than amplitudes generated in the same animal at the warm temperature range. Whereas warm acclimated crab walking leg EJP amplitudes were similar at the low and high temperature ranges, again irrespective of immobilisation, which again indicates immobilisation had no qualitative affect on double pulse EJP amplitude changes with experimental temperature.

C.pagurus.

Figure 4.6B shows warm and cold acclimated free and immobilised *C.pagurus* walking leg double EJP amplitude changes over the 6-26°C experimental temperature range. Free cold acclimated *C.pagurus* walking leg EJP amplitudes decreased with increasing experimental temperature, the decrease in amplitude over the whole experimental temperature range was approximately 81%. In contrast free warm acclimated walking leg EJP amplitudes revealed a flat EJP amplitude versus temperature curve. Near constant EJP amplitudes were recorded over 8-25°C, the EJP amplitudes decreased slightly at experimental temperatures colder than 9°C. Statistical analysis (one way ANOVA) identified free cold acclimated walking legs as generating significantly larger EJP amplitudes than free warm acclimated walking legs over 6-9°C, but

Figure 4.7A: Double pulse EJP amplitude in homothermal free and immobilised *Carcinus maenas* at low (8±1°C) and high (22±1°C) temperatures. Actual EJP amplitudes values were plotted at the high and low experimental temperature ranges (numbers of experiments are shown below each result column) to illustrate the difference between warm and cold acclimated animals (significant differences are denoted by asterisks). The percentage difference in EJP amplitude at high and low temperatures within an acclimatory group were, low to high free *C.maenas* 8= 51.8%, low to high IM *C.maenas* 8= 61.5%, low to high free *C.maenas* 22= 11%, low to high IM *C.maenas* 22= 2.13%. Mean data is plotted with standard errors of mean.

Figure 4.7B: Double pulse EJP amplitude in homothermal free and immobilised *Cancer pagurus* at low (8±1°C) and high (22±1°C). Actual EJP amplitudes were plotted at the acclimation temperatures (numbers of experiments are shown below each result column). The percentage difference between EJP amplitudes at low and high temperatures within an acclimatory group was; low to high free *C.pagurus* 8= 60.6%, low to high IM *C.pagurus* 9= 56.5%, low to high free *C.pagurus* 22= 13.57%, low to high, IM *C.pagurus* 22= 8.21%. Mean data is plotted with standard errors of mean.

Figure 4.7A: Double pulse EJP amplitudes in homothermal free and immobilised Carcinus maenas at low (8±1°C) and high (22±1°C) temperatures.

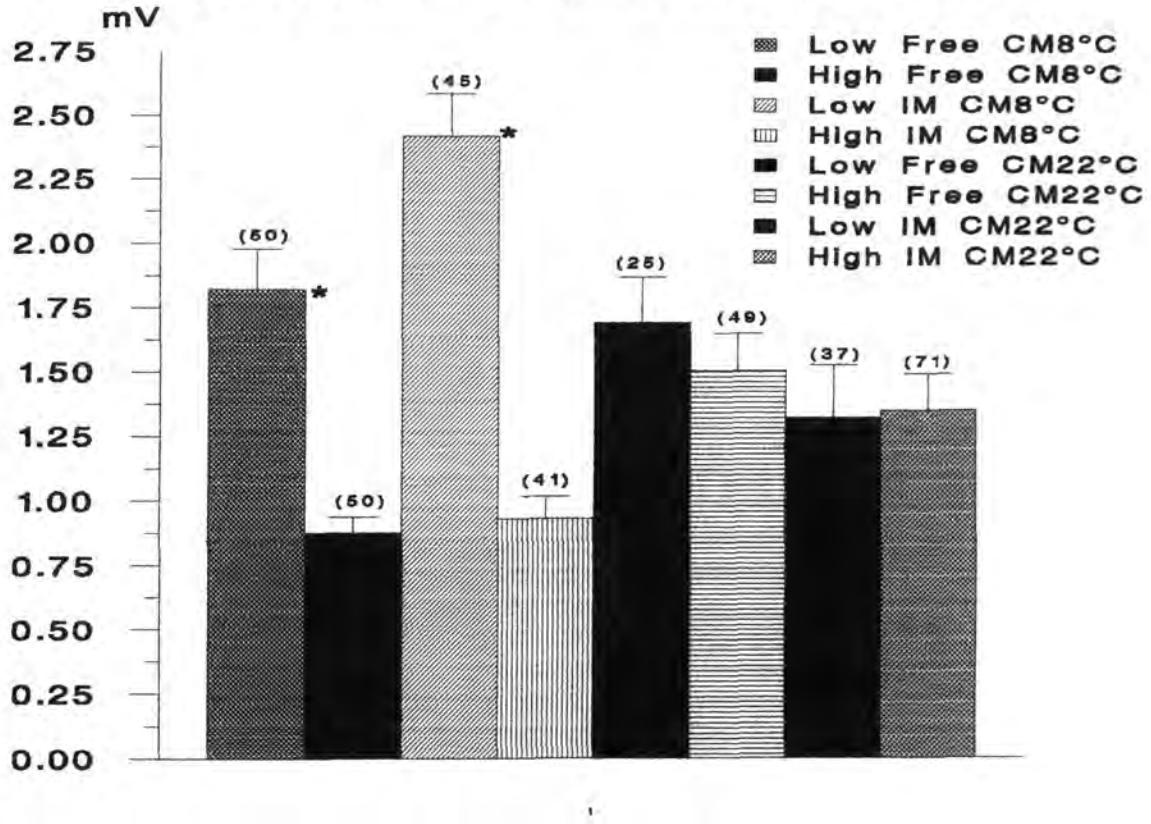
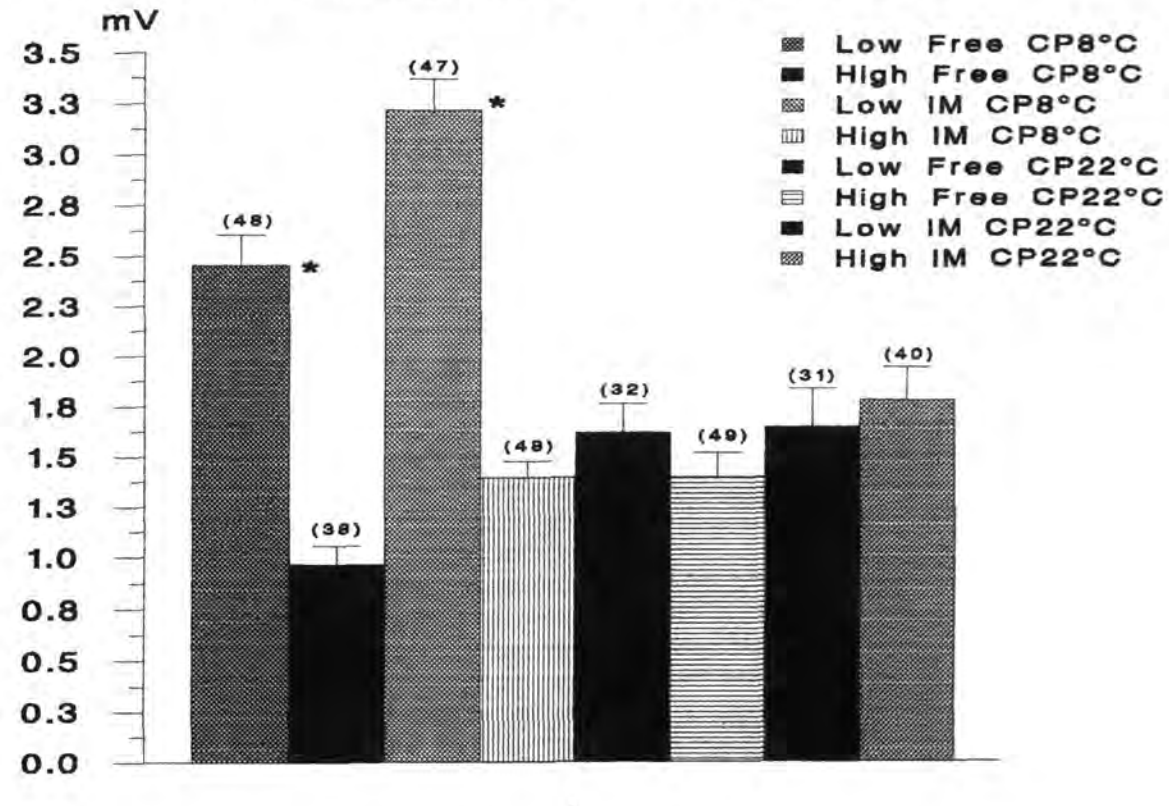


Figure 4.7B: Double pulse EJP amplitude in homothermal free and immobilised Cancer pagurus at low (8±1°C) and high (22±1°C) temperatures.



significantly smaller EJP amplitudes than free warm acclimated walking legs over 23-24°C. This revealed a clear difference between the warm and cold acclimated walking leg response to changing experimental temperatures.

Immobilised cold acclimated *C.pagurus* walking leg EJP amplitudes also decreased with increasing experimental temperature, the decrease in amplitude over the experimental temperature range was approximately 82%, this decrease in amplitude being qualitatively identical to that of free cold acclimated *C.pagurus*. Immobilised warm acclimated *C.pagurus* exhibited a slightly n-shaped EJP amplitude versus temperature curve, the amplitude at 26°C was omitted as only six out of fourteen preparations were responsive. Maximal EJP amplitudes were generated over 15-22°C, EJP amplitudes decreased slightly at experimental temperatures colder than 13°C or warmer than 21°C, which is generally similar to that previously reported for free warm acclimated *C.pagurus*. Statistical analysis (one way ANOVA) identified immobilised cold acclimated walking leg EJP amplitudes as being significantly larger than immobilised warm acclimated walking leg amplitudes over 6-17°C, which then decreased with increasing temperature to be significantly smaller than immobilised warm acclimated crab walking leg EJP amplitudes over 23-26°C, a much clearer warm and cold acclimation temperature difference.

Comparison of free and immobilised warm acclimated walking leg amplitudes revealed no significant differences, which indicates immobilisation had no affect on EJP amplitude changes with temperature. Whereas free cold acclimated walking leg EJPs were significantly smaller than immobilised cold acclimated walking leg EJPs over 6-15°C. The cold acclimated comparisons revealed a quantitative difference, indicating an immobilisation affect, however, both free and immobilised cold acclimated walking leg EJP amplitudes decreased with increasing experimental temperature in a qualitatively identical manner.

Double EJP amplitudes from free and immobilised warm and cold acclimated walking legs were compared within acclimatory groups at low (8±1°C) and high (22±1°C) temperature ranges, see Figure 4.7B. It was identified that cold acclimated walking legs (whether free of immobilised) generated significantly (Student's t-test) larger EJP amplitudes at colder experimental temperatures than the EJP amplitudes generated by the same walking legs at warmer (22±1°C) experimental temperatures. The warmer (high, 22±1°C) experimental temperatures initiated a significant decrease in cold acclimated walking leg EJP amplitudes which was not related to immobilisation. Warm acclimated walking legs generated amplitudes which were not significantly different (Student's t-test) at the low and high experimental temperature ranges, irrespective of immobilisation. It is clear that immobilisation does not affect warm acclimated but may affect cold acclimated walking leg amplitudes at colder experimental temperatures. This

however does not affect the qualitative changes in cold acclimated walking leg EJP amplitudes over the experimental temperature range.

Single EJP decay time constant.

Single EJP amplitudes were used to fit an exponential curve using a least squares fit, this identified Tau, or the EJP decay time constant (see equation 1.1).

C.maenas.

Single EJP decay time constants are presented on Figure 4.8A for warm and cold acclimated free and immobilised *C.maenas*. Free cold acclimated *C.maenas* walking leg decay time constants decreased with increasing experimental temperature, the decrease in Tau with increasing temperature indicates a decrease in membrane resistance or R_m, Tau is the time taken for an EJP to decay to 63% of its maximal value (Nicholls *et al.*, 1992). Free warm acclimated *C.maenas* walking leg time constants also decreased with increasing experimental temperature, free warm acclimated time constants were longer than free cold acclimated time constants over the whole experimental temperature range, significantly (one way ANOVA) so between 9-17 and 21-23°C. The R_m of free warm acclimated crab muscles was therefore significantly higher at colder experimental temperatures, which in part may be linked to poor exponential curve fitting to free warm acclimated single EJP amplitude results at experimental temperatures colder than 11°C. Curve fitting problems arise in single EJP amplitude results contaminated with 50Hz interference or interference from other electrical sources (e.g. centrifuge).

Immobilised cold and warm acclimated *C.maenas* walking leg muscle EJP decay time constants also decreased with increasing experimental temperature, the decrease in Tau for both immobilised warm and cold acclimated walking legs was qualitatively and quantitatively similar. Immobilised cold acclimated crab walking leg time constants were generally longer than immobilised warm acclimated crab walking leg time constants over the whole experimental temperature range. Statistical analysis (one way ANOVA) revealed no significant differences between immobilised warm and cold acclimated crab walking leg time constants except at 21°C only.

Comparison of cold acclimated free and immobilised crab walking leg time constants revealed no significant differences (one way ANOVA), indicating immobilisation had no affect on cold acclimated walking leg time constant acclimation. In comparison warm acclimated free crab walking leg time constants were significantly longer (one way ANOVA) than immobilised warm acclimated crab walking leg time constants, exhibiting significant differences over 6-26°C. The longer time constants of free warm acclimated

Figure 4.8A: Single EJP decay in *Carcinus maenas* homothermal free and immobilised controls. Numbers of experiments were; free *C.maenas* 8 (n= 13), IM *C.maenas* 8 (n= 14), IM *C.maenas* 22 (n= 24) and free *C.maenas* 22 (n= 10). Mean data was plotted with error bars shown for free *C.maenas* 22 and free *C.maenas* 8 only. The data sets were fitted with exponential curves using least squares fit, the equations of which are shown below. Exponential curves were used as changes in Tau were equivalent to changes in R_m.

Free *C.maenas* 8= 63.6*10(-0.0194x) R= 0.8736
Free *C.maenas* 22= 187.13*10(-0.0336x) R= 0.899
IM *C.maenas* 8= 68.64*10(-0.0218x) R= 0.945
IM *C.maenas* 22= 51.19*10(-0.0245x) R= 0.936

Figure 4.8B: Single EJP decay in *Cancer pagurus* homothermal free and immobilised controls. Numbers of experiments were; free *C.pagurus* 22 n= 9, IM *C.pagurus* 22 n= 12, free *C.pagurus* 8 n= 13 and IM *C.pagurus* 8 n= 16. The data sets were fitted with exponential curves using least squares fit, the equations of which are shown below.

Free *C.pagurus* 8= 62.59*10(-0.0233x) R= 0.845
Free *C.pagurus* 22= 37.27*10(-0.01296x) R= 0.653
IM *C.pagurus* 8= 76.8*10(-0.0275x) R= 0.9684
IM *C.pagurus* 22= 36.1*10(-0.01185x) R= 0.864

Figure 4.8A: Single EJP decay in Carcinus maenas homothermal free and immobilised controls.

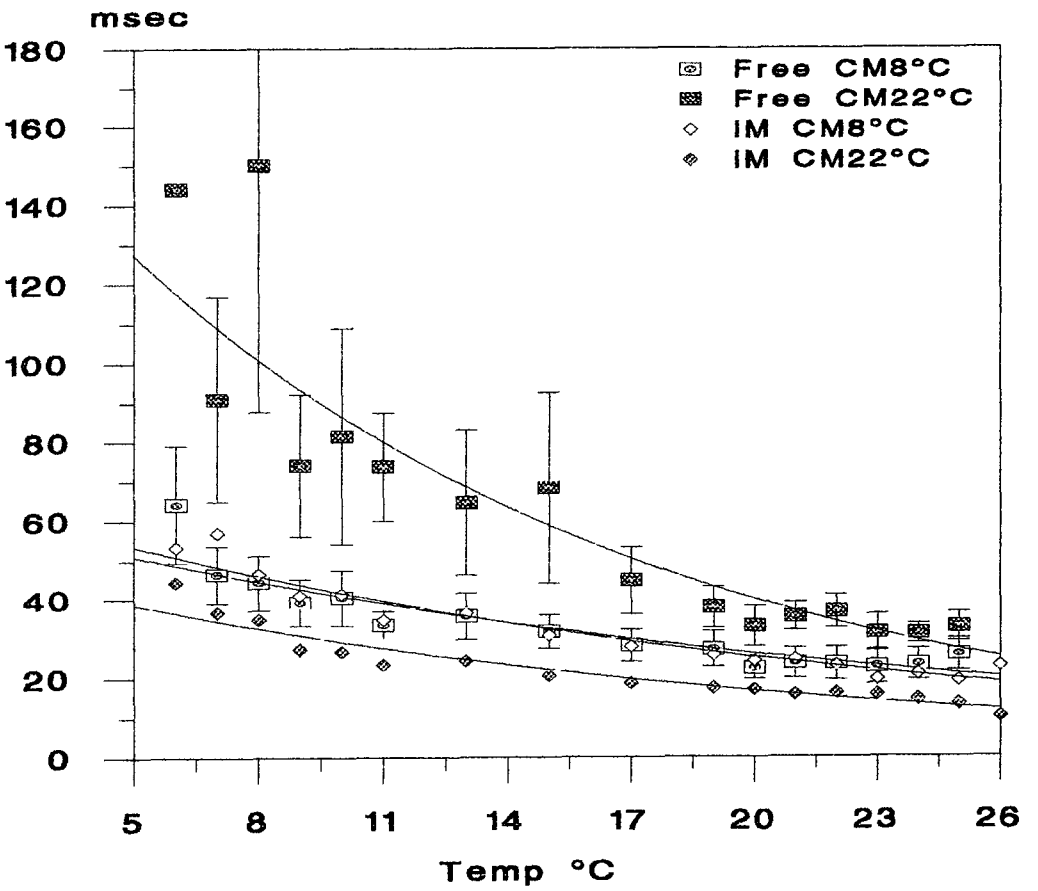
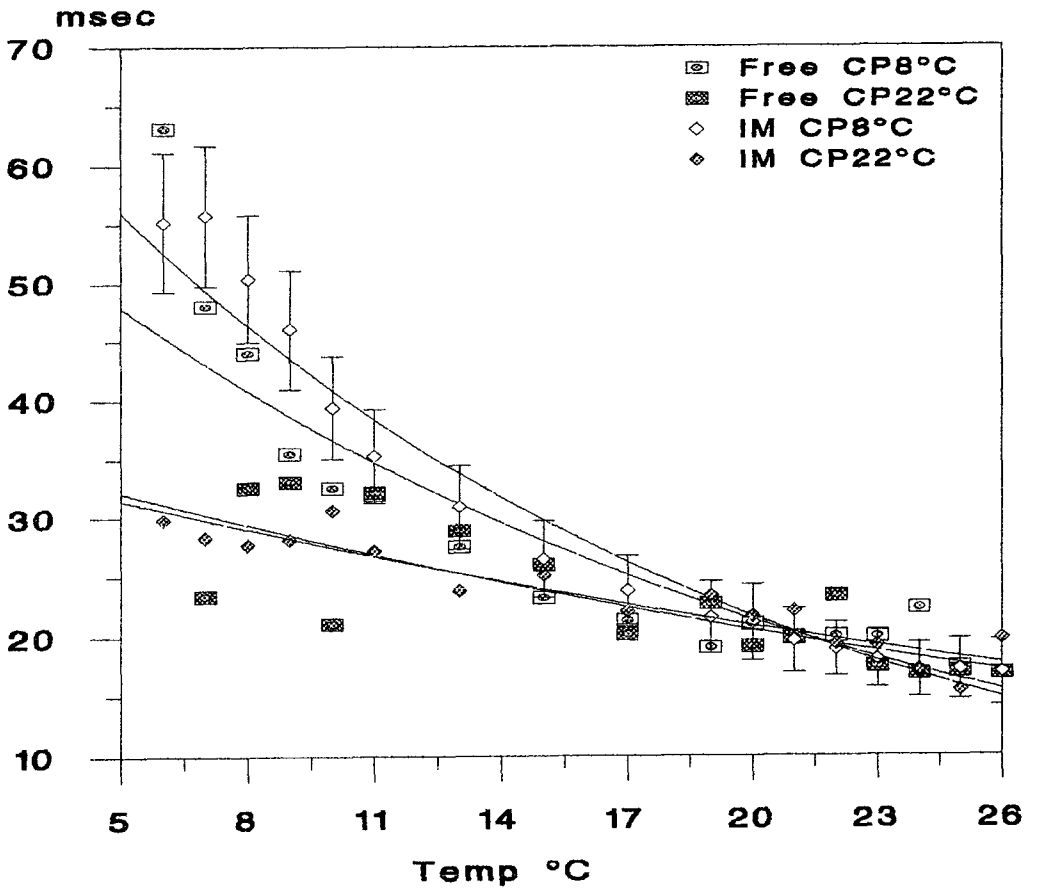


Figure 4.8B: Single EJP decay in Cancer pagurus homothermal free and immobilised controls.



C.maenas walking legs may contribute to their larger facilitation values recorded over most of the experimental temperature range (see Figure 4.5A). It is important to note that no differences in EJP amplitude were revealed between immobilised and free warm acclimated crabs, longer Tau maintains EJP amplitudes through increased R_m. This indicates curve fitting problems may account for some of the differences in Tau between free warm acclimated *C.maenas* walking legs compared to other acclimatory groups. The results indicate no immobilisation affect on cold acclimated walking legs, in contrast a potential immobilisation affect may be evident for warm acclimated walking legs. It would be interesting to know if there are any capacitance (C_m) changes, especially in un-fed immobilised warm acclimated walking legs as this has a bearing on Tau (see equation 1.1).

C.pagurus.

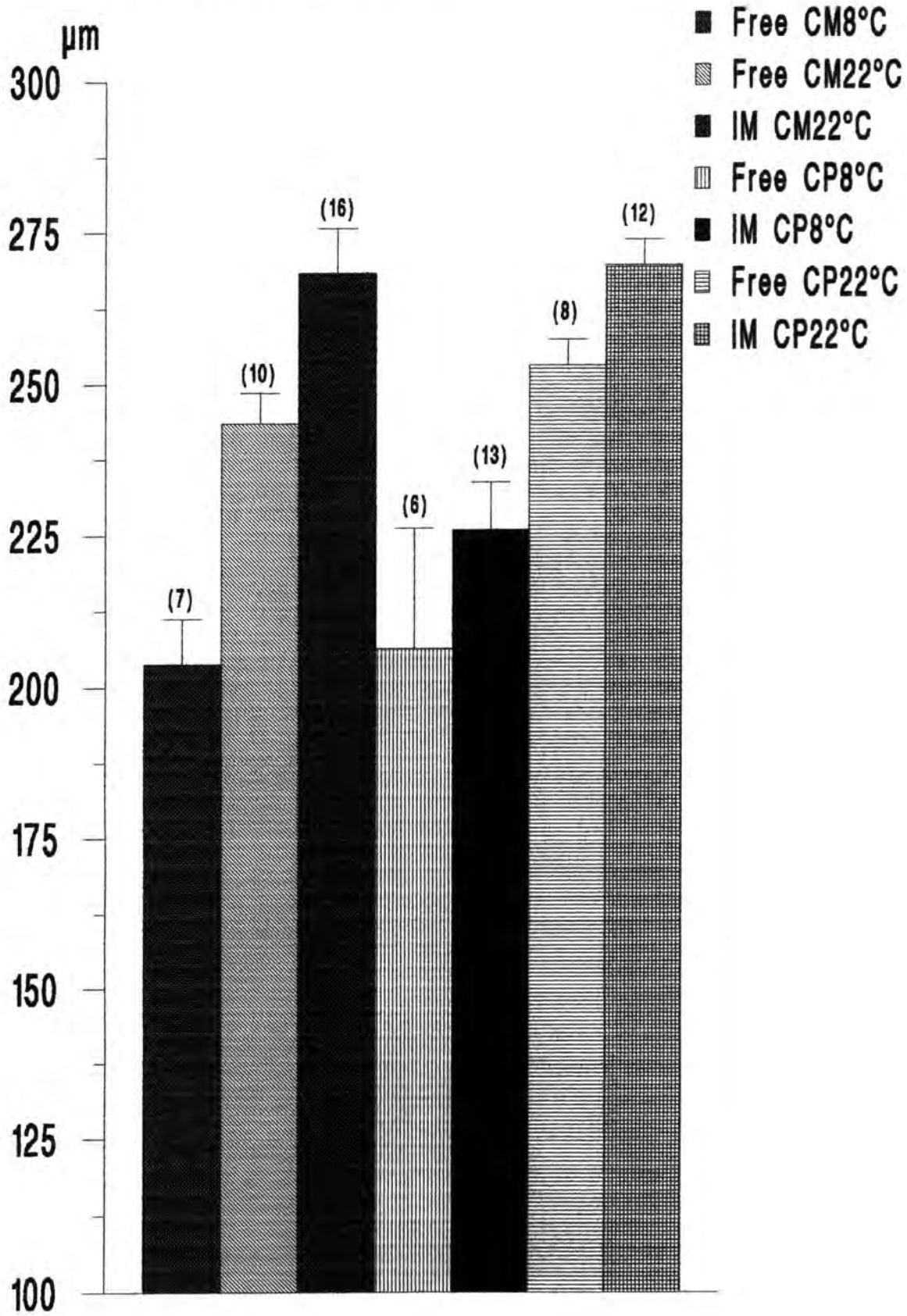
Figure 4.8B shows warm and cold acclimated free and immobilised *C.pagurus* single EJP decay time constants. Free cold acclimated crab walking leg time constants decreased with increasing experimental temperature, the decrease in Tau over the experimental temperature range was more marked than that shown for free warm acclimated crab walking legs. Although statistical analysis (one way ANOVA) revealed no significant differences between the free cold and warm acclimated *C.pagurus* walking leg time constants. It was notable that warm acclimated time constants were generally shorter at experimental temperatures colder than 11°C.

Immobilised cold acclimated crab walking leg time constants decreased with increasing experimental temperature in a qualitatively and quantitatively identical manner to that shown for free cold acclimated walking leg time constants. Immobilised warm acclimated walking leg time constants also decreased with increasing experimental temperature again in a manner identical to that previously described for free crab walking legs when warm acclimated. Statistical analysis (one way ANOVA) revealed a small number of significant differences between immobilised warm and cold acclimated crab time constants i.e. immobilised warm acclimated walking leg Tau were significantly shorter over 7-9°C only. Warm acclimated immobilised crab Tau were shorter than expected at experimental temperatures colder than 10°C.

Comparisons of free and immobilised cold acclimated walking leg time constants revealed no significant differences (one way ANOVA), which indicates no immobilisation affect. No significant differences (one way ANOVA) were found between warm acclimated immobilised and free crab walking leg time constants either, which again indicates no immobilisation affect. It has to be noted that warm and cold acclimated *C.pagurus* walking leg time constants were apparently unaffected by the

Figure 4.9: Muscle fibre diameter changes with acclimation temperature in *Carcinus maenas* and *Cancer pagurus*. Muscle fibre diameters were measured in a number of legs (numbers of experiments are shown below each result column), within each leg, the surface muscle fibres were measured (typically between 9-13 fibres) and averaged. These results were pooled into acclimatory groups and statistics done (one way ANOVA).
Mean data was plotted with standard errors of mean.

Figure 4.9: Muscle fibre diameter changes with acclimation temperature in Carcinus meanas and Cancer pagurus.



different acclimation temperatures, as no significant acclimation temperature difference was evident irrespective of the immobilised or free acclimatory condition. However, both cold acclimated free and immobilised crab walking leg time constants were generally longer at colder experimental temperatures (<11°C) than warm acclimated walking leg time constants, indicating higher R_m and a potential for greater EJP summation in cold acclimated muscle fibres. Warm acclimated free and immobilised crab walking leg time constants revealed a degree of temperature insensitivity at experimental temperatures colder than 11°C, as their EJP time constants were generally decreasing or constant over 6-11°C temperature range.

Muscle fibre diameter.

Figure 4.9 shows *C.maenas* and *C.pagurus* free and immobilised warm and cold acclimated crab walking leg muscle fibre diameters, no data was available for immobilised cold acclimated *C.maenas* walking legs. It can be seen that for both species cold acclimated walking legs had smaller diameter muscle fibres than their equivalent warm acclimated walking legs. There did appear to be an immobilisation affect (of varying significance) on muscle fibre diameters as immobilised fibre diameters from both species were larger than equivalently acclimated free walking leg muscle fibre diameters, despite the immobilised un-fed acclimation period. Specifically, free cold acclimated *C.maenas* and *C.pagurus* had significantly (Student's t-test) smaller diameter muscle fibres than free warm acclimated *C.maenas* and *C.pagurus* respectively. Immobilised cold acclimated *C.pagurus* had significantly smaller diameter muscle fibres than immobilised warm acclimated *C.pagurus*. However, immobilised and free cold and warm acclimated *C.pagurus* muscle fibre diameters were not significantly different. In contrast, immobilised warm acclimated *C.maenas* had significantly larger diameter muscle fibres than free warm acclimated *C.maenas*.

To account for some of the muscle fibre diameter differences, the lengths of walking legs of the different acclimatory groups were taken (see Table 6.8) and analysed (Student's t-test). Leg lengths were measured from the distal tip of the dactylopodite article to the proximal tip of the meropodite article, this was done to determine if fibre diameter differences were a function of leg size and not acclimation temperature.

There were no significant differences (Student's t-test) in leg length to account for the differences in muscle fibre diameters of any *C.pagurus* acclimatory group. There were some significant differences in leg lengths of *C.maenas* acclimatory groups that accounted for some muscle fibre diameter (and possibly latency and EJP decay time constant) differences. Free cold acclimated *C.maenas* (n=5) leg lengths were significantly smaller than free warm acclimated *C.maenas* (n=9), and free warm

acclimated *C.maenas* leg lengths were significantly smaller than immobilised warm acclimated *C.maenas* leg (n=16) lengths.

The results indicate immobilisation stimulated muscle hypertrophy although not significantly, there was a clear significant temperature induced hypertrophy of *C.pagurus* muscle fibres irrespective of free or immobilised acclimation.

Discussion.

In both warm and cold homothermally acclimated crab walking legs the resting potential (RP) became more negative or hyperpolarised with increasing experimental temperature, for both species whether free or immobilised. Temperature induced RP hyperpolarisation has also been seen in the tonic (E2) axon of *Pachygrapsus crassipes* (Stephens 1985b), opener muscle of *Astacus leptodactylus* (Fischer and Florey 1981) and in their closer muscle (Harri and Florey 1979). In all the acclimatory groups the RP changes with temperature were significantly greater than the 0.3163mV/°C change predicted by Nernst (MacDonald 1990; White 1983) and has been explained as a temperature induced activation of Na^+/K^+ ATPase and changes in Na^+/K^+ permeability ratios (MacDonald 1990; White 1983; Prosser and Nelson 1981). Thermal activation of membrane associated electrogenic pumps and potential changes in pump stoichiometry (Hochachka 1988a) can hyperpolarise the RP beyond the potassium equilibrium potential (Prosser and Nelson 1981). It was notable in some experiments that the RP at warm experimental temperatures approached, and in some cases exceeded the calculated value for potassium equilibrium potential (E_{K^+} being estimated as approximately -93.5mV (Nernst equation 1.2), assuming $[K]_i$=400mM and $[K]_o$=10mM at 21°C), for example RPs of free and immobilised cold acclimated *C.maenas* (and *C.pagurus*) walking legs at experimental temperatures warmer than 21°C (see Figure 4.1A/B respectively). Hyperpolarisation beyond E_{K^+} may arise through activation of the electrogenic Na^+ pumps (White 1983). The initial RP hyperpolarisation which occurs with increasing experimental temperatures from colder temperatures may be more a consequence of membrane permeability changes to Na^+/K^+ ratio (MacDonald 1990), but at even warmer experimental temperatures the continued RP hyperpolarisation beyond E_{K^+} may be due to increased active pump activity such as the Na^+/K^+ ATPase (White 1983; MacDonald 1990). To account for the Na^+/K^+ATPase activity, resting potential values were measured from muscle fibres of free cold acclimated *C.maenas* walking legs that had been incubated in the presence of 1mM ouabain (n=5) for twenty five minutes, the ouabain reduced the RP change with temperature to 0.4065mV/°C which was closer to, but still some 22% greater than that predicted by Nernst (using equation 1.2). The 22%

discrepancy may be due to changes in membrane permeability that are independent of ATPase activity (White 1983). In all acclimatory groups, RP changes with temperature were significantly different from ouabain treated muscle, except for immobilised warm acclimated *C.maenas* which was unexpected. Similar RP changes with temperature have been seen in *Procambarus clarkii* (White 1983; Colton and Freeman 1975) and other ectotherms and are reviewed by MacDonald (1990).

For both free and immobilised *C.maenas*, warm acclimation shifts the RP temperature curve along the temperature axis so that warm acclimated leg muscle RPs are depolarised as compared with cold acclimated RPs when compared at the same experimental temperatures. The efficacy of this acclimation response was 53.6% in free and 82.14% in immobilised *C.maenas*. Both free and immobilised warm acclimated *C.maenas* walking legs exhibited partial acclimation (or Type III after Precht 1958), although the immobilised crab walking leg RP acclimatory shift was more complete than that of free acclimated crab walking legs. The acclimatory shift along the temperature axis during warm acclimation is due to changes in Na^+/K^+ ATPase activity. Similar acclimatory shifts in RP have been reported by Kivivuori *et al.*, (1990) for warm (20°C) and cold (5°C) acclimated crayfish, and also for closer muscle of 12°C and 25°C acclimated *Astacus leptodactylus* (Harri and Florey 1979).

Free cold acclimated *C.maenas* RPs were significantly hyperpolarised over 8-24°C, and immobilised cold acclimated *C.maenas* RPs were significantly hyperpolarised over 6-25°C when compared to equivalent warm acclimated crab walking leg RPs (Figure 4.1A). Similar differences in RPs between warm compared to cold acclimated animals have been shown for the crayfish *Astacus astacus* giant axon acclimated to 5°C and 20°C (see also MacDonald 1990; Stephens and Atwood 1982; White 1983; Kivivuori and Lagerspetz 1982), and equivalently acclimated stretch receptor neurone of *Astacus astacus* (Kivivuori *et al.*, 1990). The RP change with temperature was 1.34mV/°C for free cold acclimated *C.maenas*, and was significantly different from the RP change with temperature of free warm acclimated *C.maenas* at 1.106mV/°C. The free warm acclimated walking leg RP change with temperature was smaller and indicated decreased sensitivity to warmer temperatures. The RP changes with temperature were similar to those of 1.13mV/°C found in 10°C acclimated opener muscle of *Astacus leptodactylus* (Fischer and Florey 1981), or 1.1-1.3mV/°C of 26°C acclimated closer muscle of *Ocypode ceratophthalma* (Florey and Hoyle 1976) and similar to 12°C and 25°C acclimated crayfish closer muscle RP change at 0.34-2.0 mV/°C (Harri and Florey 1979). The immobilised cold acclimated crab RP change with temperature was 0.878mV/°C which was significantly larger than that of immobilised warm acclimated crab (0.5955mV/°C), again, warm acclimation reduced the electrogenic component of the RP change with temperature. The RP change with experimental temperature was

1.34mV/°C and 1.106mV/°C for free cold and warm acclimated walking legs respectively, these were significantly different from equivalent RP changes of immobilised warm and cold acclimated *C.maenas* at 0.878mV/°C and 0.5955mV/°C respectively (see Table 4.2). In cold acclimated crab walking legs, the RP change with temperature was greater than that of warm acclimated walking legs, this indicated increased thermal sensitivity of cold acclimated crabs over the experimental temperature range (MacDonald 1990) compared to the RP changes of warm acclimated walking legs (whether free or immobilised). Cunningham (1995) reported RP hyperpolarisation with increasing experimental temperature for carp horizontal cells when cold and warm acclimated, the cold acclimated cells RP change with temperature was greater than the RP change of warm acclimated cells. This difference has again been related to increased Na^+/K^+ ATPase activity in cold acclimated animals compared to warm acclimated animals (Schwarzbaum *et al.*, 1991; Gladwell 1975). Some cold acclimated fish increase the density of membrane associated pumps and enzymes (Hochachka 1988a) this may affect the sensitivities of warm and cold acclimated tissue membrane pumps, thus affecting the RP change with temperature. Differences in cold compared to warm acclimated crab RP dependency on temperature may be linked to pump densities, although it remains unknown why immobilisation further reduces the electrogenic component to RP change with temperature whether warm or cold acclimated. The reduced sensitivity of immobilised walking legs must be related to the period of restraint during acclimation. Immobilisation may cause alterations in the membrane associated proteins i.e. reduced Na^+/K^+ ATPase, through muscle plasticity changes, changes in axon activity could also occur inducing synapse restructuring as shown in crayfish by Wojtowicz *et al.*, (1994), this may result in a supersensitivity (Atwood and Nguyen 1995). Supersensitivity is associated with an increased number of post-synaptic receptors and increased pre- and post-synaptic Na^+ channels, which may result in increased EJP amplitudes and faster pre- and post-synaptic responses (Hille 1992). However, supersensitivity generally results in an up-regulation of receptors and pumps etc., the RP change with temperature shown in immobilised crabs indicates a smaller contribution to RP from the electrogenic pumps indicating no supersensitivity.

However, cold acclimated free and immobilised crab walking leg RP versus temperature curves overlapped almost perfectly, indicating immobilisation had no quantitative or qualitative effect on *C.maenas* walking leg RPs. Warm acclimated walking legs revealed some significant differences between free and immobilised crab RP versus temperature curves. Free warm acclimated *C.maenas* RPs were significantly hyperpolarised over 21-24°C compared to immobilised warm acclimated *C.maenas*, these small differences indicate that immobilisation may affect acclimation to a small extent at higher experimental temperatures only. The difference in this case being

relatively small and perhaps accounted for by experimental variability. It is known that membrane resistance decreases with increasing experimental temperature which indicates an increase in the number of open channels (Stephens 1985b; Fischer and Florey 1981; Prosser and Nelson 1981; Castillo and Machne 1953), evidence for which will be discussed later (see single EJP decay). Acclimation to a warm temperature changes the lipid composition (Lee and Chapman 1987) of the membranes, this may affect channel conductance and therefore resistance (Green and Andersen 1991; Harri and Florey 1979). For example, changes in calcium dependent potassium channel conductance were found with changes in membrane composition, as reported by Bolotina *et al.*, (1989). Changes in channel function may therefore affect muscle RP, especially at warmer temperatures where R_m is lower. White (1983) reported loss of cell function through AP block due to increases in $[K^+]_o$ through increasing membrane leak at warmer temperatures. Decreased R_m could therefore contribute to immobilised warm acclimated crabs RP being relatively depolarised at experimental temperatures warmer than 20°C. The results indicate no differences between RPs of cold acclimated crabs whether free or immobilised, and a small number of differences between warm acclimated free and immobilised crabs at warmer temperatures. The small number of differences found for immobilised warm acclimated *C.maenas* are not expected to invalidate the protocol.

Warm and cold acclimated free and immobilised *C.pagurus* RP changes with experimental temperature were quantitatively and qualitatively similar to those of equivalently acclimated *C.maenas*, revealing a cold and warm acclimation temperature difference not dependent on the free or immobilised condition. There were no significant differences between *C.pagurus* free and immobilised cold acclimated crab walking leg RPs, and some RP differences between free and immobilised warm acclimated *C.pagurus* over 19-25°C only. The change in *C.pagurus* RP with experimental temperature were also similar to those already reported for *C.maenas*. Cold acclimated free and immobilised crab RP changes with temperature were significantly larger than the RP changes with temperature estimated for equivalent free and immobilised warm acclimated crabs. The trend of decreased RP sensitivity with warm acclimation and immobilisation was also apparent, although the difference between cold acclimated free and immobilised crabs were not significant, whereas the differences between warm acclimated free and immobilised crab RP changes with temperature were significant. The results indicate *C.pagurus* was as sensitive to experimental temperature change as *C.maenas*, but *C.pagurus* revealed decreased sensitivity to restraint.

The latency of nerve stimulated EJPs incorporates, from the point of stimulation, action potential conduction time to the synaptic cleft, diffusion of transmitter in the synaptic cleft, post-synaptic response to the neurotransmitter and subsequent t-tubule or muscle fibre depolarisation (Florey and Hoyle 1976; Nicholls *et al.*, 1992).

It has been shown previously that synaptic events are particularly thermolabile (Prosser and Nelson 1981; MacDonald 1990), changes in experimental temperature if large enough can block conduction at a synapse (Montgomery and MacDonald 1990). Furthermore axonal block can occur at neuronal branch points (White 1983; Atwood and Nguyen 1995). Latency includes conduction velocity or the time taken for an AP to travel from the source of stimulation to the axon terminal. Conduction velocity (CV) is reported to account for most of the latent period delay; whereas synaptic delay is relatively small irrespective of the acclimation temperature (MacDonald 1988), therefore, large changes in latency are related more to the CV component than synapse changes. Conduction velocity is dependent on axon diameter and the number of Na^+ channels; larger diameter axons conduct at faster velocities (Nicholls *et al.*, 1992), CV is increased further by increases in Na^+ channel number as long as C_m does not change (MacDonald 1990).

The latent periods of free and immobilised warm and cold acclimated *C.maenas* decreased with increasing experimental temperature, all data was fitted to a log/linear plot. Latency at warmer experimental temperatures (25-26°C) was approximately 3-3.5 fold smaller than the latency at colder experimental temperatures (6-7°C). Similar decreases in latency with increasing temperature were reported by Florey and Hoyle (1976) in warm and cold acclimated ghost crabs, by Cunningham (1995) using warm and cold acclimated carp and by Cuculescu (1996) who worked on warm and cold acclimated *C.maenas* and *C.pagurus*. The decrease in latency with increasing experimental temperature was attributed to the thermal effect on conduction, which incorporates temperature affects on molecule Brownian motion, thermal activation of Na^+ conductance (Prosser and Nelson 1981), and decreased electrical resistance (Montgomery and MacDonald 1990; Fatt and Katz 1953). Decreased refractory periods are also reported to occur with increasing experimental temperature and would be important at higher frequencies of stimulation, see Florey and Hoyle (1976), but in this case the frequency of stimulation at 0.5Hz was too low to be affected by refractory period changes. Once an action potential (AP) is initiated its conduction to the axon terminal is relatively assured (MacDonald 1990), AP conduction is comparatively thermotolerant (Atwood *et al.*, 1994) although blocks can occur at axon branch points at extreme temperatures (White 1983). Cuculescu (1996) has shown that the speed of AP conduction in isolated *C.maenas* and *C.pagurus* leg nerves was from 1.5m/sec at low experimental temperatures (6-7°C) up to 5m/sec at warmer experimental temperatures

(23-26°C), her data does not include synaptic or post-synaptic delays. This difference in AP conduction velocities (3-3.5 fold) was similar to the latent period change over a similar temperature range shown in Figure 4.2A, which further supports the idea that CV accounts for most of the latent period. Furthermore, Florey and Hoyle (1976) reported conduction velocities of APs in Hawaiian ghost crab (*Ocypode ceratophthalma*; acclimatized) axon, CV was 2.1m/sec and 5.3m/sec at experimental temperatures of 11°C and 31°C respectively, an approximate 3 fold change over that temperature range. The results of Cuculescu (1996), and Florey and Hoyle (1976) above clearly show a 3-3.5 fold change in CV, this indicates that over the 6-26°C experimental temperature range changes in CV had more affect on the latent period than did changes in synapse function. Synapse changes will have an increased affect on latency at temperature extremes (Prosser and Nelson 1981).

Statistical analysis showed latent periods from warm acclimated crab walking legs were significantly shorter than their equivalent cold acclimated crab latent periods, whether free or immobilised. Warm acclimation may change the relative contribution of pump activity and pump density (Hochachka 1988a; Schwarzbaum *et al.*, 1991), if axonal pump density increased during cold acclimation, decreased pump density and conductance might be expected during warm acclimation, therefore slowing conduction. Cunningham (1995) reported longer latent periods with warm acclimation in carp horizontal cells, which indicated an acclimatory compensation in that system. No latent period acclimation was found in warm acclimated *C.maenas*, whether free or immobilised. Shorter latent periods with warm acclimation similar to those reported here have been reported in other systems, Easton and Swenberg (1975) reported shorter latent periods in warm compared to cold acclimated squid, Cuculescu (1996) reported shorter latent periods in warm compared to cold acclimated *C.maenas* (and *C.pagurus*). Warm acclimation has been shown to cause increases in general cell size (Treistman and Grant 1993), increases in axon diameter with warm acclimation would reduce the latent period and account for the differences between warm and cold acclimated crab latent periods (whether free or immobilised).

Immobilised cold acclimated crab latent periods were significantly longer than free cold acclimated walking leg latent periods over the whole experimental temperature range. Changes in C_m could occur with acclimation through changes in Na^+ channel number (MacDonald 1990; Prosser and Nelson 1981), increased C_m slows AP conduction. Adams (1987) reported membrane capacity increased in lizard iliofibularis muscle fibres with increasing temperature (>35°C), which would act to lengthen EJP decay times as well as increase the latent period. C_m has been shown to increase slightly with increased membrane unsaturation (MacDonald 1990), increased unsaturation is normally associated with cold acclimation, it may be that a difference in lipid

composition contributes to the differences in latency found between cold acclimated free and immobilised crabs.

Immobilised warm acclimated crab latent periods were significantly shorter than free warm acclimated crab latent periods over the whole experimental temperature range, the reason for this is unknown but it may be linked to axon hypertrophy although no data is available.

The differences between free and immobilised latent periods indicated that immobilisation changed some, as yet unknown, factor(s) (e.g. axon diameter, Na^+ channel density, C_m) that resulted in altered conduction times.

Latent periods from *C.pagurus* were of a generally similar duration as those reported for *C.maenas*. All latency versus temperature curves were successfully fitted with exponential curves with correlation coefficients greater than 0.95 and graphed on a log/linear plot. The relative decreases in latency for immobilised, free warm and cold acclimated crab walking legs with increasing experimental temperatures were similar to those of *C.maenas,* showing a 3-3.5 fold decrease in latency over the whole experimental temperature range.

No differences in latent period were found between the free warm and cold acclimated crab walking legs, or between the immobilised warm and cold acclimated crab walking leg latent periods. This indicated latency was not changed with acclimation temperature, which was unexpected, and therefore may indicate no acclimation induced changes in axon diameter etc. in either warm or cold acclimated crabs, whether free or immobilised. Immobilisation significantly altered latency to the same extent for both acclimation conditions. The reason for the clear latent period difference between free and immobilised crabs in unknown. The shorter latent periods of immobilised walking legs may be associated with a supersensitivity, resulting in increased Na^+ channel number which would increase conduction velocity (if no changes in membrane capacity occur; MacDonald 1990), shortening the latent period. Activity induced changes have been reported to occur in crayfish opener muscle, but in that case were linked to neurotransmitter release and not the latent period (Wojtowicz *et al.*, 1994). Because latency is changed with immobilisation future comparisons between heterothermal and free acclimated crabs may be equivocal.

Single EJP amplitudes do not include any facilitative component. Post-synaptic events or EJP amplitudes are dependent on the successful exocytosis of neurotransmitter, the amplitude of the EJP is dependent on the amount of transmitter released (Matthews 1996; Fatt and Katz 1953) and the number and sensitivity of post-synaptic receptors (Nicholls *et al.*, 1992; White 1983). Muscle membrane resistance also influences EJP

amplitudes, especially the EJP amplitude duration or decay properties which is important in summation and therefore muscle movement (MacDonald 1990; Montgomery and MacDonald 1990).

Both free and immobilised cold acclimated *C.maenas* walking leg EJP amplitudes decreased significantly with increasing experimental temperature, indicating increased lability to warmer experimental temperatures. Immobilised cold acclimated *C.maenas* walking leg EJP amplitudes were significantly larger than free cold acclimated *C.maenas* amplitudes at temperatures colder than 15°C, this difference does not obscure the similarities of the temperature dependencies of free and immobilised cold acclimated *C.maenas* EJPs. The small immobilisation affect could be due to increased transmitter release, increased numbers of receptors or their sensitivity, increases in transmitter half life in the synaptic cleft, all of the above would contribute to increased EJP amplitudes in immobilised cold acclimated crabs. Immobilised and free cold acclimated *C.maenas* EJPs were qualitatively similar, such that an immobilisation affect was discounted. The decreasing EJP amplitudes of cold acclimated crabs reveal increased temperature sensitivity which indicates decreased muscle function especially at temperatures warmer than 17°C.

Free and immobilised warm acclimated *C.maenas* walking leg EJP amplitude versus temperature curves were generally n-shaped, exhibiting stable EJP amplitudes over most of the experimental temperature range. No significant qualitative or quantitative differences were found between EJP amplitudes from free and immobilised warm acclimated crabs, indicating no immobilisation affect. The stable EJP amplitudes recorded from warm acclimated free and immobilised *C.maenas* walking legs indicates maintained muscle function to warmer experimental temperatures, although small decreases in EJP amplitude at temperatures colder than 9°C or warmer than 22°C indicated increasing cold/warm sensitivity respectively of warm acclimated crabs, whether free or immobilised. The important acclimation temperature difference to note is the increased temperature sensitivity of cold acclimated *C.maenas* walking legs which was independent of the method of acclimation.

Cold acclimated free and immobilised *C.pagurus* EJP amplitudes were qualitatively similar, showing significantly decreased EJP amplitudes with increasing experimental temperature, the decrease in amplitude being marked at temperatures warmer than 15°C, whether free or immobilised. Both immobilised and free cold acclimated *C.pagurus* EJP amplitudes were significantly larger than the amplitudes from the equivalent warm acclimated immobilised and free *C.pagurus*. That is, free cold acclimated *C.pagurus* had larger EJPs at temperatures colder than 9°C than free warm acclimated *C.pagurus*, where immobilised cold acclimated *C.pagurus* EJPs were larger than immobilised warm

acclimated EJPs at temperatures colder than 17°C. Free and immobilised cold acclimated *C.pagurus* EJP amplitudes decreased to be significantly smaller than equivalent warm acclimated free and immobilised *C.pagurus* EJP amplitudes at temperatures warmer than 23°C.

The immobilised cold acclimated *C.pagurus* EJP amplitudes were significantly larger than free cold acclimated *C.pagurus* EJP amplitudes at temperatures colder than 17°C, which was similarly reported for *C.maenas*. These quantitative differences were small and clearly had no effect on immobilised cold acclimated *C.pagurus* walking leg responses to direct temperature and were considered not to be crucial. The clear decreases in EJP amplitude in cold acclimated crabs indicates increased temperature sensitivity whether free or immobilised, and in that respect are similar to the results reported for *C.maenas*.

Both immobilised and free warm acclimated *C.pagurus* revealed n-shaped EJP amplitude versus temperature curves. The EJP amplitude dependency on temperature indicated near constant EJP amplitudes over 11-25°C, the immobilised and free warm acclimated *C.pagurus* EJPs decreased at temperature's colder than 11°C indicating a degree of cold sensitivity. No significant differences were found between free and immobilised warm acclimated *C.pagurus* EJP amplitudes, indicating no immobilisation affect on warm acclimated crabs.

Both species results indicate decreasing muscle function with increasing experimental temperature when cold acclimated, whereas warm acclimated *C.pagurus* exhibited more stable muscle function over the experimental temperature range, whether free or immobilised.

For discussions and comparisons of potential changes in neurotransmitter, membrane resistance and other factors which may alter EJP amplitudes see double pulse EJP amplitude discussion below.

The facilitation values recorded from both *C.maenas* and *C.pagurus* acclimatory groups were similar, all preparations except free cold acclimated *C.maenas* exhibited generally u-shaped facilitation versus temperature curves, as such the *C.maenas* and *C.pagurus* results will be discussed together. Facilitation was not found to increase, as was expected, at warmer experimental temperatures in free cold acclimated *C.maenas*. Similar u-shaped facilitation versus temperature curves have been reported by Stephens and Atwood (1982) in *Pachygrapsus crassipes*, by Cuculescu (1996) in *C.maenas* and *C.pagurus* and closer muscle fibres of *Eriphia spinifrons* (Rathmayer and Hammelsbeck 1985) Facilitation is directly related to EJP amplitude (Stephens 1985a). Facilitation is thought to occur as a result of latent Ca^{2+} from a previous EJP contributing to the

amplitude of a subsequent EJP (Stephens and Atwood 1982; Baldo *et al.*, 1983). In almost every individual case positive facilitation values were recorded indicating no inhibitory component to the stimulation.

There were no significant differences between any *C.maenas* facilitation values irrespective of acclimation temperature or free and immobilised condition, except for data from free warm acclimated *C.maenas*. The free warm acclimated crabs gave facilitation values significantly larger than all other *C.maenas* acclimatory groups over almost all the experimental temperature range, particularly between 7-26°C.

Cuculescu (1996) reported u-shaped facilitation versus temperature curves for warm and cold acclimated *C.maenas* and *C.pagurus*, but also reported a small shift with warm acclimation. The ascendant portion of the facilitation versus temperature curve for warm acclimated walking legs, at colder experimental temperatures, moved towards warmer temperatures, although no acclimatory shifts were found in this study. All experimental groups in this study exhibited increased facilitation at approximately the walking leg acclimation temperatures, increased facilitation was also recorded at experimental temperature extremes furthest from their acclimation temperature, this being in agreement with results reported by Cuculescu (1996).

C.pagurus facilitation values were quantitatively and qualitatively similar to those of *C.maenas* (excluding free warm acclimated *C.maenas*), following the same trends with changing experimental temperature. There were no significant differences in facilitation between any *C.pagurus* acclimatory group, the u-shaped response curves of *C.pagurus* were more evident than those of *C.maenas*.

Increased facilitation at an animal's acclimation temperature has been reported in the crayfish *Astacus leptodactylus* (Harri and Florey 1979; Fischer and Florey 1981). Whereas, in other work facilitation was minimal at an animal's acclimation temperature, as shown for stretcher muscle of *Pachygrapsus crassipes* (Stephens and Atwood 1982), and in bender muscle of *Pachygrapsus crassipes* (Stephens 1985a). Facilitation is extremely variable, it can change in a particular muscle fibre depending on the frequency of stimulation (Rathmayer and Maier 1987; Rathmayer and Hammelsbeck 1985; Günzel *et al.*, 1993).

In cold acclimated *C.pagurus* and *C.maenas* facilitation decreased as the experimental temperature rose from 6-9°C. This correlated with smaller double EJP amplitudes (see Figures 4.6A/B) over the same experimental temperature range. Decreasing facilitation over a similar temperature range was also reported from the stretcher muscle of *Pachygrapsus crassipes* (Stephens and Atwood 1982). At warmer experimental temperatures still (i.e. warmer than 17°C), cold acclimated walking leg EJP amplitudes decreased markedly, independent of increasing facilitation. Increasing facilitation at temperatures warmer than 17°C is clearly evident for *C.pagurus*, whether free or

immobilised, but is less clear for *C.maenas* (except free warm acclimated *C.maenas*) acclimatory groups. The decrease in EJP amplitude at temperatures warmer than 17°C might be reduced by the increased facilitative component to EJP depolarisation at the warmer experimental temperatures.

In warm acclimated *C.pagurus* and *C.maenas* facilitation decreased with decreasing experimental temperature despite increases in Ca^{2+} sensitivity reported by Stevens and Godt (1990). Facilitation decreased to minimal values around the mid-temperature range (15°C). It is interesting to note that EJP amplitudes from both warm acclimated *C.maenas* and *C.pagurus* whether free or immobilised were maintained or near maximal around mid-temperatures, 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, these factors being synergisitically co-ordinated to maintain EJP amplitude (see Montgomery and MacDonald 1990; White 1983; Cuculescu 1996). Facilitation then increased in both free and immobilised warm acclimated *C.maenas* and *C.pagurus* with further decreases in experimental temperature, possibly due to continued increasing Ca^{2+} sensitivity and increasing membrane resistance which both maintain EJP amplitudes. At these colder experimental temperatures, decreased neurotransmitter release is known to occur (White 1983) which may be due to decreased calcium currents which would account for the decreasing EJP amplitudes recorded from warm acclimated *C.maenas* and *C.pagurus* walking legs at the colder experimental temperature range, a view supported by MacDonald (1990); and Prosser and Nelson (1981). Facilitation is particularly important at experimental temperature extremes in contributing to EJP amplitude, but it is itself dependent on other temperature dependent factors (calcium changes, transmitter release etc). It was clear that no facilitation acclimatory shifts were revealed for either cold and warm acclimated *C.maenas* and *C.pagurus* whether free or immobilised, which indicates facilitation's contribution to muscle function is exhibited over the short term experimental temperature range, and not through long term acclimation.

Double pulse stimulated EJP amplitudes were measured over the experimental temperature range 6-26°C. The first and second EJP amplitudes were measured (the second EJP was initiated approximately 40msec after the first and was corrected for non-linear summation (c.f Figure 2.4, after Stephens 1985a)) and the ratio of which provided facilitation values over the same experimental temperature range (Stephens and Atwood 1982). Synaptic transmission is recognised as consisting of a number of important phases; release of neurotransmitter, diffusion across the synaptic cleft, binding to post-synaptic receptor/channel, opening/closing response, removal of transmitter and finally

transmitter recycling or synthesis (MacDonald 1990; Nicholls *et al.*, 1992). Neurotransmitter exocytosis is a Ca^{2+} dependent event (Froehner 1993; Matthews 1996), Stevens and Godt (1990) have reported a general decrease in Ca^{2+} sensitivity with increasing experimental temperature. Furthermore Hidalgo and Donoso (1995) reported significant decreases in sarcoplasmic reticulum Ca^{2+} release at pH <6.5, it is known that pH is inversely related to temperature (Meis *et al.*, 1996), therefore decreasing Ca^{2+} release and sensitivity occurs with increasing experimental temperature which may indicate a potential for reduced transmitter exocytosis with increasing temperature. Although Montgomery and MacDonald (1990) and White (1983) all reported increased quantal content and release with increasing direct temperature. Thermal neuromuscular block is linked to cessation of transmitter release rather than a block of axonal conduction (Prosser and Nelson 1981; Montgomery and MacDonald 1990). Once an AP is initiated it is relatively assured to reach a synaptic terminal (White 1983), where Ca^{2+} influx initiates neurotransmitter release (Froehner 1993; Matthews 1996).

Carcinus maenas EJP amplitudes exhibited two populations of results, cold acclimated walking leg double EJP amplitudes from free and immobilised crabs were not significantly different, both showed greatest EJP amplitudes at cold experimental temperatures which decreased significantly with increasing experimental temperature. Similar decreases of EJP amplitude with increasing experimental temperature have been reported for cold acclimated opener muscle of the crayfish *Astacus leptodactylus* (Fischer and Florey 1981) and 10°C acclimated *Procambarus clarkii* (White 1983). In the crustacean nervous system there are multiple branch points and bottlenecks of axons (Atwood 1976; Atwood and Nguyen 1995) where, at warmer experimental temperatures, due to $[K^+]_o$ accumulation, an action potential may be blocked (White 1983). This block may not stop the successful release of neurotransmitter as crustacean muscles are polyterminally innervated (Wiersma and Ripley 1952; Atwood 1976), but may reduce the amount of transmitter exocytosed and the subsequent post synaptic depolarisation (Stephens and Church 1988; Atwood *et al.*, 1994).

In contrast warm acclimated free and immobilised *C.maenas* were able to maintain their EJP amplitudes over the experimental temperature range, their EJP amplitude versus temperature curves were slightly n-shaped, but amplitudes were more variable at lower experimental temperatures. There were no significant differences between EJP amplitudes from warm acclimated walking legs of free or immobilised *C.maenas* over the experimental temperature range. The amplitude results indicate that immobilisation did not affect the walking leg EJP amplitude changes with experimental temperature, since the EJP amplitude versus temperature curves almost overlapped and were qualitatively identical. Similar n-shaped amplitude versus temperature curves have been

shown in stretcher muscle of 21°C acclimated *Pachygrapsus crassipes* (Stephens and Atwood 1982) and crayfish closer muscle acclimated to 25°C (Harri and Florey 1979).

Comparisons between cold and warm acclimated *C.maenas* acclimatory groups did exhibit a small number of significant differences. Free and immobilised cold acclimated *C.maenas* exhibited progressively decreasing EJP amplitudes with increasing direct temperature, whereas free and immobilised warm acclimated *C.maenas* did not. The most obvious distinction between the cold and warm acclimated crabs with respect to double pulse EJP amplitude was the shape of their temperature response curves. Irrespective of free or immobilised acclimation, cold acclimated *C.maenas* showed increased sensitivity to warm temperatures. Whereas warm acclimated *C.maenas* showed a decreased thermal sensitivity through maintained EJP amplitudes, which was again unaffected by immobilisation.

The decrease in EJP amplitude with temperature shown by cold acclimated crabs occurs in spite of Whites (1983) reports from crayfish that quantal content/release increase with increasing experimental temperature (Montgomery and MacDoanld 1990). The initial decreases in EJP amplitude in cold acclimated crabs were in spite of contributions to amplitude from facilitation and Tau principally over 6-13°C. Furthermore, it has already been reported that muscle fibre RPs hyperpolarised with increasing experimental temperature, hyperpolarisation of RPs has been reported for several ectothermic animals (see White 1983; MacDonald 1990 etc.) and is considered to reduce muscle fibre excitability (Hille 1992). The reduction in muscle excitability lowers the sensitivity of post-synaptic membranes to transmitter therefore possibly contributing to the reduction in EJP amplitude shown by cold acclimated crabs with increasing temperature (whether free or immobilised). White (1983), Fatt and Katz (1953) and Stephens (1985a) etc. have all reported decreasing R_m with increasing experimental temperature, similarly reported for *C.maenas* and *C.pagurus* here (see below), which indicates the number of open channels increases with increasing temperature (Hille 1992). The decreased R_m at warmer temperatures reduces the capacity for facilitation and particularly for summation, which again contributes to decreasing EJP amplitudes with increasing experimental temperature in cold acclimated crabs. Apart from increasing quantal content/release only increases in facilitation contribute to cold acclimated crab EJP amplitudes at warmer experimental temperatures. The decrease in EJP amplitude at warmer temperatures may have been more profound if not for the contribution from facilitation, especially at temperatures warmer than 17°C. It is also clear that facilitation contributes significantly to cold acclimated crab EJP amplitudes at temperatures colder than 13°C, where increased R_m also has an important role. Facilitation and resistance play an important role in the EJP amplitudes at low temperatures especially if quantal content/release decrease with decreasing temperatures.

It may also be the case that Ca^{2+} sensitivity which is increased at lower temperatures according to Meis *et al.*, (1996); Stevens and Godt (1990); Hidalgo and Donoso (1995), could contribute to transmitter release at colder experimental temperatures despite the coincident decreases in quantal content/release.

The question arises, what might have changed with warm acclimation that enables crabs to maintain muscle EJP amplitudes and therefore function to warmer experimental temperatures? It is clear that warm acclimation induces an acclimatory shift in RP, so that near constant RPs are recorded at either warm or cold acclimation temperatures, whether free or immobilised. This acclimatory shift maintains the muscle fibres excitability and contributes to maintaining EJP amplitudes at warm acclimation and experimental temperatures. Cold acclimation increases the density/activity of pumps and enzymes according to Hochachka (1988a) and Schwarzbaum *et al.*, (1991), therefore warm acclimation might reduce the density/activity of pumps etc. This was seen to occur through reduced RP changes with temperature (slopes) in all warm acclimated crab experiments, the decrease in pump activity with warm acclimation may reduce the sensitivity of the membrane to heat induced RP changes. This decreased sensitivity may contribute to warm acclimated crabs maintaining appropriate muscle excitability at even warmer temperatures. It could also be that changes in the half-life of exocytosed glutamate occur, this could be counterbalanced by changes in receptor density/sensitivity, although no information is available about glutamate half-life changes with acclimation.

It may be expected that warm acclimated crab EJP amplitudes would increase with decreasing experimental temperature due to increases in Tau and Ca^{2+} sensitivity, this was seen to occur very slightly from 26°C to 17°C in some warm acclimated experimental preparations (i.e. single EJP amplitudes). The slight decrease in warm acclimated crab EJP amplitudes at temperatures colder than 11°C could be due to decreased neurotransmitter release, through the beginning of cold block as quantal content and release decrease with decreasing experimental temperatures (Montgomery and MacDonald 1990). The decreasing EJP amplitudes of warm acclimated crab walking legs at cold experimental temperatures indicates cold sensitivity, the EJP amplitudes could be partially maintained by increased membrane resistance and RP depolarisation, but only if neurotransmitter is successfully exocytosed. The main contributor to maintained EJP amplitude at cold experimental temperatures in warm acclimated crab walking legs is increased summation and facilitation (Stephens and Atwood 1982). Warm acclimated crab walking leg EJP amplitudes decreased slightly in some cases at experimental temperatures warmer than 22°C, probably due to decreased membrane resistance which is supported in our work by decreasing Tau values (see Figure 4.8A), decreased Tau results in decreased summation and potential conduction block at neural branch points (White 1983).

Double EJP amplitude results from free and immobilised warm and cold acclimated *C.pagurus* were qualitatively similar to those reported for *C.maenas*. Cold acclimated *C.pagurus* walking leg EJP amplitudes were greatest at cold experimental temperatures near to their acclimation temperature, whether free or immobilised. Cold acclimated free and immobilised *C.pagurus* walking leg EJP amplitudes decreased significantly with increasing experimental temperature in a similar manner up to a blocking temperature of 25-26°C. Free cold acclimated *C.pagurus* EJP amplitudes were significantly smaller than amplitudes from immobilised cold acclimated *C.pagurus* at temperatures colder than 15°C, although their EJP amplitude versus temperature curves were a similar shape.

Free warm acclimated *C.pagurus* walking legs showed maintained EJP amplitudes over a 8-23°C range, free warm acclimated *C.pagurus* EJP amplitudes were not significantly different from immobilised warm acclimated *C.pagurus* amplitudes, the amplitudes decreased only slightly at the ends of the experimental temperature range (see Figure 4.6B). Similar n-shaped or constant EJP amplitude versus temperature curves have also been reported in environmentally acclimatized closer muscle of *Ocypode ceratophthalma* (Florey and Hoyle 1976) living at 26-28°C, and stretcher muscle of 21°C acclimated *Pachygrapsus crassipes* (Stephens and Atwood 1982).

Comparison of warm and cold acclimated *C.pagurus* walking leg EJP amplitudes identified some significant differences between free and immobilised crabs. Free cold acclimated *C.pagurus* EJP amplitudes were significantly larger than free warm acclimated *C.pagurus* at cold temperatures, but cold acclimated crab amplitudes decreased to be significantly smaller at warmer experimental temperatures. The amplitudes from cold acclimated *C.pagurus*, whether free or immobilised, show increased temperature sensitivity to warm temperatures, whereas warm acclimated crabs show maintained EJP amplitudes indicating temperature tolerance. The maintained amplitudes of warm acclimated *C.pagurus* indicate an acclimatory compensation, but this was generally at the expense of increased muscle EJP amplitude variability at experimental temperatures colder than 9°C.

The warm and cold acclimation differences, whether free or immobilised, were clearer for *C.pagurus* than *C.maenas*. Warm acclimated *C.pagurus* walking leg EJP amplitude versus temperature curves indicated both species exhibited partial acclimation (Type III after Precht 1958), *C.pagurus* acclimated more completely than *C.maenas* as maximum amplitude EJPs were generally recorded at slightly warmer experimental temperatures for *C.pagurus* than for *C.maenas* (see Table 6.7). Both species exhibited successful acclimation as both species functioned at the warm acclimation temperature, whether free or immobilised, although it was surprising that *C.pagurus* appeared to acclimate more successfully than *C.maenas*. The capacity acclimation shown by

C.pagurus must have incorporated significant resistance acclimation, it may be that the temperature space available to *C.pagurus* is larger than might be expected.

By fitting exponential curves (least squares fit) to the EJP decay phase single EJP decay time constants (Tau) could be determined, Tau gives a measure of membrane resistance (as capacitance is constant) or the number of open channels (Hille 1992; Fatt and Katz 1953).

Figure 4.8A shows changes in Tau (R_m) over the experimental temperature range for free and immobilised warm and cold acclimated *C.maenas*. For all acclimatory groups it can be seen that Tau decreased with increasing experimental temperature, which indicates decreasing R_m or the number of open channels is increasing (Fatt and Katz 1953; Nicholls *et al.*, 1992), lower R_m results in decreasing summation. Free warm acclimated *C.maenas* Tau values were significantly different from free cold acclimated *C.maenas* Tau values over most of the experimental temperature range, the increased R_m recorded from free warm acclimated *C.maenas* may contribute to their larger facilitation values recorded over almost all of the experimental temperature range (see Figure 4.5A). In worm giant axons, cold and warm acclimated preparations showed input resistance changes. Lower input resistances in cold (5°C) acclimated worms were observed compared to those from warm (23°C) acclimated worms when measured at 20°C (Prosser and Nelson 1981). This may be linked to homeoviscous adaptation, because differences in membrane lipid composition may alter channel conductance (Bolotina *et al.*, 1989) and therefore membrane resistance.

Immobilised warm and cold acclimated *C.maenas* Tau were not significantly different from each other, both exhibited decreasing Tau with increasing experimental temperature. No significant differences were revealed between cold acclimated free or immobilised walking leg Tau values, indicating no immobilisation affect. Whereas free and immobilised warm acclimated walking leg Tau values were significantly different over 6-26°C, indicating an immobilisation affect, free warm acclimated *C.maenas* Tau were quite variable at temperatures colder than 13°C. The variability was due to a decrease in the number of single EJP amplitudes from free warm acclimated walking legs which could be successfully fitted with an exponential (decay) curve, which may explain the increased R_m found in free warm acclimated *C.maenas* walking legs over the colder temperature range.

The acclimation temperature differences were not clear, although, free warm acclimated *C.maenas* EJP time constants were longer than free cold acclimated *C.maenas* walking legs, it is not clear if this is an acclimatory response or not. No clear acclimation temperature differences were found for Tau from immobilised cold and

warm acclimated *C.maenas* walking legs, and excluding free warm acclimated *C.maenas* Tau values no consistent immobilisation affect was revealed.

Tau values from warm and cold acclimated free and immobilised *C.pagurus* are shown in Figure 4.8B. All acclimatory groups revealed decreasing Tau with increasing experimental temperature, therefore reducing the potential for summation and to a lesser extent facilitation which normally contributes to muscle depolarisation (Castillo and Machne 1953; Fatt and Katz 1953), especially at physiological frequencies of stimulation. There were no significant differences between the Taus of any *C.pagurus* acclimatory group, irrespective of warm or cold acclimation or the free and immobilised condition. The only qualitative difference was that both warm acclimated free and immobilised Tau values were generally shorter (not significantly) than the Tau of cold acclimated free and immobilised *C.pagurus* walking legs at temperatures colder than 12°C. The Tau values indicate immobilisation had no affect on EJP decay time constants, but also that warm and cold acclimation had little effect on *C.pagurus* EJP decay. It is notable that both free and immobilised warm acclimated *C.pagurus* walking leg Tau values were generally insensitive to temperatures colder than 11°C, which may indicate warm acclimated crabs were incapable of maintaining EJP amplitudes through summation at colder temperatures. The temperature sensitivity may be linked to changes in membrane fluidity affecting channel opening and therefore membrane resistance (see Figure 1.3; Bolotina *et al.*, 1989).

It is interesting to note that for both *C.maenas* and *C.pagurus* acclimatory groups, facilitation was minimal over the mid temperature range (approximately 13-17°C), where near maximal (or constant) EJP amplitudes were recorded from warm acclimated free and immobilised crabs. Near maximal or constant amplitudes over this range were not potentiated by facilitation or by any significant changes in Tau (R_m). It is not known if capacitance (C_m) changes had occurred between the different acclimatory groups, no C_m changes were expected over the short term experimental temperature range (MacDonald 1990). Any changes in C_m that do occur between the different acclimatory groups were expected to be small as homeoviscous adaptation only has a very slight affect on C_m (MacDonald 1988; 1990). Therefore changes in transmitter release were responsible for the maintained EJP amplitudes at mid temperature ranges whether free or immobilised, although changes in receptor density/sensitivity cannot be ruled out.

Muscle fibre diameter and the surface area of a muscle are the principal controlling factors in R_m and C_m respectively, increases in either R_m or C_m lengthen Tau and therefore increase the potential for EJP summation. Larger muscle fibres have lower R_m

(Tau), but C_m is not expected to change significantly although no critical investigation of C_m was done. To identify possible changes in R_m (apart form Tau), muscle fibre diameters were measured as an indication of temperature induced muscle fibre hypertrophy.

Free warm acclimated *C.maenas* muscle fibre diameters were significantly larger than equivalent cold acclimated *C.maenas* fibre diameters, indicating muscle hypertrophy, this however does not account for the lower Tau values from cold acclimated walking leg muscle fibres. This difference between warm and cold acclimated *C.maenas* fibre diameters is nullified by the fact that warm acclimated crabs had significantly larger legs than cold acclimated *C.maenas* (see Table 6.8). Free warm acclimated *C.maenas* fibre diameters were significantly smaller than fibres from warm acclimated immobilised *C.maenas*, this result again is not significant due to immobilised warm acclimated *C.maenas* walking legs being significantly larger than free warm acclimated *C.maenas*. The differences in leg size account for the fibre diameter differences and the generally smaller Tau found in immobilised warm acclimated *C.maenas*, what is important to note is that no significant hypertrophy of muscle fibres was found with respect to the immobilised condition.

There were no significant differences in the leg lengths of any *C.pagurus* acclimatory group to account for cold acclimated walking legs having significantly smaller muscle fibre diameters than warm acclimated walking legs, whether free or immobilised. This indicates fibre hypertrophy (Rome 1990; Treistman and Grant 1993) occurred at warmer acclimation temperatures which was supported by the qualitatively reduced (not significant) decay time constants found in warm acclimated free and immobilised *C.pagurus* walking legs at the lower experimental temperature range (6-10°C). These differences must therefore be due to the different acclimation temperatures. It was particularly interesting that immobilised walking legs exhibited muscle hypertrophy despite their un-fed state, probably through utilisation of internal stores. Additionally no significant hypertrophy was related to the immobilised condition.

Summary.

It can be concluded that there are clear acclimation temperature differences between warm and cold acclimated *C.maenas* and *C.pagurus,* whether free or immobilised. These acclimatory differences were similar to those already shown for *C.maenas* and *C.pagurus* by Cuculescu (1996) i.e. acclimatory shift in RP for warm acclimated crabs, decreasing EJP amplitudes with increasing experimental temperature in cold acclimated crabs, maintained EJP amplitudes to warmer experimental temperatures near to the warm

acclimation temperature, and u-shaped facilitation versus temperature curves for both warm and cold acclimated crabs. Similar results have also been reported for *Astacus leptodactylus* (Harri and Florey 1979; Fischer and Florey 1981) and *Pachygrapsus crassipes* (Stephens and Atwood 1982; Stephens 1985b).

The actual differences between stenothermic *C.pagurus* and eurythermic *C.maenas*'s capacity to acclimate were small, a conclusion which is supported by results reported by Cuculescu (1996) for warm and cold acclimated *C.maenas* and *C.pagurus*. Electrophysiology results strictly identified *C.pagurus* as acclimating more completely than *C.maenas* to the warm acclimation temperature, with respect to RP acclimation. No species differences were revealed for double EJP amplitude acclimation, but warm acclimated *C.pagurus* single EJP amplitudes did appear more temperature sensitive than *C.maenas* at temperatures colder than 11°C, indicating warm acclimation was at the expense of muscle function at colder temperatures.

Immobilised crab results were in most cases unaffected by the period of restraint during acclimation when compared to equivalently acclimated free crabs. There was no immobilisation affect on EJP amplitude, muscle fibre diameter and membrane potential (except at high experimental temperatures when warm acclimated). Some immobilisation differences were found in single EJP decay time constant and facilitation for warm acclimated *C.maenas* only. Only latency was grossly changed through immobilisation, indicating that future latency times of restrained crabs may be equivocal if compared to free crabs. It can therefore be concluded that other electrophysiological differences of heterothermally (restrained) acclimated crabs (Chapter Six) should be wholly due to the acclimation temperature (or CNS derived trophic factor) differences and not principally due to the immobilisation period during heterothermal acclimation.

Chapter Five.

Comparison of neuromuscular parameters in seasonally acclimatized *Carcinus maenas* and *Cancer pagurus*.

Introduction.

Seasonal changes have been shown to affect a variety of physiological parameters in ectotherms. In temperate regions seasonal changes obviously concern temperature and day length, but in aquatic environments changes may also occur in oxygen tension, pH and salinity, to which an organism maybe responding.

Layne *et al.*, (1985; 1987) have shown that CTMax of the crayfish *Orconectes rusticus* was greater in summer than winter caught animals, a change that may simply reflect the 20°C difference in environmental temperature. However, Fodor *et al.*, (1995) have demonstrated seasonal changes in membrane composition and order in carp (*Cyprinus carpio*) liver that corresponds to homeoviscous adaptation (Sinensky 1974). Stephens (1952) reported in a study that extended from September through June that crayfish exposed to normal day/night photoperiods showed cycles of ovarian development which were dependent on the day length and temperature.

Many studies have been carried out on summer and winter caught animals which were subsequently subjected to laboratory acclimation regimes (e.g. Matikanien and Vornanen 1992; Layne *et al.*, 1987; Pruitt 1988), and so true seasonal effects are difficult to establish.

There are numerous reports that the synapse is the most thermally sensitive structure in the nervous system (Prosser and Nelson 1987; Lagerspetz 1974 etc). It is also a relatively plastic structure in that well defined changes occur in response to age, growth and activity (Lnenicka 1993; Wojtowicz *et al.*, 1994; Nguyen and Atwood 1994). In consequence synaptic events may well be subject to alteration in response to seasonal changes in the environment.

The crustacean nervous system has been well studied in this respect. Lnenicka and Zhao (1991) have shown in *Procambarus clarkii* that nerve terminals of claw closer muscle undergo seasonal changes in the amount of transmitter released by claw closer muscles. These workers argued that as there were no changes in input resistance of the muscle fibres then the seasonal change in EJP amplitude was owing to different levels of neurotransmitter release. A similar conclusion was reached by Atwood *et al.*, (1994) who reviewed work on plasticity of quantal release in crustacean motor terminals. Lnenicka (1993) also determined that in summer

animals the synaptic varicosities contained more mitochondria and synaptic vesicles than were found in winter animals, which conferred a greater resistance to fatigue in summer animals when stimulated at 5Hz. However, at lower stimulation frequencies (0.1Hz) the winter preparations gave the larger EJP amplitudes due to winter animal's transmitter release being greater than summer.

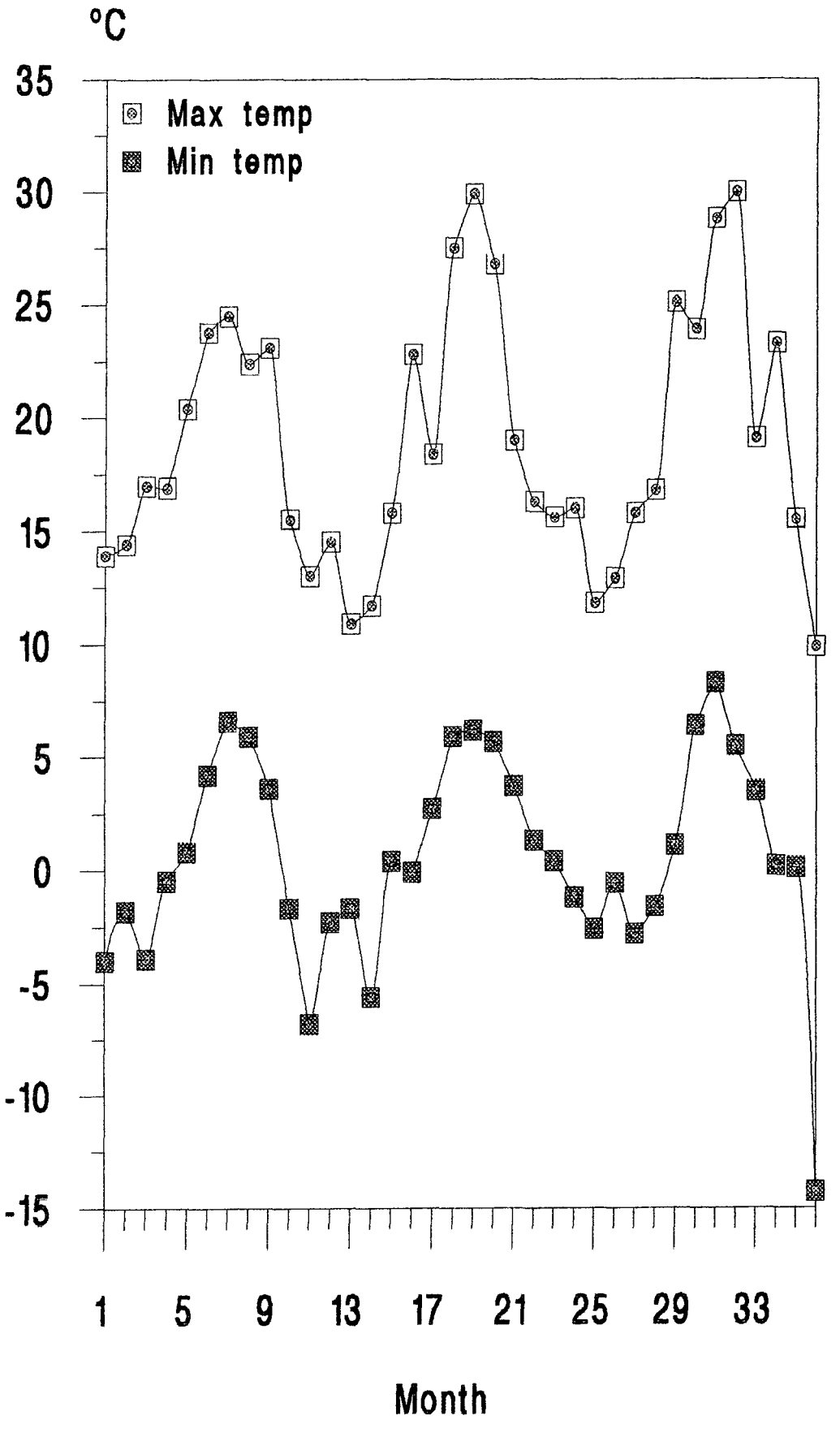
In *Procambarus clarkii* the described neuromuscular plasticity changes in motor terminal morphology are likely to be in response to higher summer levels of neuron activity, rather than temperature *per se*. Lnenicka and co-workers used immature *Procambarus clarkii* and so reproductive hormones could not have played a role in the observed neuron plasticity (Lnenicka 1993; Lnenicka and Zhao 1991). There is, however, evidence of a hormonal influence in motor terminal physiology in crustaceans. Kravitz (1988) has shown the neurohormones serotonin and octopamine alter motor neuron activity and affect transmitter release for lobster motor terminals. Furthermore, in crayfish the co-transmitter proctolin potentiates the activity of voltage-dependent Ca^{2+} channels, the activity of proctolin being greater in summer than winter animals (Bishop *et al.*, 1991). Skerrett *et al.,* (1995) examined the effects of two identified neuropeptides (NF_1 and DF_2) on *Procambarus clarkii* heart rate and neuromuscular junction (NMJ) function, and concluded the neuropeptides were neurohormones, both increased the release of neurotransmitter quanta at the NMJ.

Seasonal changes in the environment might be expected, therefore, to be transduced by the nervous and humoral systems into adaptive responses in cell function and structure. With respect to thermal physiology however such centrally driven changes might be additive to the effects at the tissue level, caused by the direct and all pervasive influence of temperature. In fact little direct evidence exists for hormonal influences on temperature acclimation. The most significant evidence indicated that protein synthesis in cultured catfish (*Ictalunis punctalus*) hepatocytes was dependent on fish serum, the serum from 15°C acclimated fish enhanced synthesis to a greater extent than serum from 25°C acclimated fish (Prosser *et al.*, 1991). There is however little doubt that it would be expected that the nervous system would be capable of responding to seasonal changes in the environment, and that such responsiveness would impact widely.

The aim of the work in this chapter was to determine the extent of the differences in neuromuscular physiology between summer and winter caught *C.maenas* and *C.pagurus* without subjecting the animals to further acclimation.

Figure 5.1: Minimum and maximum monthly air temperatures from January 1993 to December 1995. Individual minimum and maximum air temperatures measured at Whitby are shown, data was supplied by the Meteorological Office. The results gave an indication of the temperature extremes an intertidal animal may be exposed to if emersed. The data also gave a general indication of surface sea and rock pool temperatures, which would obviously be within the temperatures shown.

Figure 5.1: Minimum and maximum monthly air temperatures from January 1993 to December 1995.



Method.

Crabs caught from the sea were used immediately. *C.maenas* and *C.pagurus* summer acclimatized crabs were caught and experimented upon in the month of August, the winter acclimatized animals were caught and used in February and March. The average summer and winter environmental intertidal sea temperatures were approximately 12-16°C and 4-6°C respectively. Where possible *C.pagurus* were used within hours of being caught, otherwise they were used within three days of capture having been held in clean, aerated sea water at 8°C. They were always used within three days of capture. *C.maenas* were also used within hours of capture but when this was not possible they were also held in clean aerated sea water at 8°C. They were always used within six days of capture. No consistent differences in electrophysiological parameters were observed between crabs used immediately and those held for a few days. It is therefore concluded that no significant laboratory acclimation occurred.

The electrophysiological parameters investigated over the 6°C to 26°C experimental temperature range were, latency, membrane potential, single and double pulse stimulated EJP amplitudes, facilitation and single pulse EJP decay time constant.

Results.

Environmental Temperatures.

Intertidal sea temperatures were approximately 4-6°C in winter and 12-16°C in summer. The temperature of deeper sublittoral waters were 5-10°C in summer and 4-8°C in winter (personal communication Dr Hyde). No systematic data was available for habitat temperatures over an annual period. *C.maenas* migrates into the littoral zone around the coast of Britain during the summer months where it often becomes stranded in rock pools, in contrast, *C.maenas* migrates to sublittoral water in winter months (Taylor and Wheatly 1979). No data is available on seasonally dependent migration of *C.pagurus*.

Figure 5.1 shows systematic data for air temperature from January 1993 to December 1995 (supplied by Meteorological Office) around Whitby. The temperatures give an indication of the temperatures *C.maenas* or *C.pagurus* might be exposed to if caught emersed between tides.

Figure 5.2A: Changes in resting potential in summer and winter acclimatized *Carcinus meanas*. Summer and winter acclimatized RP change with temperature are shown, the data is presented as mean±S.E. mean; numbers of experiments were, CMwin n= 10, CMsum n=11. Statistical analysis (Student's t-test) determined some significant differences at higher temperatures between summer and winter crabs, denoted as asterisks on the figure. Equations for straight line plots shown in the figure are shown below. However, equations for a potential discontinuity are shown for CMwin, although this was not pursued.

CMsum: y=-73.9-1.0656x R= 0.986

CMwin y=-74.1-0.83x R= 0.846

Or: at 6-17°C; y=-66.6-1.595x R= 0.9954

at 17-25°C; y=-92.9-0.0531x R= 0.0448.

Figure 5.2B: Changes in resting potential in summer and winter acclimatized *Cancer pagurus*. Changes in RP with temperature in summer and winter *C.pagurus* are shown. Data is presented as mean±S.E. mean where error bars are shown for CPwin only. Statistical analysis determined no significant differences between the seasonal groups; numbers of experiments were, CPwin n=9, CPsum=11.

CPsum; y=-66.7-1.11x R= 0.988

CPwin; y=-66.92-1.08x R= 0.966.

Figure 5.2A: Changes in resting potential in summer and winter acclimatized Carcinus meanas.

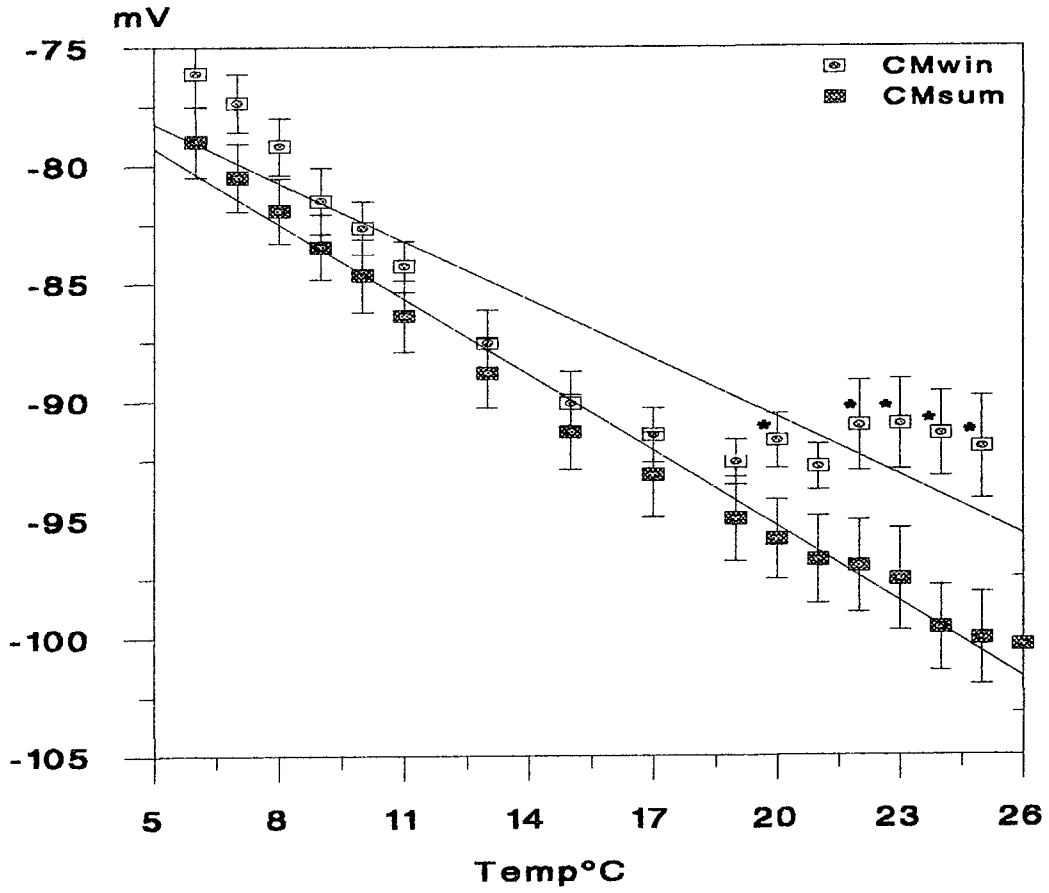
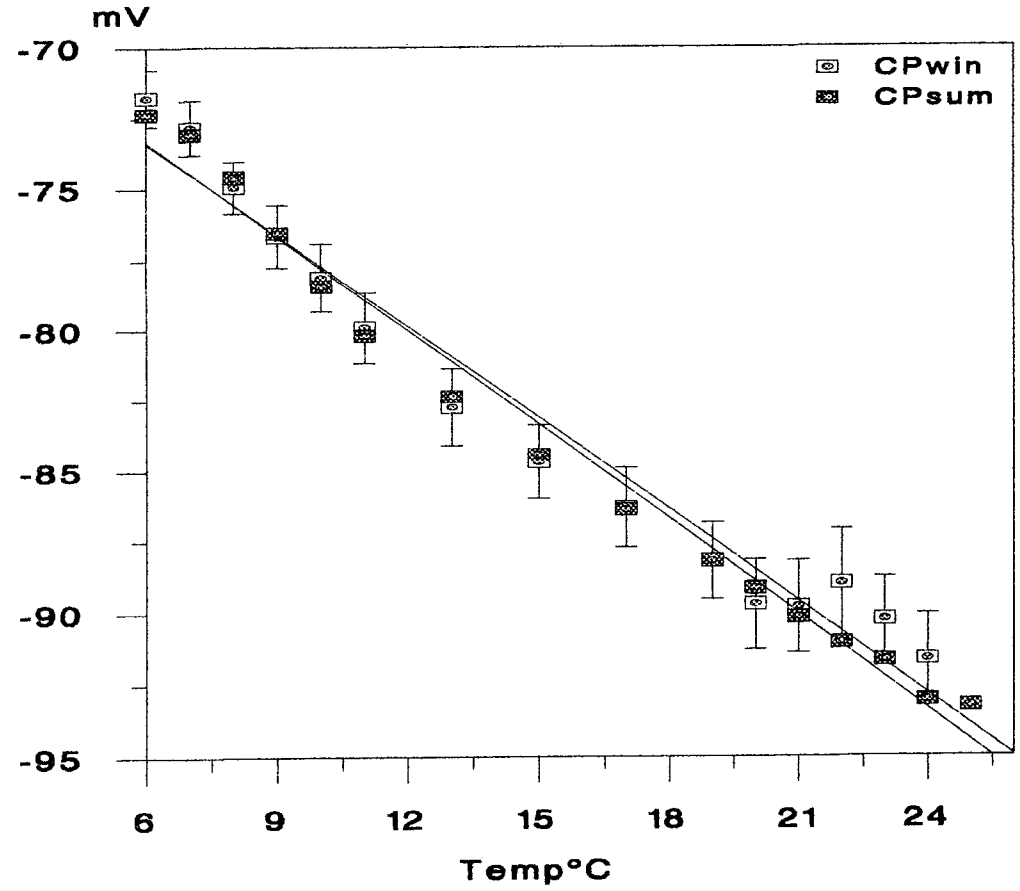


Figure 5.2B: Changes in resting potential in summer and winter acclimatized Cancer pagurus.



Resting Potential.

Figure 5.2A shows the RP change with temperature for summer and winter acclimatized *C.maenas*. It can be seen that the RP hyperpolarised with increasing experimental temperature, and that there was no seasonal acclimatory shift between summer and winter data sets. However, statistical analysis (Student's t-test) of the data points did determine significant differences between summer and winter *C.maenas* above 20°C. The RP of winter animals at these temperatures was significantly more depolarised than the RP of summer animals. The change in RP with temperature was significantly greater than that predicted by Nernst, the relationship for summer acclimatized *C.maenas* RP change with temperature was 1.07mV/°C where winter acclimatized *C.maenas* was 0.83mV/°C over the whole experimental temperature range. However, considering in detail the RP temperature relationship of winter *C.maenas,* a discontinuity was apparent, the RP change with temperature was 1.6mV/°C over 6-17°C and 0.0531mV/°C over 17-25°C. These values indicate RP in winter crabs was more temperature sensitive over the lower temperature range as the RP change with temperature was marked, but at temperatures warmer than 17°C the winter curve exhibited thermal insensitivity as the change with temperature was notably less than that predicted by Nernst. The decreased thermal sensitivity of RP at warmer experimental temperatures in winter crabs may indicate lability to warmer temperatures for the cold acclimatized winter animals.

Figure 5.2B shows RP changes with temperature in summer and winter acclimatized *C.pagurus*. It was seen that the summer and winter results were not significantly different (Student's t-test). The RP change with temperature was 1.11mV/°C for summer *C.pagurus* and 1.08mV/°C for winter *C.pagurus*, both seasons RP change with temperature was greater than that predicted by Nernst and were not significantly different.

Latent period.

Data on latent periods for preparations from summer and winter acclimatized animals of both species were successfully fitted with exponential curves with correlation coefficients greater than 0.99, these are shown in Figures 5.3A and 5.3B.

Figure 5.3A shows latent periods of summer and winter acclimatized *C.maenas* over the experimental temperature range. Both summer and winter acclimatized latent periods decreased with increasing experimental temperatures.

Figure 5.3A: Latent period to first EJP in summer and winter acclimatized *Carcinus meanas*. The data is presented as mean±S.E. mean; numbers of experiments were, CMwin n=10, CMsum n=11. Statistical analysis (Student's t-test) determined significant differences between almost all summer and winter seasonal comparisons, significant differences are denoted by asterisks. Equations for exponential (least squares fit) plots are shown below.

CMsum: y=18.2*10(-0.0266x) R= 0.995

CMwin: y=25.6*10(-0.0261x) R= 0.996.

Figure 5.3B: Latent period to first EJP in summer and winter acclimatized *Cancer pagurus*. Data is presented as mean±S.E. mean; numbers of experiments were, CPwin n=9, CPsum n=11. Statistical analysis determined significant differences between summer and winter seasonal groups over the whole experimental temperature range, significant differences are denoted by asterisks. equation foe exponential curves (least squares fit) are shown below.

CPwin: y=31.95*10(-0.0261x) R= 0.993

CPsum: y=17.4*10(-0.0288x) R= 0.9963.

Figure 5.3A: Latent period to first EJP in summer and winter acclimatized Carcinus meanas.

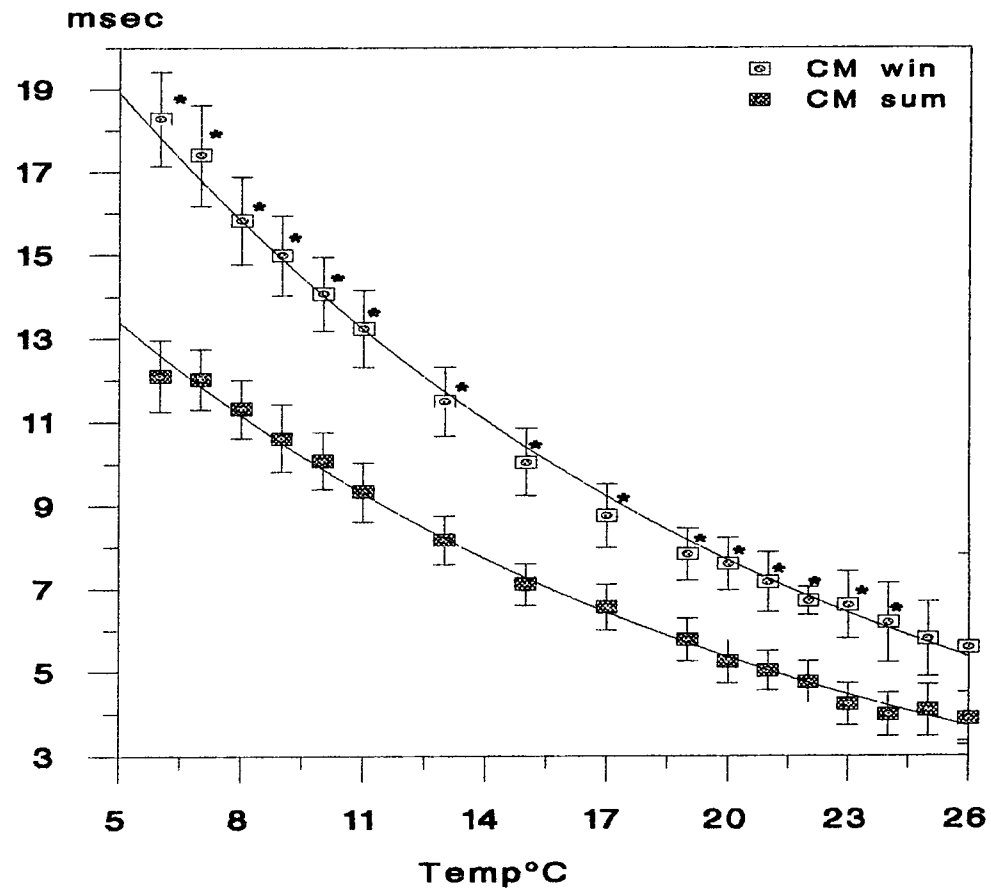


Figure 5.3B: Latent period to first EJP in summer and winter acclimatized Cancer pagurus.

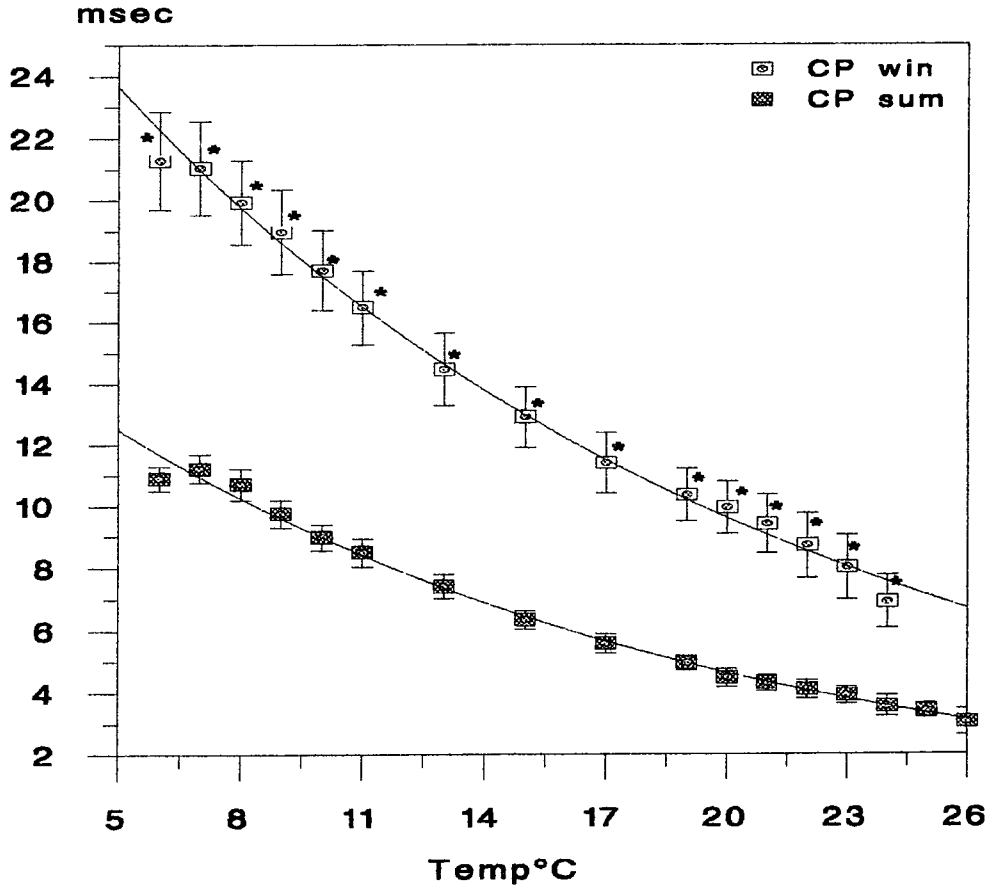


Figure 5.4A: Single pulse stimulated EJP amplitude in summer and winter acclimatized *Carcinus meanas*. Single EJP amplitudes (corrected for non-linear summation) are shown for summer and winter acclimatized *C.maenas*. Mean data is presented as mean±S.E. mean, where error bars are shown for CMsum only; numbers of experiments were, CMwin n=10, CMsum n=11. Statistical analysis (Student's t-test) determined no significant differences between seasonal groups, data points were joined by interpolation.

Figure 5.4B: Single pulse stimulated EJP amplitudes in summer and winter acclimatized *Cancer pagurus*. Single pulse EJP amplitudes are shown for summer and winter *C.pagurus*, where the data is presented as mean±S.E. mean; numbers of experiments were, CPwin n=9, CPsum n=11. Statistical analysis determined some significant differences between summer and winter seasonal groups denoted as asterisks on the figure. Data points were joined by interpolation.

Figure 5.4A: Single pulse stimulated EJP amplitude in summer and winter acclimatized Carcinus maenas.

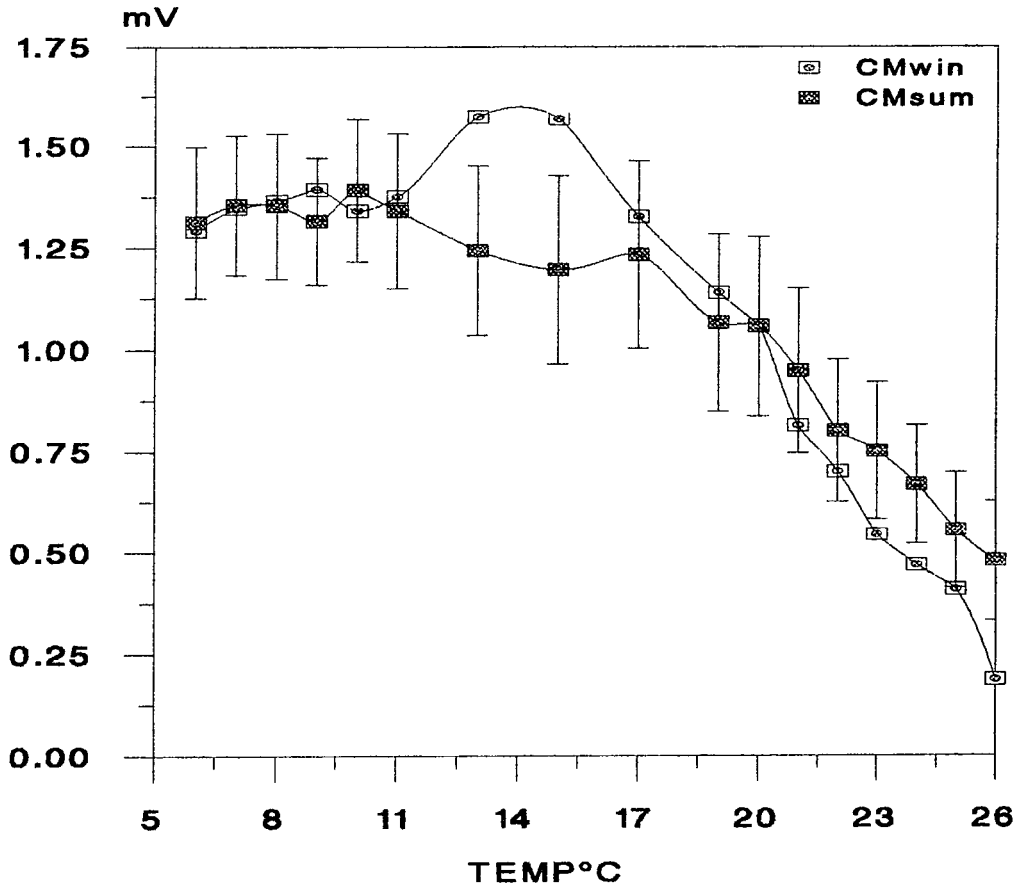
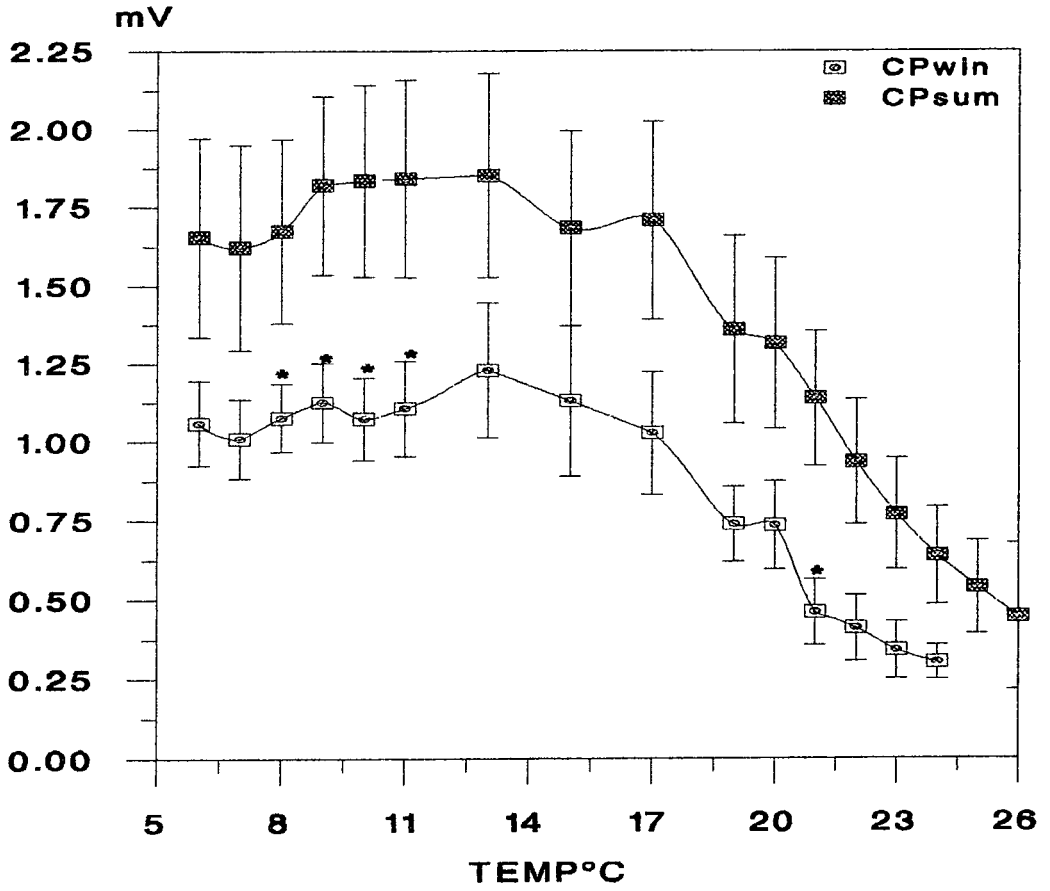


Figure 5.4B: Single pulse stimulated EJP amplitude in summer and winter acclimatized Cancer pagurus.



Statistical analysis (Student's t-test) determined significant differences between seasonal groups over 6-24°C of the experimental temperature range; thus, summer crabs had significantly shorter latent periods than winter animals over the whole experimental temperature range.

Figure 5.3B shows latent periods of summer and winter acclimatized *C.pagurus*, the latencies were similar to those of *C.maenas* in that latent periods of summer crabs were significantly shorter than those of winter animals over 6-24°C. It is notable that for winter acclimatized *C.pagurus* no results could be obtained at temperatures warmer than 24°C, which indicated increased thermolability. Similar sized crabs of both species were used in summer and winter.

Single pulse stimulated EJP amplitude.

The change in single EJP amplitudes with experimental temperature for *C.maenas* are shown on Figure 5.4A. The single EJP amplitudes of summer and winter acclimatized *C.maenas* overlapped. The EJP amplitudes for both seasons were generally maintained up to 17°C but decreased rapidly at temperatures warmer than 17°C. No significant differences were determined between summer and winter group comparisons (Student's t-test).

Figure 5.4B shows the changes in single EJP amplitudes of summer and winter acclimatized *C.pagurus*. Summer acclimatized animals generated larger EJP's over the whole temperature range, significantly so at 8-11 and 21°C when compared to winter acclimatized *C.pagurus*. It was noted that summer and winter acclimatized single EJP amplitudes were maximal around 6-15°C, but decreased rapidly at experimental temperatures warmer than 17°C. Comparison of mean temperatures at which maximal EJP amplitudes were initiated during individual experiments are shown in Table 5.3.

Table 5.1 shows single EJP amplitudes at low (8±1°C) and high (22±1°C) experimental temperatures for both species and seasons. The amplitudes generated at experimental temperatures of 8±1°C and 22±1°C were significantly different (Student's t-test) when compared within species and seasonal groups. This indicated that both species could not maintain their EJP amplitudes to warmer experimental temperatures. It was noted that the decrease in amplitude at 22±1°C was greater for *C.pagurus* than *C.maenas*. Comparison of the single EJP amplitudes generated by summer and winter acclimatized animals at the same experimental temperatures revealed no seasonal differences for *C.maenas*, but did reveal *C.pagurus* as generating significantly larger amplitudes in summer than in winter.

Figure 5.5A: Facilitation in summer and winter acclimatized *Carcinus meanas*. Facilitation is shown for summer and winter *C.maenas*, where mean data is presented and no error bars are shown; numbers of experiments were, CMwin n=10, CMsumn=11. Statistical analysis (Student's t-test) determined no significant differences between seasonal groups. Data points were joined by interpolation.

Figure 5.5B: Facilitation in summer and winter acclimatized *Cancer pagurus*. Facilitation values calculated for summer and winter *C.pagurus* are shown. Mean data is presented where no error bars are shown; numbers of experiments were, CPwin n=9, CPsum n=11. Statistical analysis determined no significant differences between seasonal groups. Data points were joined by interpolation.

Figure 5.5A: Facilitation in summer and winter acclimatized Carcinus maenas.

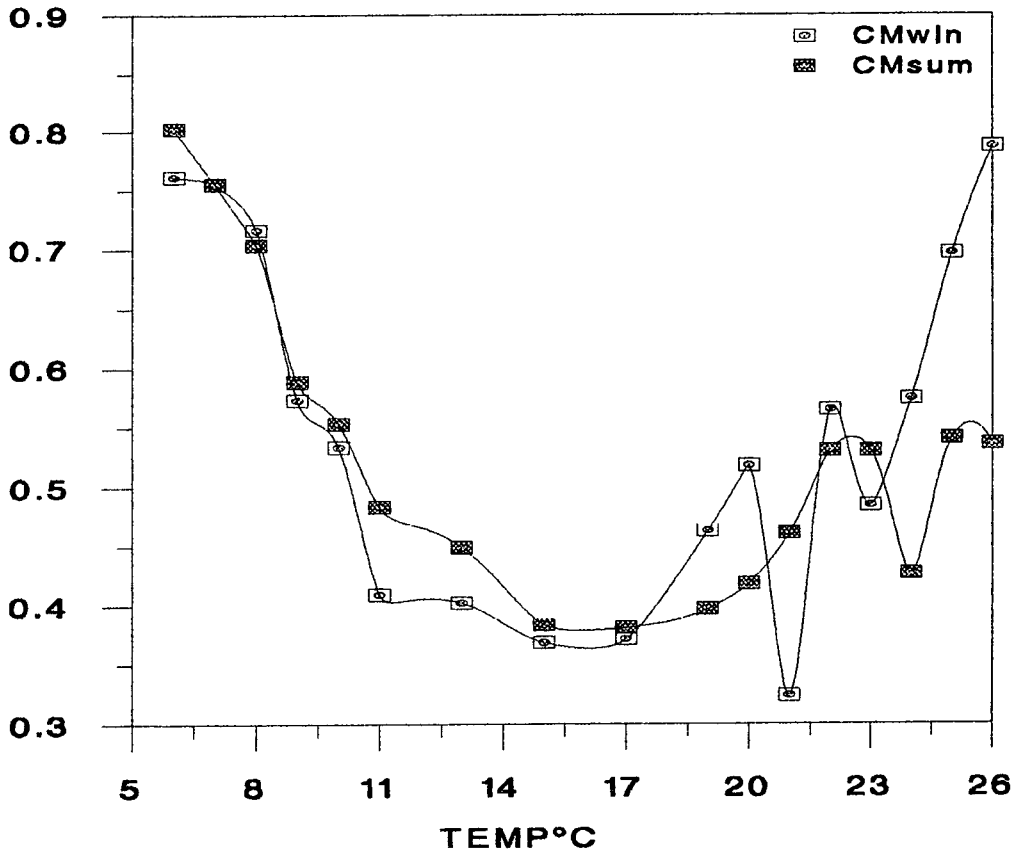


Figure 5.5B: Facilitation in summer and winter acclimatized Cancer pagurus.

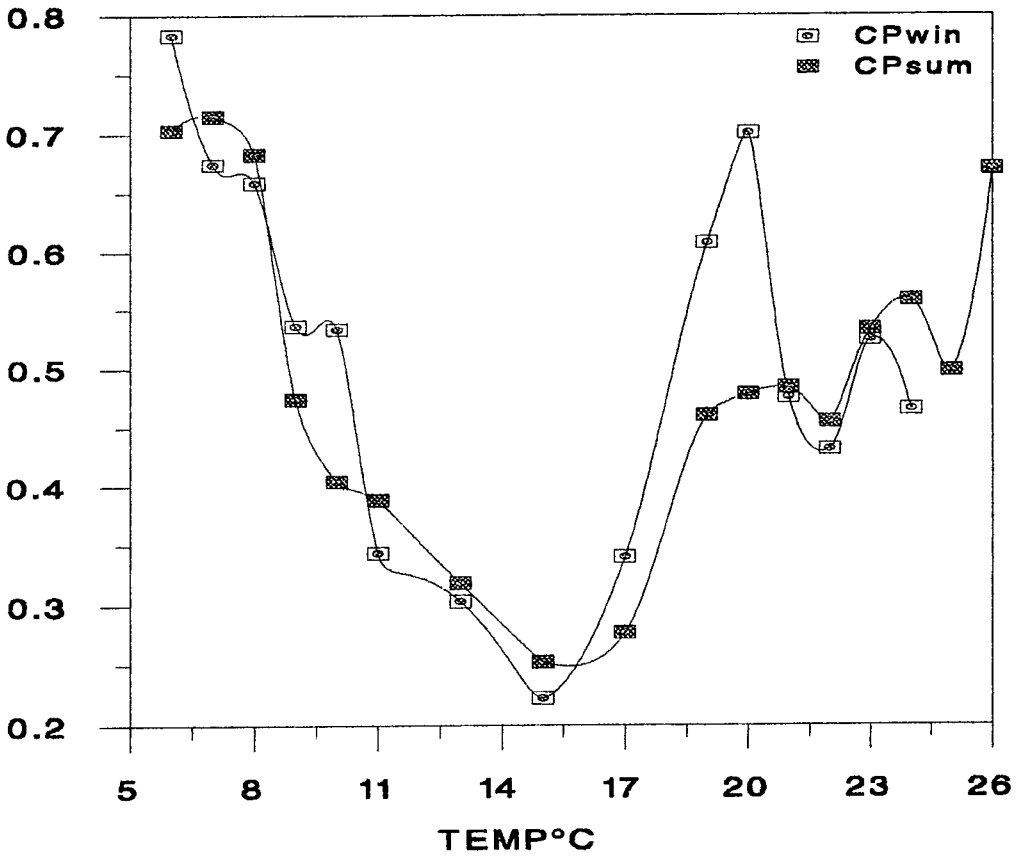


Table 5.1: Comparison of single pulse stimulated EJP amplitudes (Mean±S.E. mean) measured at low (8±1°C) and high (22±1°C) temperatures in summer and winter acclimatized *C.maenas* and *C.pagurus*.

	C.maenas		*C.pagurus*	
Experimental Temp (°C).	Winter acclimatized.	Summer acclimatized.	Winter acclimatized.	Summer acclimatized.
8±1	1.37±0.139 (n=30)	1.34±0.09 (n=33)	1.07±0.064 (n=27)	1.71±0.16 (n=33)
22±1	0.7±0.065 (n=24)	0.84±0.099 (n=32)	0.41±0.052 (n=22)	0.95±0.619 (n=32)

Facilitation.

Facilitation shown in Figures 5.5A for *C.maenas* and 5.5B for *C.pagurus* were similar irrespective of season or species. The facilitation versus temperature curves were u-shaped for both species and seasons. Facilitation for summer and winter acclimatized crabs of both species exhibited greatest facilitation around 6-7°C which decreased with increasing temperature to minimal values around 15°C, facilitation then increased with increasing experimental temperatures to equally as large values at temperatures warmer than 20°C. Statistical analysis (Student's t-test) determined no significant differences between summer and winter comparisons for both species.

Double pulse stimulated EJP amplitude.

Double EJP amplitude changes with temperature are shown in Figure 5.6A for *C.maenas* and 5.6B for *C.pagurus*. Both *C.maenas* and *C.pagurus* revealed decreasing EJP amplitudes with increasing experimental temperature, irrespective of summer or winter acclimatization. The EJP amplitude versus temperature curves from summer and winter acclimatized *C.maenas* overlapped. Whereas EJP amplitudes from summer acclimatized *C.pagurus* were larger than EJP amplitudes from winter *C.pagurus* over the whole experimental temperature range. However, comparisons (Student's t-test) of summer and winter acclimatized EJP amplitudes at the same experimental temperatures determined no significant differences for either species.

Figure 5.6A Double pulse stimulated EJP amplitude in summer and winter acclimatized *Carcinus maenas*. Double pulse EJP amplitudes are shown for summer and winter acclimatized *C.maenas*, where the data presented was mean±S.E. mean and error bars are shown for CMsum only; numbers of experiments were, CMsum n=11, CMwin n=10. The amplitudes were corrected for non-linear summation and statistical analysis (Student's t-test) determined no significant differences between seasonal groups. Data points were joined by interpolation.

Figure 5.6B: Double pulse stimulated EJP amplitudes in summer and winter acclimatized *Cancer pagurus*. Double pulse EJP amplitudes are shown for summer and winter acclimatized *C.pagurus*, where data is presented as mean±S.E. mean; numbers of experiments were, CPwin n=9, CPsum n=11. Statistical analysis determined no significant differences in EJP amplitude between seasonal groups. Data points were joined by interpolation.

Figure 5.6A: Double pulse stimulated EJP amplitude in summer and winter acclimatized Carcinus meanas.

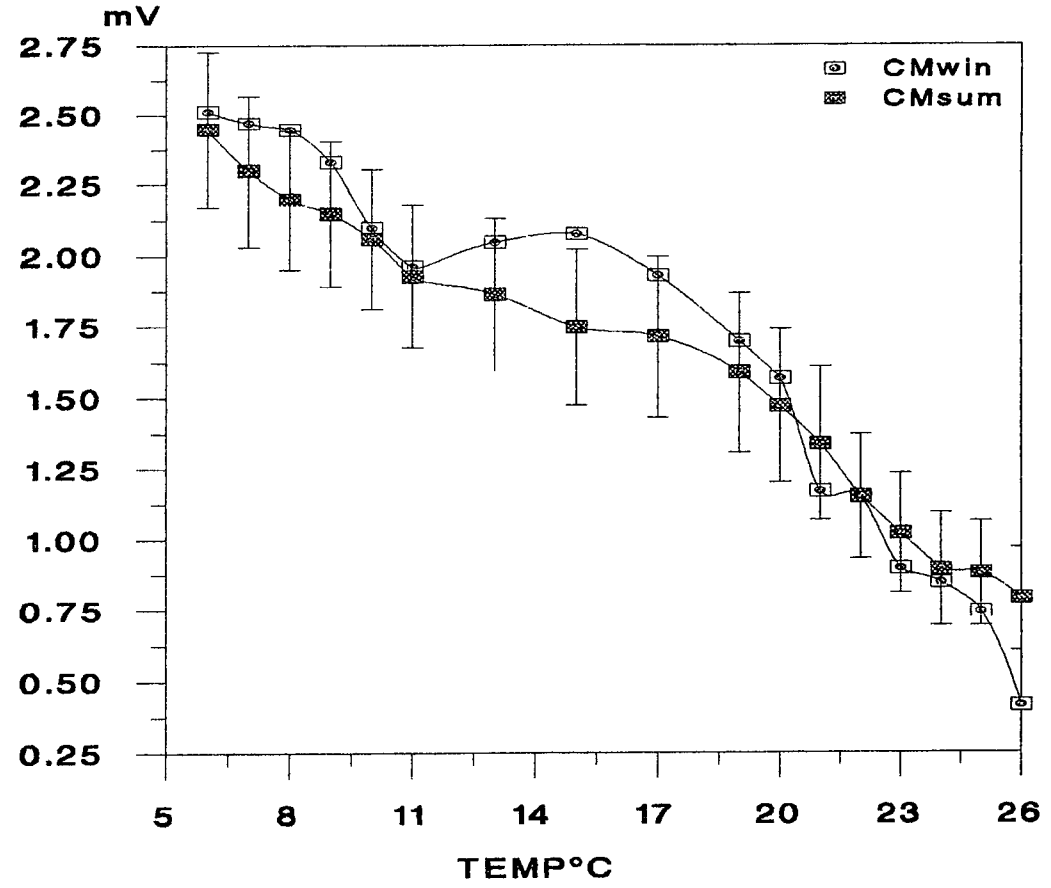


Figure 5.6B: Double pulse stimulated EJP amplitude in summer and winter acclimatized Cancer pagurus.

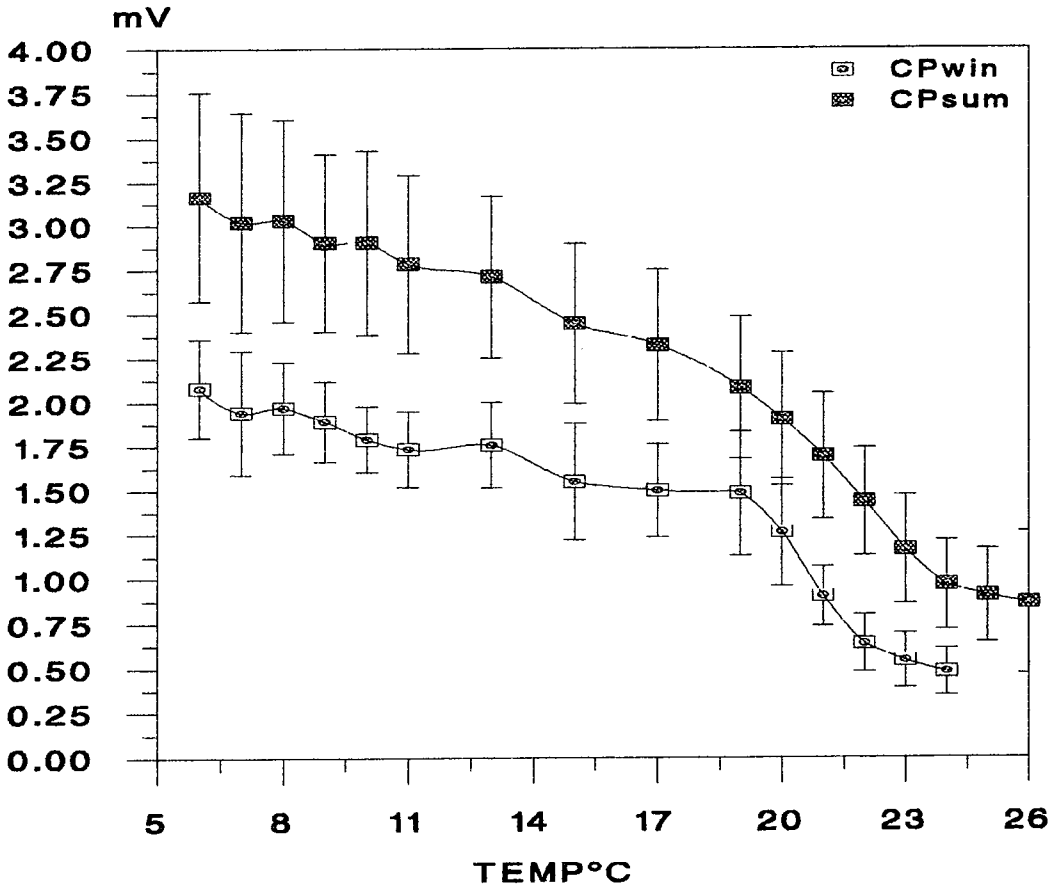


Table 5.2: Comparison of double pulse stimulated EJP amplitudes (Mean±S.E. mean) measured at low (8±1°C) and high (22±1°C) temperatures in summer and winter acclimatized *C.maenas* and *C.pagurus*.

	C.maenas		*C.pagurus*	
Experimental Temp (°C).	Winter acclimatized.	Summer acclimatized.	Winter acclimatized.	Summer acclimatized.
8±1	2.4±0.244 (n=30)	2.22±0.138 (n=33)	1.93±0.127 (n=27)	2.99±0.304 (n=33)
22±1	1.08±0.122 (n=22)	1.18±0.128 (n=32)	0.705±0.089 (n=22)	1.44±0.18 (n=32)

The EJP amplitudes at low (8±1°C) and high (22±1°C) experimental temperatures for summer and winter acclimatized *C.maenas* and *C.pagurus* are shown in Table 5.2. Statistical analysis of amplitudes generated at low to high temperatures (Student's t-test) within seasonal groups identified a significant decrease in EJP amplitude at high experimental temperatures for both species and seasonal groups. The EJP amplitudes generated by summer and winter acclimatized *C.maenas* at 8±1°C were not significantly different (Student's t-test), where *C.pagurus'* were. Furthermore the EJP amplitudes generated by summer and winter acclimatized *C.maenas* at 22±1°C were not significantly different where *C.pagurus'* were.

The difference between the EJP amplitudes of summer and winter acclimatized *C.pagurus* was larger than that of equivalent summer and winter acclimatized *C.maenas,* indicating *C.pagurus'* increased thermal sensitivity.

Temperatures at which maximal EJPs were initiated.

Table 5.3 shows the experimental temperatures at which maximal EJP amplitudes were generated for summer and winter acclimatized *C.maenas* and *C.pagurus*. *C.maenas* generated maximal EJP amplitudes at temperatures colder than 11°C, and *C.pagurus* generated maximal amplitudes at temperatures colder than 13°C. Statistical analysis (Student's t-test) of the temperatures at which maximal EJP amplitudes were generated for *C.maenas* and *C.pagurus* revealed no significant differences between seasonal pair comparisons. This indicated summer animals maintained maximal muscle function at colder temperatures than may be expected, especially for the comparatively eurythermic *C.maenas*.

Figure 5.7A: Single EJP decay time constant in summer and winter acclimatized *Carcinus meanas*. Single EJP decay time constants are shown for summer and winter acclimatized *C.maenas*, the data presented is mean±S.E. mean; numbers of experiments were, CMwin n=10, CMsum n=11. Statistical analysis (student t-test) determined significant differences between almost all summer and winter data point seasonal comparisons, significant differences are denoted by asterisks on the figure. All data was fitted with exponential curves (least squares fit), the equations of which are shown below.

CMwin: y=74.66*10(-0.0226x) R= 0.937

CMsum: y=34.852*10(-0.0186x) R= 0.949.

Figure 5.7B: Single EJP decay time constants in summer and winter acclimatized *Cancer pagurus*. Single EJP decay time constants are shown for summer and winter *C.pagurus*, where the data is presented as mean±S.E. mean; numbers of experiments were, CPwin n=9, CPsum n=11. Statistical analysis determined a significant difference at 6°C only which is denoted by an asterisk. All data points were fitted with exponential curves (least squares fit), the equations of which are shown below.

CPwin: y=69.03*10(-0.0217x) R= 0.897

CPsum: y=53.23*10(-0.0193x) R= 0.881.

Figure 5.7A: Single EJP decay time constant in summer and winter acclimatized Carcinus maenas.

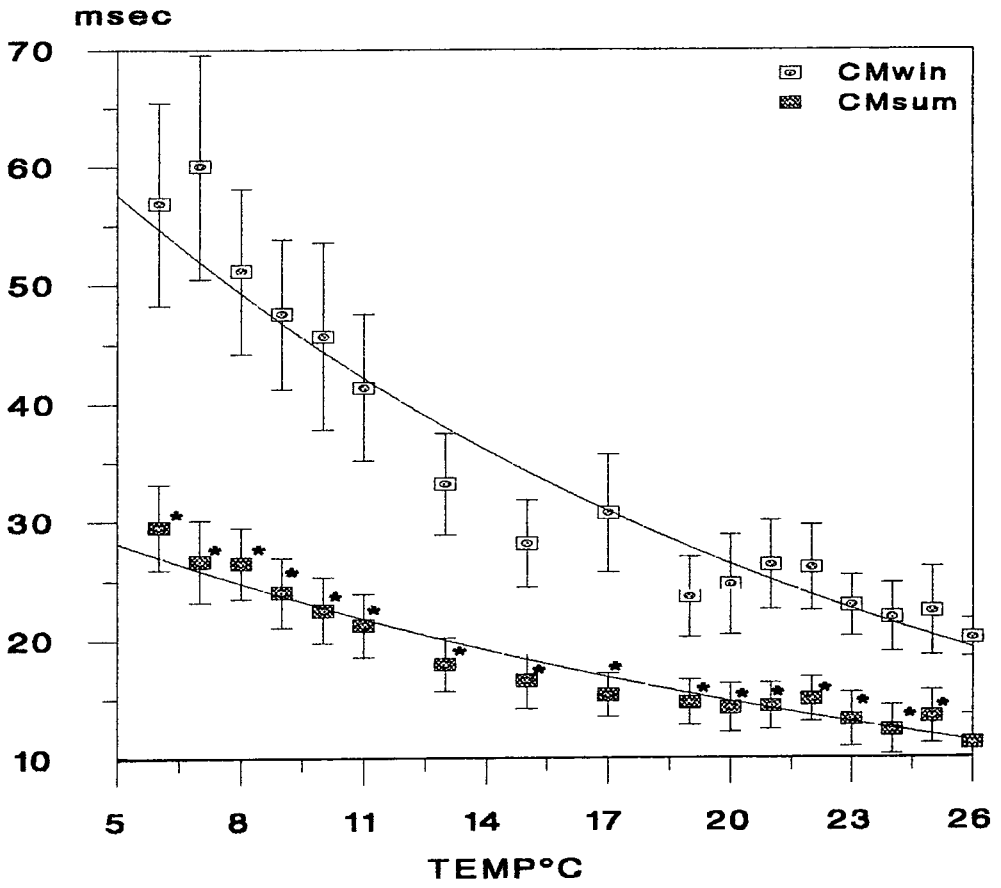


Figure 5.7B: Single EJP decay time constant in summer and winter acclimatized Cancer pagurus.

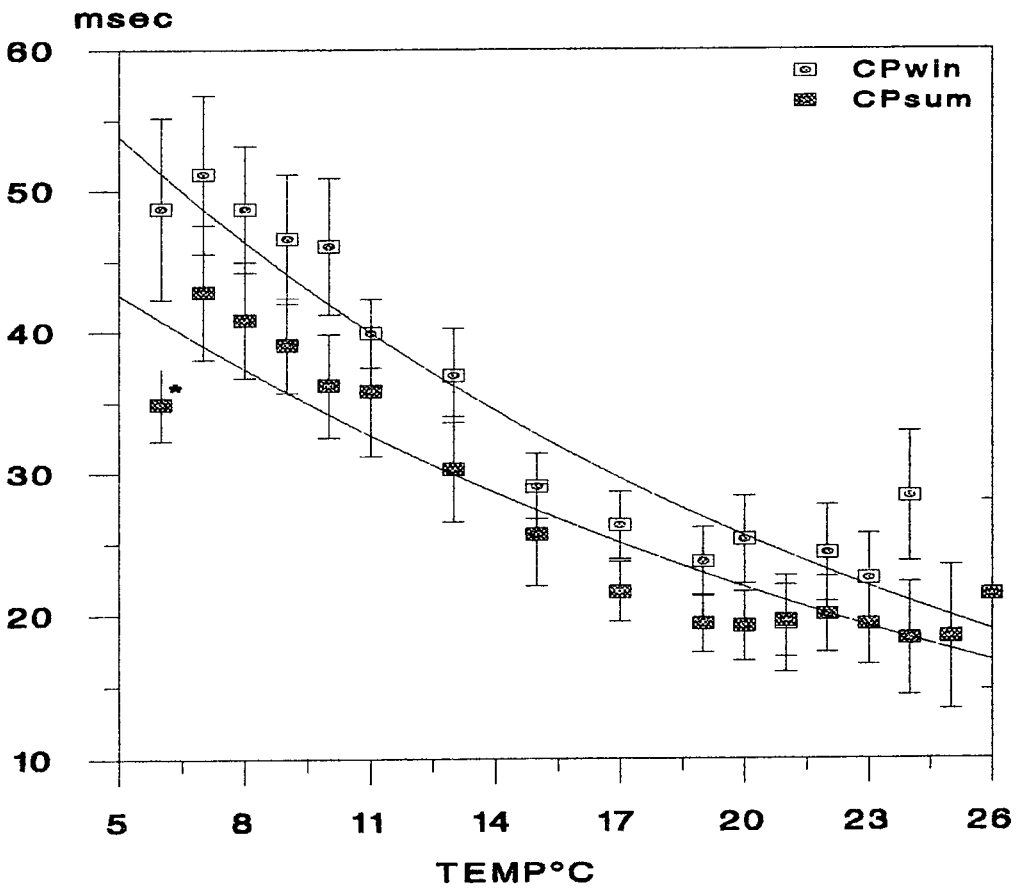


Table 5.3: Comparison of experimental temperatures (Mean±S.E.mean) at which maximal double and single pulse stimulated EJP amplitudes were recorded from summer and winter acclimatized *C.maenas* and *C.pagurus*.

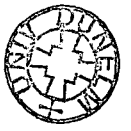
Temperature at which maximal EJP amplitudes were initiated °C.		
Animal.	Double EJP amplitude.	Single EJP amplitude.
Summer *C.maenas*	6.5±0.23 (n=12)	9.833±1.34 (n=12)
Winter *C.maenas*	7.09±0.667 (n=11)	10.4±1.14 (n=10)
Summer *C.pagurus*	8.18±0.971 (n=11)	12.45±1.23 (n=11)
Winter *C.pagurus*	9.89±1.57 (n=9)	11±1.45 (n=9)

Double EJP amplitudes were generated at colder experimental temperatures (not significantly colder) than single EJP amplitudes for both species and seasons, this being due to the facilitative component included in double EJP amplitudes.

Single EJP decay time constant (Tau).

Figure 5.7A shows the changes in single pulse stimulated EJP decay time constants with experimental temperature for summer and winter acclimatized *C.maenas*. It was noted that Tau decreased with increasing temperature, the change in Tau over the whole experimental temperature range was approximately three fold for both summer and winter animals. However, summer *C.maenas* Tau values were significantly (Student's t-test) shorter than those of winter crabs over the whole experimental temperature range.

Figure 5.7B shows Tau changes with temperature for summer and winter acclimatized *C.pagurus*. *C.pagurus* Tau decreased with increasing experimental temperature, the relative change over the whole experimental temperature range was approximately 2.5 fold for both summer and winter acclimatized animals. Statistically there (Student's t-test) was a significant difference between summer and winter Tau at 6°C only, although it can be noted that Tau from summer animals was shorter than winter acclimatized animals over the whole experimental temperature range and was similar to the summer and winter *C.maenas* results.



Discussion.

Figure 5.2 shows that muscle membrane resting potential became hyperpolarised with increased measuring temperature for both species of crab. This also has been described for muscle membranes of other crustaceans, e.g. *Menippe mercenaria* and *Callinectes sapidus* (Blundon 1989). It is of interest that the slope of the RP temperature plot was significantly greater than the 0.316mV/°C predicted from the Nernst equation (Table 4.2). Other workers have reported a similar RP-temperature relationship, and have related the hyperpolarisation to the thermal activation of the electrogenic Na^+/K^+ ATPase (Prosser and Nelson 1981; White 1983; Kivivuori *et al.*, 1990), and also to changes in the Na^+/K^+ permeability ratio at different temperatures. An active electrogenic component to the membrane resting potential would explain the hyperpolarisation shown, and Table 4.2 reports that indeed the membrane potential of both species is ouabain sensitive. A functional consequence of the hyperpolarisation of RP with increased temperature would be reduced muscle fibre excitability.

In *C.pagurus* the RP-temperature plots of summer and winter caught animals were identical and there was no acclimatization effect. In *C.maenas* however the RP-temperature plot for winter caught crabs showed deviation from linearity at temperatures above 20°C. Indeed, the muscle membrane was significantly depolarised at these temperatures as compared to the membrane RP from summer caught crabs. This relative insensitivity of the membrane RP, at the equivalent of summer temperatures, suggests an acclimatization effect in the winter crabs. A further point of interest is that recordings of RP could not be obtained in winter caught *C.maenas* at 26°C and in *C.pagurus* above 24°C, whereas RPs were maintained at slightly higher temperatures in summer caught animals. Overall the data suggests that no compensation is made for RP with respect to season, this means that winter acclimatized muscle RP is significantly depolarised as compared to the summer condition. The consequence of this is to increase muscle excitability in winter months when physical activity and the capacity for sustained transmitter release are reduced (Lnenicka 1993).

Latency is a compound phenomenon involving axonal conduction time and synaptic delay, events that may have different temperature dependencies. Figure 5.3 shows this dependency has the same pattern for both species irrespective of whether they were winter or summer caught, although the dependency was more pronounced in winter caught crabs in both species. A marked seasonal effect was also seen for both species, where the latency determined was significantly longer for the winter

caught animals across the whole of the temperature range measured. It is of interest that Cuculescu (1996) has shown that axonal conduction is 30% faster in summer as compared with winter *C.pagurus* across the temperature range, whereas in *C.maenas* it is not different in winter and summer caught crabs. The longer latencies in winter caught *C.maenas* must therefore be a result of changes in synaptic delay or axon diameter, whereas the more pronounced seasonal difference found in *C.pagurus* could be due to the additive effects of both synaptic delay and axonal conduction times. In both species however, the effect of seasonal acclimatization on latency is non-compensatory for the direct effect of temperature.

The single EJP amplitudes recorded from *C.maenas* muscle showed that same temperature dependency in both winter and summer caught crabs, thus no seasonal acclimation was evident in this parameter. In both cases consistent single EJP amplitudes were obtained between about 1.1-1.5mV at temperatures between 6°C and 17°C, at higher temperatures amplitudes fell progressively to be between about 0.25 and 0.5mV. The single EJPs obtained from *C.pagurus* muscle showed a similar temperature dependency for amplitude as described for *C.maenas*, that is they gave consistent values between 6°C and 17°C, but declined to about 0.25-0.5mV at higher experimental temperatures. A clear seasonal effect in *C.pagurus* could be demonstrated. Once again the summer caught crab muscle yielded larger EJP amplitudes across the whole range of temperatures than muscle from winter caught animals, indeed the data obtained at some individual temperatures (8-11, 21°C) were significantly different. A decrease in the amplitude of a single EJP is related to a decrease in Tau. Tau decreases with increasing experimental temperature indicating the number of open channels is increasing which therefore reduces membrane resistance and reduces the EJP amplitudes (Nicholls *et al.*, 1992; Fatt and Katz 1953). The decrease in single EJP amplitude is interpreted as a reduction in neurotransmitter release in summer and winter caught *C.pagurus* as no differences in Tau were found although changes in transmitter processing and receptor sensitivity cannot be ruled out (Atwood *et al.*, 1994). Other factors which may effect transmitter release are axonal block which may occur at axon branch points (White 1983) and result in reduced transmitter exocytosis, or hyperpolarisation of muscle RP which reduces muscle excitability (Prosser and Nelson 1981; Hille 1992; Montgomery and MacDonald 1990).

In the case of *C.pagurus*, where seasonal differences in the temperature dependency of EJP amplitude occurred, it is possible that in summer animals there is an increased quantal release of neurotransmitter. White (1983) reported increasing temperature increased the quantal content and release of transmitter at crayfish motor terminals which was similarly reported by Montgomery and MacDonald (1990).

This being supported further by Lnenicka (1993) who reported summer crayfish as having a greater capacity for transmitter release. However, that may not be the only explanation because Cooper *et al.*, (1993) reported that differences in muscle fibre input resistance accounted for 25% of the recorded differences in EJP amplitude in a population of crayfish muscle fibres, which correlates here with seasonal changes in Tau for *C.maenas* but not *C.pagurus*.

Figure 5.5 shows that there were no differences in the extent of facilitation observed at different temperatures between summer and winter caught crabs of either species. However, facilitation was markedly temperature sensitive, the response curves obtained in all cases were u-shaped, with minimal values being obtained in the 10-17°C range. The decrease in facilitation observed between 6°C and 15°C may be associated with a fall in calcium sensitivity (Stevens and Godt 1990), where Hochachka *et al.*, (1988a) considered the increased facilitation that occurred at higher experimental temperatures was because of a failure in calcium buffering. Figures 5.4 and 5.6 show that EJP amplitudes are maintained at low environmental temperatures just as well as they are in the mid-temperature range, in both species, irrespective of whether the crabs were summer or winter caught. This suggests that facilitation plays an important role in EJP responses at low temperature but make little contribution to the maintenance of EJP amplitude in the mid-temperature range. At warmer experimental temperatures EJP amplitudes fell to minimal values for all groups, and although facilitation increased it was clearly incapable of maintaining EJP amplitude. It is possible that differential transmitter release accounts for some of the temperature dependent effects, because quantal content and it's release are reported to rise with increasing experimental temperature (Montgomery and MacDonald 1990). Other factors associated with neurotransmitter metabolism may also be involved in the temperature dependence, including clearance from the synaptic cleft (Otis *et al.*, 1996; Wolosker *et al.*, 1996b; Xu *et al.*, 1996), as well as changes in autoreceptor function which influence pre-synaptic events (Parnas *et al.*, 1994).

Single EJP decay time constants (Tau) for winter and summer caught crabs of both species (Figure 5.7) were fitted with exponential curves using a least squares fit. The value of Tau obtained was found to decrease by between two and three fold across the temperature range 6°C to 26°C. A clear seasonal effect is seen in *C.maenas* where Tau values from winter animals were significantly longer at all measuring temperatures. The same pattern was obtained from *C.pagurus* preparations but in this case the Tau values at individual temperatures were not significantly different, with the exception of values at 6°C. In *C.maenas* in particular, the increased values for Tau in muscle from winter animals would

contribute to the maintenance of EJP amplitudes, especially at the low prevailing seasonal temperatures. In the case of *C.pagurus* however, the lack of a significant seasonal effect on Tau strongly supports the view that the seasonal differences found in EJP amplitude must be accounted for by seasonal changes in transmitter release or receptor sensitivity. Lnenicka (1993) has reported a greater capacity for neurotransmitter release in crayfish in summer, and MacDonald (1990) also found Tau was dependent on membrane resistance and capacitance (Nicholls *et al.*, 1992). The clear dependency of Tau on season in *C.maenas* indicated that the muscle membrane must change in a way that increased Tau in winter. MacDonald (1990) reported that capacitance increased slightly with increased membrane unsaturation. Increased membrane unsaturation is a commonly reported phenomenon in cold acclimation in ectothermal animals (Cossins and Bowler 1987), and so modulation of membrane lipid structure may be a factor in seasonal acclimatization in *C.maenas* but not in *C.pagurus*. It is also recognised that seasonal activity levels alter muscle phenotypes in some crustaceans, this has been demonstrated in crayfish where a phasically innervated muscle in winter became more tonic like in summer (Lnenicka 1993; Atwood and Nguyen 1995). Thus, the maintenance of similar EJP amplitudes between summer and winter *C.maenas*, principally as a consequence of a change in Tau, identified a functional acclimatization effect. In *C.pagurus*, on the other hand, an inverse effect of acclimatization on EJP amplitude was found, where EJPs for summer animals were significantly greater than for winter animals and so no compensation for seasonal temperatures was evident. It is also significant that muscle excitability was lost above 24°C in winter *C.pagurus* but in summer animals it was maintained to 26°C the highest experimental temperature used. No high temperature block on muscle excitability was found in *C.maenas* preparations.

The data in Chapter Four clearly shows acclimation temperature compensation of RP and EJP amplitude so as to maintain muscle function around the acclimation temperature. Seasonally induced compensation was reported in this chapter for *C.maenas* but not *C.pagurus*, the seasonal changes are influenced by temperature and probably hormonal factors. *C.pagurus* is probably exposed to a narrower seasonal temperature range, it is possible that *C.pagurus* is able to maintain sufficient appropriate muscle function in winter at a time of year where physical activity is reduced (Taylor and Wheatly 1979) without the need of metabolically expensive adaptation. Cuculescu (1996) reported changes in fatty acid composition of membrane lipids from seasonally acclimatized crabs. Spring crabs had more saturated fatty acids and less polyunsaturated fatty acids in their plasma membranes than autumn crabs, when measured after a period of laboratory acclimation. Cuculescu (1996) also reported that membrane order of summer crabs at

temperatures warmer than 15-20°C was more ordered than those of spring and autumn crabs, which suggested seasonal adaptation would enable summer crabs to cope with rapid increases in temperature.

Chapter Six.

Heterothermal Acclimation.

Introduction.

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). These changes have been described both at the level of the whole organism and also at the individual tissue level. To take the special case of skeletal muscle, in fish Ushio and Watabe (1993) reported increased Ca^{2+}ATPase and Ca^{2+} uptake activities in cold acclimated carp muscle sarcoplasmic reticulum, Guderley and Johnston (1996) reported cold acclimation doubled the activity of pyruvate oxidation when investigating sculpin muscle mitochondria, Sidell (1980) reported a decrease in goldfish red muscle tissue, and an increase of myofibrillar ATPase activity with cold acclimation. This is supported by Heap *et al.*, (1985) who reported increased myofibrillar ATPase activity at cold acclimation temperatures when compared to the myofibrillar ATPase activity of warm acclimated carp, tench and roach. Egginton and Sidell (1989) reported an increase in mitochondria volume and proliferation of cellular organelles in striped bass muscle when acclimating from 25°C to 5°C. Atwood and Nguyen (1995) reported neuronal changes in crayfish which changed the muscle phenotype, such changes have been related to seasonal changes in temperature and activity by Lnenicka and Zhao (1991). Layne *et al.*, (1987) reported changes in crayfish critical thermal maxima (CTMax) and minima dependent on the season and acclimation temperature, summer crayfish had higher CTMax irrespective of cold or warm acclimation compared to winter crayfish.

The above work simply allowed comparisons to be made between differently acclimated individuals, providing information primarily on a variety of measurable physiological and biochemical parameters that were changed by acclimation. Such experimental approaches have also been used to make comparisons between species with respect to their eurythermicity (Blundon 1989; Lahdes *et al.*, 1993).

Heterothermal acclimation, the condition where regions of the animal are held at different temperatures simultaneously (Fahmy 1972; 1973), has been used as an experimental protocol to determine to what extent acclimation is controlled by central systems (hormonal and CNS). This approach has been adopted in this study. Crabs were heterothermally acclimated using an off-centre positioned membrane so that one side and the CNS were influenced by one temperature whilst the contralateral side was held at a different temperature of acclimation (see Figure 6.0). Thus if a governing CNS

influence was present the contralateral side, as described above, would not show acclimation responses consistent with its local thermal experience, but its responses would be consistent with the temperature experienced by the CNS. Prosser *et al.*, (1991) have reported a central hormonal factor that overrode local thermal effects in catfish hepatocytes. Fahmy (1973) was unable to rule out a governing CNS influence from his work on trout, but concluded no hormonal influence effected trout CTMax responses. In contrast Silverthorn (1975) reported an apparent hormonal influence on acclimation status of respiration in fiddler crabs (*Uca pugilator*). He reported eye-stalk extracts from 25°C acclimated crabs decreased respiration when injected into 15°C acclimated crabs, whereas eye-stalk extracts from 15°C acclimated crabs significantly increased respiration in 25°C acclimated crabs. This was interpreted as the response to two antagonistically acting hormones on crab metabolic rates. Lagerspetz (1974) has also reported that different levels of spinal cord tonic discharges caused enzymatic changes which persisted in isolated muscles, and consequently that metabolic compensation during temperature acclimation may result indirectly from adaptive changes in the nervous system.

The data presented in Chapter Four have demonstrated clear acclimation responses to a variety of physiological parameters in the leg neuromuscular system of both species of crab. Acclimation temperature induced changes were found for resting potential, single and double pulse stimulated EJP amplitude, all of which were shifted so as to maintain appropriate muscle function at the different acclimation temperatures. In Chapter Five seasonal differences in the same physiological factors were studied. Seasonal acclimatization was most clearly seen for *C.maenas* as longer Tau in winter crabs so as to maintain muscle depolarisation, *C.pagurus* showed little seasonal acclimatization. The seasonal acclimatization and temperature acclimation responses were different, temperature acclimation of both species of crab induced no compensatory changes in Tau. The question arises what controls acclimation, temperature or hormonal influences?

In this chapter *Carcinus maenas* and *Cancer pagurus* were heterothermally acclimated to assess more definitively the effect of any potential neural or humoral factor on acclimation. The test strategy used was to identify whether the CNS acclimation temperature was important in influencing contralateral walking leg acclimation. Comparison of neurophysiological parameters such as latency, facilitation, membrane potential etc. were measured and tested for significant (one way ANOVA) differences between the different heterothermal acclimatory groups. The experimental protocols chosen will also permit comparisons to be made between the two species. This is pertinent in view of the generally held view that *C.pagurus* is relatively stenothermal as compared with *C.maenas*. Heterothermal acclimation may highlight

Figure 6.0: Heterothermal acclimation apparatus.

A= Apparatus insulation,
B= Water cooler set to 8°C,
C= Water heater set to 22°C,
D= Rubber partitioning diaphragm,
E= Acclimating crab (*Carcinus maenas*).

Pictured is a heterothermally acclimating crab (*C.maenas*), the crab is acclimating with most of it's body including it's CNS oriented within the inner warm compartment. Therefore this acclimation type nomenclature was **H**ot Central **N**ervous System with **H**ot **L**egs or HN/HL for walking legs collected from within the inner compartment, and the contralateral heterothermal acclimation was HN/CL for walking legs collected from the outer cold compartment.

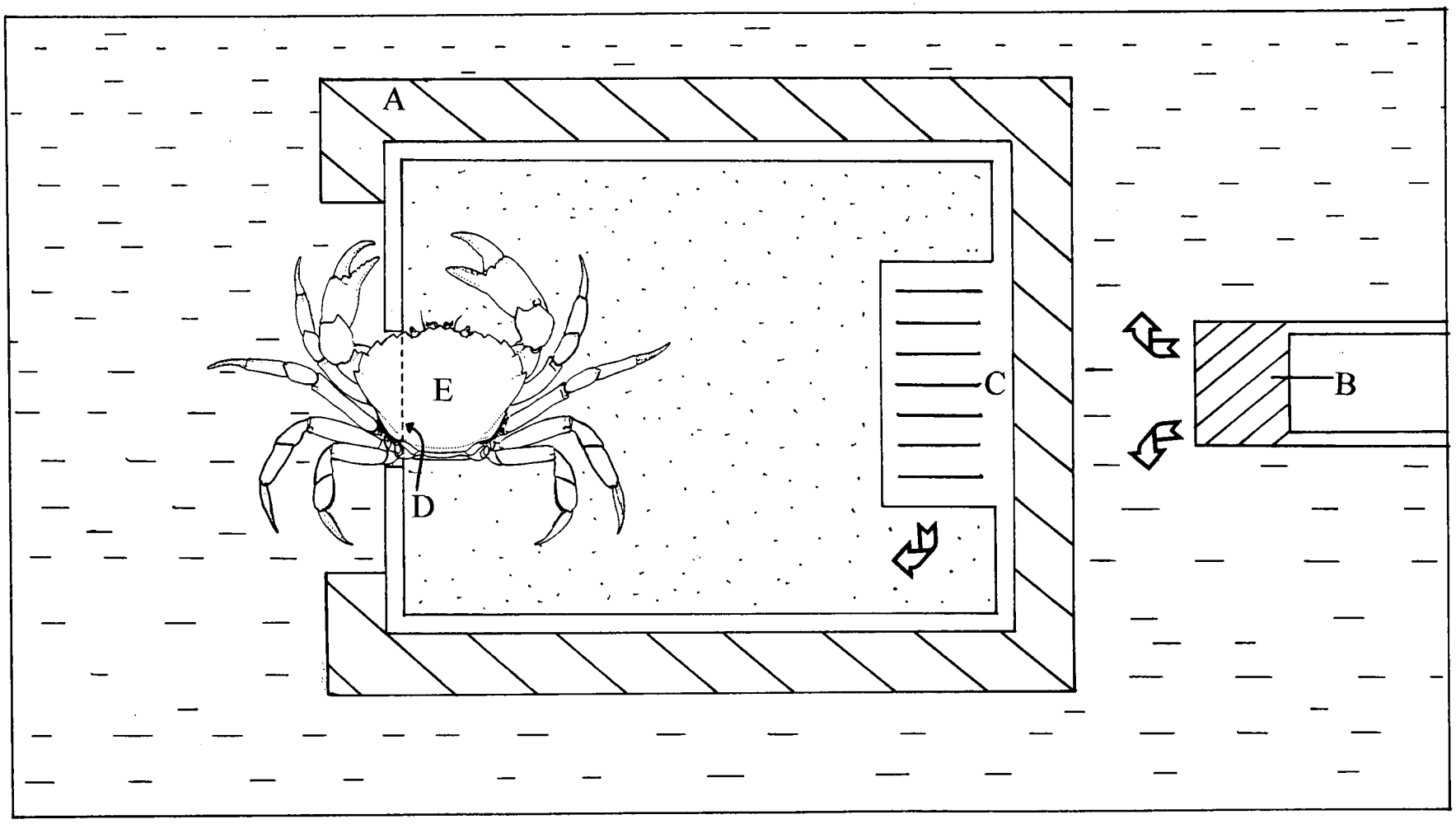


Figure 6.0

differences between the two species of crab in-light of the stenothermal and eurythermal titles.

Method.

Heterothermal acclimation.

The acclimation temperatures selected for heterothermal acclimation were the same as those used in the control homothermal free (Stephens 1985a; Florey and Hoyle 1976) and immobilised acclimation temperature experiments i.e. 8°C and 22°C.

Crabs to be heterothermally acclimated were, after capture, maintained in a 8°C environmental room for twenty four hours to clear their gills, and fed mussel and frozen fish. The next day crabs of appropriate size were chilled to approximately 4°C for a period of three hours to facilitate handling. A chilled crab was then selected and three rubber partitioning diaphragms put around its body longitudinally. The diaphragms were slightly off centre so that they were between a claw and the mouth parts (see Figure 6.0), this arrangement would isolate a three quarter body section to one acclimation temperature, the contralateral body quarter was isolated at the other acclimation temperature. The crab was then sealed in place using elastic bands and clamped securely in the heterothermal apparatus (made from Perspex). After the crabs were securely clamped in place, the apparatus was lowered into a large tank and filled with fresh sea water to a level that covered the animals. Four or six crabs were acclimated heterothermally at a time. Setting up the heterothermal apparatus took approximately three hours. The crabs were positioned so that either the three quarter section was oriented within the inner compartment, the inner compartment was heated to 22°C, or positioned toward the outer compartment which was maintained at 8°C (see Figure 6.0). If the larger body portion which included the CNS was cold acclimating (outer compartment), the acclimation nomenclature for that three quarter section would be **C**old Central **N**ervous System/**C**old **L**eg or CN/CL. The opposite heterothermal one quarter section of the same crab would be **C**old CNS/**H**ot **L**eg or CN/HL, in which the walking legs were at the warmer acclimation temperature and the CNS was at the lower temperature. Therefore, if most of the body and CNS was oriented within the inner compartment the nomenclature would be HN/HL with a heterothermal HN/CL.

The sea water was aerated using portable rotary air pumps with 2cm aeration stones, once the heterothermal set-up was completed the crabs were left for a period of 24 hours. Thereafter the water heater (Techne Tempette junior TE-8J) inside the

heterothermal apparatus (see Figure 6.0) was used to increase the sea water temperature of the inner compartment, as shown in Table 6.1.

Throughout the acclimation period the crabs were checked to determine that they were still responsive, this was done by clasping a leg and gently pulling against it, and noting the retraction reflex. If the leg was not retracted then eye-stalk movement was investigated, the eye-stalk normally respond to a touch stimulus by retracting. If neither of these reflexes were present the crab was removed. Once removed the heterothermal temperatures were maintained by sealing the vacant position where the crab had been with a sheet of plastic.

Table 6.1 Heterothermal acclimation protocol.

Day.	Event.
1	Set crabs in apparatus (8°C).
2	Increase interior compartment temperature from 8°C to 11°C.
6	Replace sea water with new water.
7	Increase interior compartment temperature from 11°C to 16°C.
12	Replace sea water with new water.
13	Increase interior compartment temperature from 16°C to 19°C.
17	Replace sea water with new water.
18	Increase interior compartment temperature from 19°C to 22°C.
23	Change sea water.
28	Change sea water.
32	Acclimation complete.

Crabs were not fed whilst in the apparatus except for their initial feed after capture. Electrophysiological parameters of leg neuromuscular activity were measured according to the protocol given in Chapter Two.

Measurement of crab internal temperature.

Water bath temperatures were routinely measured using thermocouples. Living crabs had their internal body temperature measured with a thermocouple (Type-K from RS) at the end of an experimentation period. The temperatures were measured by drilling a 2mm diameter hole in the carpopodite article of a claw, using an engravers hand drill (Minicraft MB140) then inserting a thermocouple approximately 1cm inside allowed accurate temperatures to be recorded. Data is shown in Figures 6.2A/B.

CNS temperatures were measured in living *C.maenas*, three crabs were placed in a heterothermal apparatus (see Figure 6.0) as HN/HL, and three crabs as CN/CL.

Thermocouple probes were placed approximately 8mm along the animal's oesophageal tracts, this location was determined by dissection to be exactly adjacent to their brain. Once the 8°C and 22°C heterothermal temperatures were achieved readings from each crab were taken every ten minutes for a period of approximately five hours. This was done to determine the exact CNS temperature, and to identify if intrathoracic temperature fluctuations could occur due to the crab changing the direction of sea water flow through its gills to modulate its intrathoracic temperature. The data are shown in Figure 6.1A/B.

Sea water conductivity changes.

Whilst acclimating crabs within the heterothermal apparatus the sea water conductivity was routinely measured using a hand held conductivity meter (Hanna instruments Type HI 9033). Normal fresh sea water conductivity was measured after collection and was determined to be 52.5±0.5 mSiemens (n=5). Because the inner warm compartment in the heterothermal apparatus was small and constantly heated, evaporation was expected. This might result in increasing the salinity of the sea water thereby increasing its conductivity when measured. The heterothermal water conductivity was measured daily and the sea water was changed approximately every four to five days (see Table 6.1) to reduce the effect of raised ionic concentrations on the crabs' physiology. The mean measured sea water conductivity during the heterothermal acclimation period measured between water changes was 53.5±0.3 mSiemens (n=21), which was not significantly different (Student's t-test) from the conductivity readings of freshly obtained sea water (52.5mSiemens).

Results

The hypothesis developed would predict that, in heterothermally acclimated crabs, the results obtained from the walking legs receiving the same acclimation experience as the CNS (i.e. CN/CL and HN/HL) would be analogous to the data obtained from walking legs of crabs homothermally acclimated to those temperatures. In that case the CN/CL and HN/HL results can be used as same animal controls for the contralateral heterothermal CN/HL and HN/CL data respectively. These comparisons allowed an assessment to be made of the extent of a central influence on the development of thermal acclimatory responses of walking legs.

Figure 6.1A: Measured CNS temperature in cold acclimating *Carcinus maenas*. Temperature values from three *C.maenas* CNS are shown with respect to the inner (warm) and outer (cold) compartment sea water temperatures. The animals were set up as CN and as such their CNS temperature should match the cold outer compartment sea water temperature. The CNS temperature was always slightly warmer than required due to the CN/HL contralateral side leaking water. Changes in CNS temperature were caused by a compartments water temperature changing, and not due to the animal changing the direction of water flow through the gills as a method of modulating it's CNS temperature.

Figure 6.1B: Measured CNS temperature in hot acclimating *Carcinus maenas*. CNS temperature values from three *C.maenas* (mean±S.E mean) are shown. The animals were set up as HN, with an opposite HN/CL. The first 30-60 minutes were quite variable, due to thermal steady state not being reached. However, it is clear that the hot CNS was cooler than required, again due to cold water (from contralateral HN/CL side) mixing. Over the 270 minute period that the CNS temperature was recorded, any shift in CNS temperature was mirrored by a compartment water temperature change. This matching of body temperature to ambient water temperature is typical of ectothermic animals.

Figure 6.1 A: Measured CNS temperature in cold acclimating Carcinus maenas.

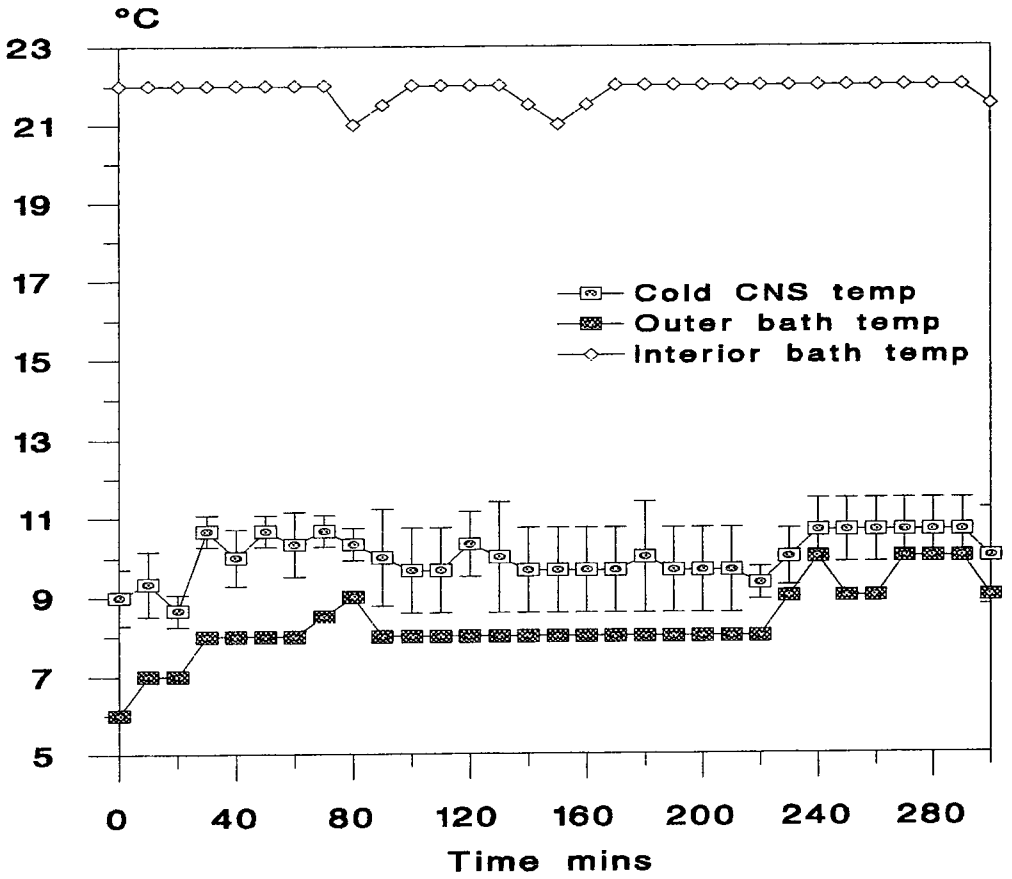
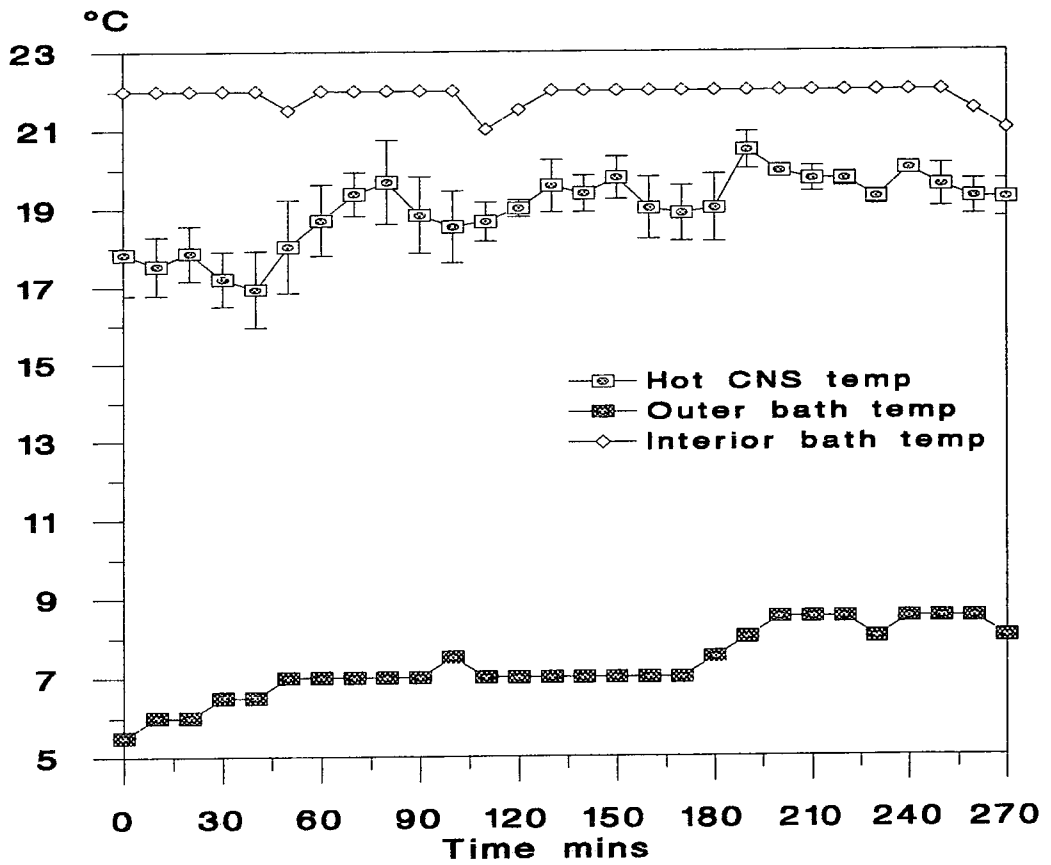


Figure 6.1B: Measured CNS temperature in hot acclimating Carcinus maenas.



Measurement of electrophysiological parameters were made, over the temperature range 6-26°C, on preparations from crabs of both species heterothermally acclimated as described above. The electrophysiological parameters investigated were the same as those described in Chapter Four. The figure legends show, in the form of statistical matrices the actual individual temperature points where significant differences (one way ANOVA, $P<0.05$) were found when comparing the different acclimatory groups.

Central Nervous System acclimation temperature.

The data shown in Figure 6.1A/B show an example of the temporal changes in temperature experienced by heterothermally acclimated crabs in relation to the bath temperatures. The actual mean CNS temperatures from three warm acclimating *C.maenas* or HN (Figure 6.1B) and three cold acclimating *C.maenas* or CN (Figure 6.1A) are shown compared to the sea water compartment temperatures. Before taking temperature readings the crabs and apparatus were left for two hours to equilibrate to the acclimation temperatures. However, it was noticed that the first sixty minutes of results shown in Figure 6.1A and 6.1B were quite variable due to temperature steady state not being achieved. The mean temperature difference between the cold acclimating CNS and the inner warm compartment sea water temperature over the whole time range was 11.8±0.1°C (n=32). The mean temperature difference between the warm acclimating CNS compared to the cold outer compartment sea water temperature was 11.7±0.2°C (n=28). These differences were identical to those reported between warm and cold acclimated claw temperatures compared to the inner and outer compartment temperatures shown for *C.pagurus* in Figures 6.2B, and quite similar to those of *C.maenas* shown in Figures 6.2A.

Figure 6.1A and 6.1B clearly illustrate that any shift in the cold or warm compartment sea water temperature resulted in a change in the CNS acclimation temperature toward the temperature change of that compartment. The temperature difference between individual *C.maenas* heterothermally acclimated animals was approximately 11.2°C for chelae, comparing *C.maenas* CN/CL to the inner warm compartment sea water temperature (using data from Figure 6.2A). Where also *C.maenas* HN/HL temperature difference was 12.3°C (claw) when compared to the cold outer compartment sea water temperature. The equivalent differences for *C.pagurus* (using Figure 6.2B) heterothermally acclimated animals were, CN/CL 12.85°C (claw) and HN/HL was 12.3°C (claw). The temperature difference between control warm and cold acclimated crabs (irrespective of free or immobilised) was 14°C, which was greater than that of heterothermally acclimated crabs. It can be seen from Figure 6.2A/B for

Figure 6.2A: Claw acclimation temperatures of heterothermally acclimating *Carcinus maenas*. All data shown is from living crabs and mean±S.E mean, experimental numbers are shown above each result column.

Figure 6.2B: Claw acclimation temperatures of heterothermally acclimating *Cancer pagurus*. Mean temperatures±S.E. mean are shown from living crabs, experimental numbers are shown above each column.

Figure 6.2A: Claw acclimation temperatures of heterothermally acclimating Carcinus maenas.

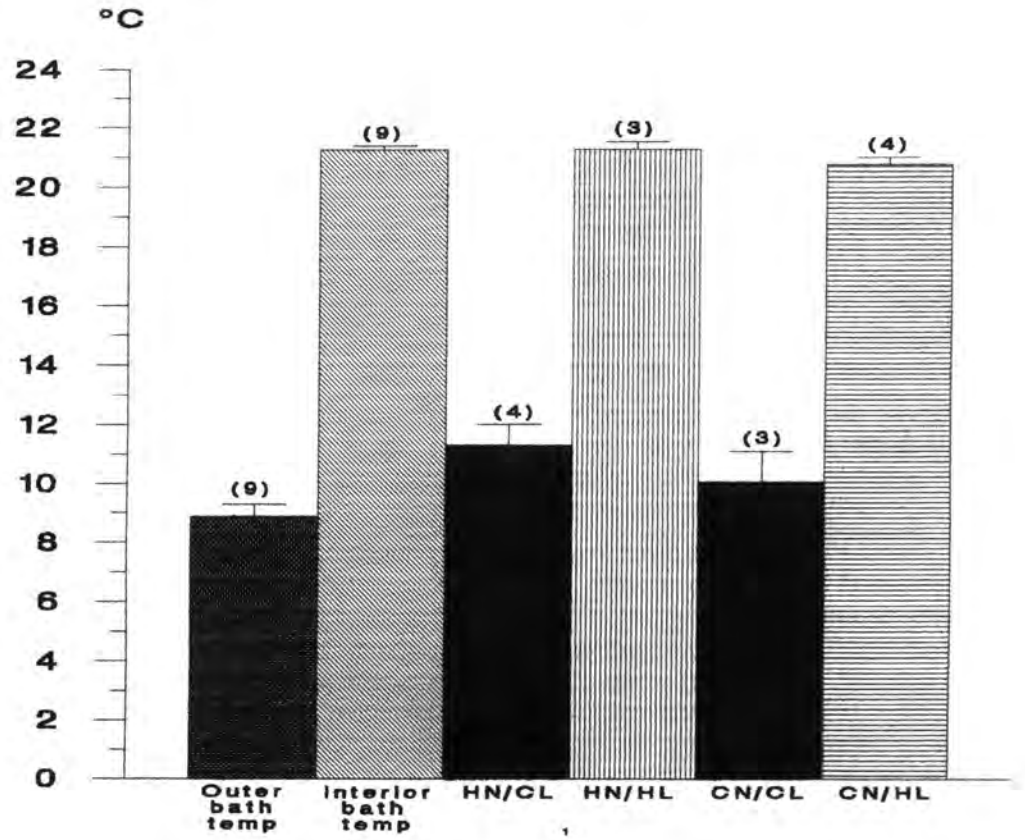
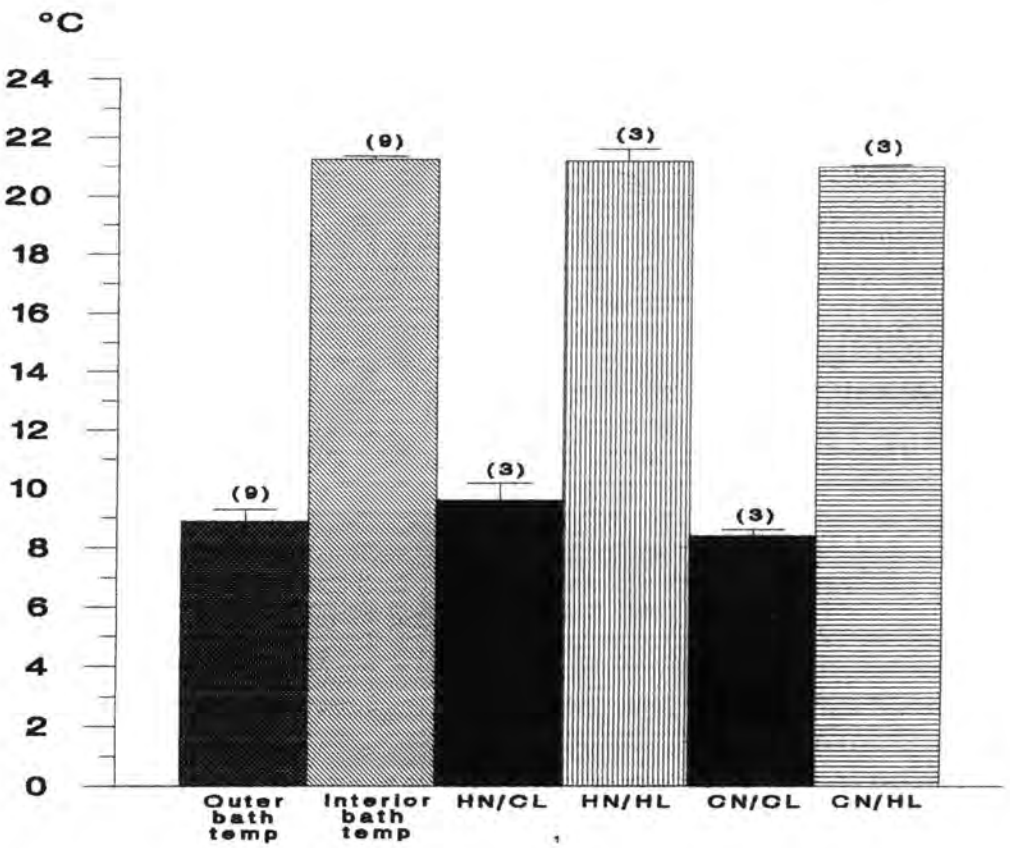


Figure 6.2B: Claw acclimation temperatures of heterothermally acclimating Cancer pagurus.



both *C.pagurus* and *C.maenas*, it is possible to establish a pronounced thermal gradient between the temperatures of the claws on the right and left sides of a heterothermally acclimated crab. In these cases claw temperatures were found not to be significantly different from their respective bath temperatures. Figure 6.1A/B also show that it is possible to maintain CNS temperature close to either the warm or cold bath temperatures depending on the position of the partition. A consistent feature however was that CNS temperature was displaced towards the other bath temperature by about ±2°C. It was considered that these small differences in temperature of the CNS from the bath temperature did not significantly nullify the rationale of the experimental protocol stated in the introduction.

Claw acclimation temperatures.

C.maenas

Figure 6.2A shows the sea water compartment temperatures and claw temperatures of living heterothermally acclimated *Carcinus maenas*. The claw (chela) temperature gave an approximate measure of the walking leg acclimation temperature. The average cold outer compartment sea water temperature was 8.9±0.4°C (n=9). The average interior compartment sea water temperature was 21.25±0.12°C (n=9), the temperature difference between the inner and outer compartments was 12.35°C, which was less than the 14°C temperature difference between the warm and cold acclimated free and immobilised control animals, see Chapter Four.

Crabs whose claws were acclimating with their major body portion at an acclimation temperature, had claw temperatures closer to that compartment temperature than the contralateral body portion. The cold exterior sea water compartment acclimation temperature was significantly colder by 2.42°C than the claw acclimation temperature of HN/CL crabs. No significant differences in temperature were found between the warm inner compartment and warm walking leg acclimation temperatures.

In all cases the cold acclimated chela were acclimating at significantly (one way ANOVA) colder temperatures than the contralateral warm acclimated chela.

C.pagurus

Figure 6.2B shows inner and outer compartment sea water temperatures and claw acclimation temperatures of heterothermally acclimating *C.pagurus*. No significant differences were found between the warm inner compartment and warm walking leg

acclimation temperatures, or between the contralateral outer cold compartment and cold acclimating walking leg acclimation temperatures. *C.pagurus* claw acclimation temperatures were also slightly nearer the required acclimation temperature when acclimated to the temperature of the ipsilateral CNS. In all cases the warm acclimating body parts were acclimating at significantly warmer temperatures than the contralateral cold acclimating body portions.

Both *C.maenas* and *C.pagurus* claw temperatures were very close to the required acclimation temperature when their walking legs were acclimated to the CNS acclimation temperature. Although with the side affect of altering the contralateral heterothermal chela temperature away from its required acclimation temperature.

Axon identification.

It was necessary to identify the low threshold or tonic motor axon in each experiment as it has been shown previously that the tonic axon innervates muscle fibre types I and II only (Rathmayer and Erxelben 1983; Rathmayer and Maier 1987), thereby increasing result homogeneity.

Table 6.2: Voltage (V) thresholds required to elicit an EJP in the low and high threshold motor axons innervating the walking leg closer muscle. Values from cold acclimated walking leg axons were determined around 7±1°C, whereas warm acclimated walking leg axon voltage thresholds were determined at 19±2°C for both species (n= number of different preparations).

Species and type of acclimation.	Low threshold (V±S.E.mean).	High threshold (V±S.E.mean).	n
C.maenas CN/CL	3.11±0.305	6.46±0.144	12
C.maenas CN/HL	2.29±0.450	6.55±0.210	8
C.maenas HN/HL	2.63±0.259	6.34±0.136	17
C.maenas HN/CL	2.83±0.298	6.60±0.164	13
C.pagurus CN/CL	2.25±0.350	6.27±0.228	17
C.pagurus CN/HL	3.07±0.345	6.38±0.221	17
C.pagurus HN/HL	3.10±0.284	6.54±0.127	15
C.pagurus HN/CL	2.25±0.220	6.33±0.265	14

The voltage values of the low threshold motor axons of *C.maenas* were compared between the various heterothermal acclimation groups, and no significant differences

were found (one way ANOVA). The voltage values of low threshold motor axons of *C.pagurus* did show some significant differences when compared between acclimation groups. The threshold values obtained from HN/HL and CN/HL acclimated crabs were not different, but both were significantly larger than the values obtained from the HN/CL and CN/CL acclimatory groups (one way ANOVA). In neither species were the voltage values for the high threshold motor axons different between any acclimation condition.

A reason for some of the differences between some of the low threshold axons may be accounted for by axon damage during dissection, or the size of the animal, as smaller diameter axons require higher stimulation voltages (Adams 1987). The differences between low threshold axons of the different acclimatory groups were small and did not affect the successful identification of the low threshold or tonic motor axon. Thus, it can be concluded that low and high threshold axons could be reliably selected irrespective of the acclimation condition imposed.

Resting membrane potential.

C.maenas

Figure 6.3A and Figure 6.3B show mean resting potential (RP) changes over the 6-26°C experimental temperature range for heterothermally acclimated *C.maenas*. Table 6.3 showed that the degree of linear correlation was high and so the data were fitted with a single straight line. The RP change with temperature were significantly greater than that predicted by Nernst for all acclimatory groups.

Table 6.3: Mean (±S.E.mean) RP change with temperature (mV/°C) and mean (±S.E.mean) RP intercepts for heterothermally acclimated *C.maenas* (n= number of preparations).

Species and acclimation type.	Correlation coefficient.	Intercept (mV).	RP change with Temp mV/°C.	n
Free *C.maenas* 8 +Ouabain (1mM).	0.9962	-75.2±0.1	-0.4065±0.08	5
C.maenas CN/CL	0.91226	-69.9±1.6	-1.099±0.1	18
C.maenas HN/CL	0.97268	-66.5±1.8	-1.19±0.09	20
C.maenas HN/HL	0.92089	-70.1±2.4	-0.899±0.05	15
C.maenas CN/HL	0.89264	-67.5±2.6	-0.93±0.095	14

Figure 6.3A: Resting potential change in heterothermal *Carcinus maenas* acclimated with a cold CNS Mean data including standard error of mean are presented for all groups. See statistical matrix[1] below for full statistical analysis (one way ANOVA). The acclimatory shift calculated for CN/CL (n=17) to CN/HL (n=17) was 4.03°C= 37.5%.

Carcinus maenas Resting Potential statistical matrix[1] identifies the individual temperatures at which significant differences between acclimatory groups were found.

	Free *C.maenas* 22°C	IM *C.maenas* 8°C	IM *C.maenas* 22°C	CN/CL	HN/CL	HN/HL	CN/HL
Free *C.maenas* 8°C	8-24		7-24		7-8	15, 19-23	11, 19, 21-24
Free *C.maenas* 22°C		8-24	21-24	9-22	13-20, 22-23		
IM *C.maenas* 8°C			6-25		7-8	11-13, 19-23	11-13, 21-24
IM *C.maenas* 22°C				9-24	11-25	22, 24	17-23, 25-26
CN/CL					10, 21	11-22	10-13, 17, 22
HN/CL						20-23	21-24
HN/HL							

Figure 6.3B: Resting potential change in heterothermal *Carcinus maenas* acclimated with a hot CNS Mean data is presented for heterothermally acclimated *C.maenas*, error bars are shown for both acclimatory groups. See statistical matrix for full acclimatory group (one way ANOVA) comparisons. The acclimatory shifts were calculated; HN/HL (n=17) to HN/CL (n=20) was 2.52°C= 25.3%.

[1] Key to using statistical matrix. Locate the acclimation condition first, the column (or row) contains individual temperature significant differences (one way ANOVA) when compared to a different row (or column) i.e. a different acclimation condition. E.g. free *C.maenas* 8°C RPs were significantly different from HN/CL acclimated walking leg RPs at 7-8°C; whereas CN/CL acclimated walking leg RPs were significantly different from HN/HL acclimated walking leg RPs at 11-22°C.

Figure 6.3A: Resting potential change in heterothermal Carcinus maenas acclimated with a cold CNS.

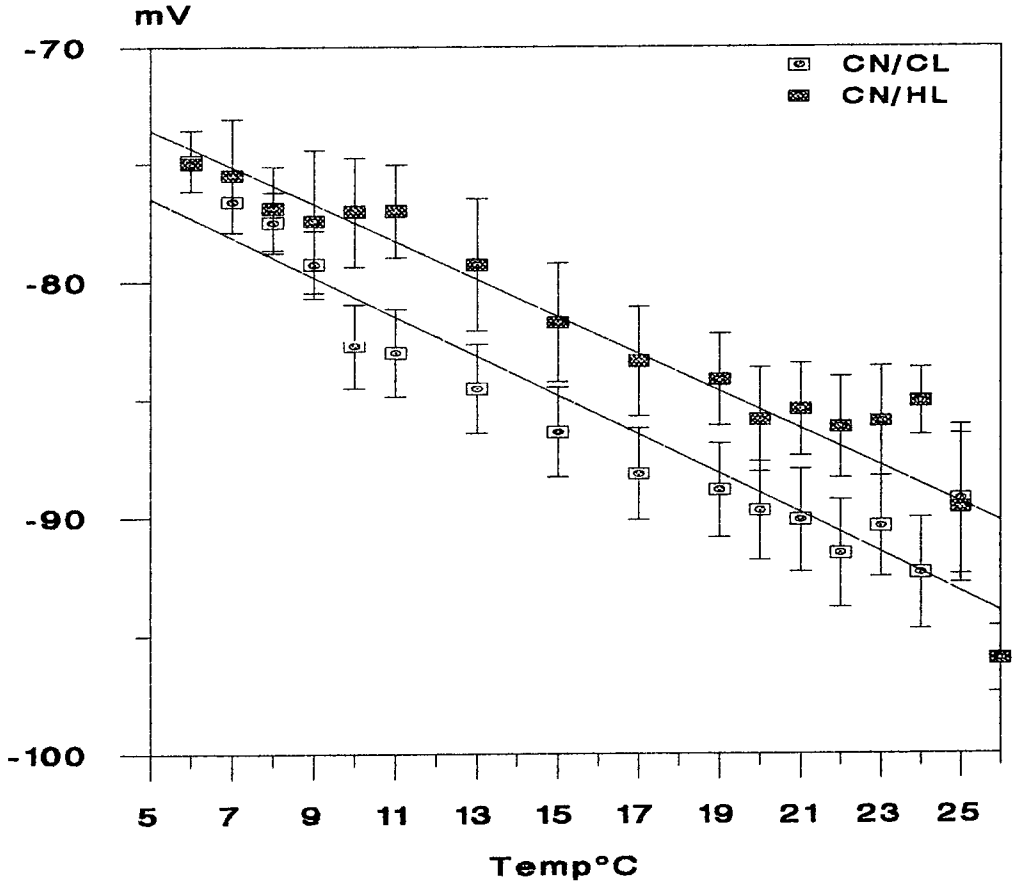


Figure 6.3B: Resting potential change in heterothermal Carcinus maenas acclimated with a hot CNS.

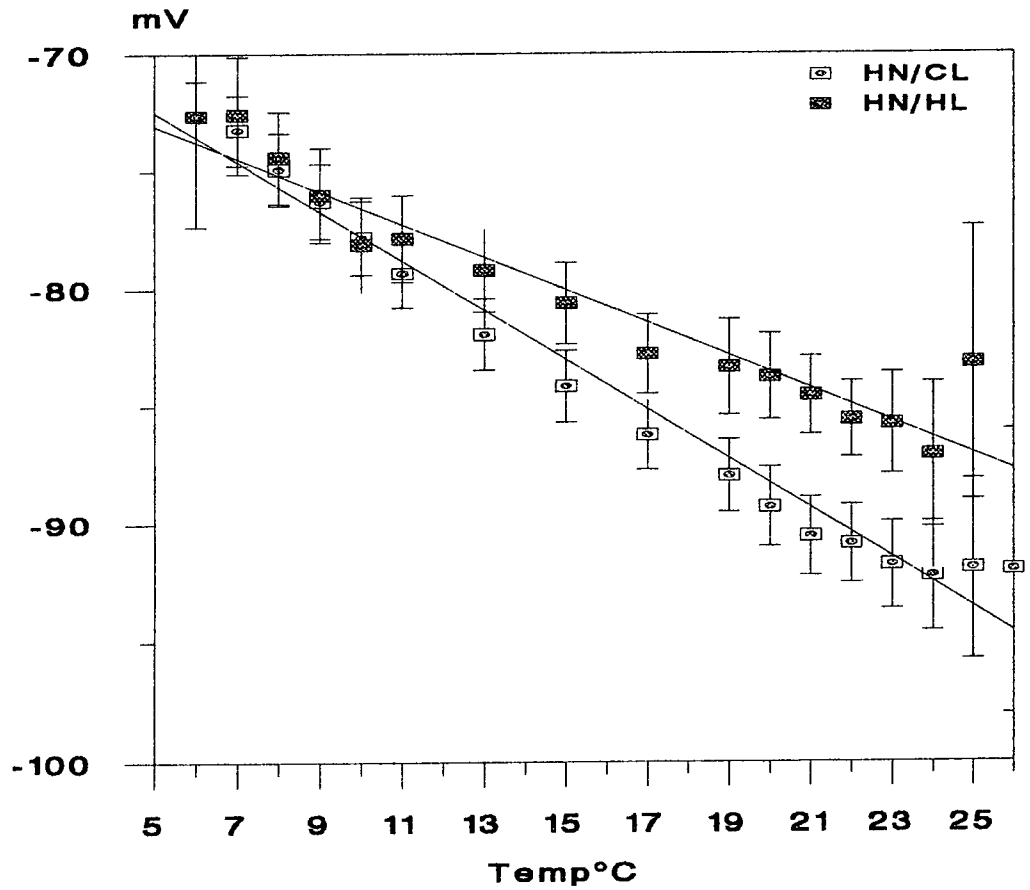


Figure 6.3A shows RP changes of cold and warm acclimated walking legs which were acclimated with a cold CNS. The cold acclimated walking leg (CN/CL) RPs were hyperpolarised compared to the RP values of warm acclimated walking legs (CN/HL), indicating an acclimatory shift (see below for statistical comparison). The acclimatory shifts of the different acclimatory groups for *C.maenas* were calculated (see Table 6.4), for example, the RP of *C.maenas* acclimated as CN/CL at their 10.1°C acclimation temperature was -80.7mV. Then by traversing along the temperature axis at -80.7mV, noting the experimental temperature at which that RP value intercepted the straight line RP plot of CN/HL acclimated crabs. The CN/HL intercept temperature gave an indication of that crabs acclimatory shift, in that case being calculated as 37.5% (see Table 6.4) with respect to its acclimation temperature.

Both CN/CL and CN/HL acclimated walking legs exhibited hyperpolarising RPs with increasing experimental temperature, although no significant differences (one way ANOVA) in the RP intercepts or RP change with temperature were found when comparing CN/CL and CN/HL acclimated walking legs. Comparisons of the RP values at individual experimental temperatures revealed some significant differences; CN/CL acclimated walking leg RP values were significantly (one way ANOVA) hyperpolarised over 11-13, 17, 22°C compared to CN/HL acclimated walking legs.

Figure 6.3B shows RP changes of cold and warm acclimated walking legs which were acclimated with a hot CNS. The warm acclimated walking leg (HN/HL) RP were generally depolarised compared to the RP of cold acclimated walking legs (HN/CL), notably at temperatures warmer than 10°C, the HN/CL RP values exhibited a 25.3% (see Table 6.4) acclimatory shift (partial acclimation or Type III after Precht, 1958).

Table 6.4: Estimated acclimatory shift for cold to warm homothermally and heterothermally acclimated walking legs of *C.maenas*.

	Percentage acclimatory Shift (%)
Acclimation type.	*Carcinus maenas.*
Free homothermal 8°C→ 22°C	53.6
Homothermal immobilised 8°C→ 22°C	82
CN/CL → CN/HL	37.5
HN/HL → HN/CL	25.3

Both HN/HL and HN/CL exhibited hyperpolarising RPs with increasing experimental temperatures, although no significant differences (one way ANOVA) in the RP intercepts were found. Whereas the RP change with temperature (Table 6.3)

when comparing HN/HL to HN/CL were significantly different, HN/CL RP slope was significantly (one way ANOVA) greater. Comparisons of the RP values at individual experimental temperatures revealed HN/HL RP values were significantly depolarised over 20-23°C when compared to HN/CL RP values, which was an unexpectedly small number of differences.

Comparison of RP values shown on Figures 6.3A and 6.3B revealed significant differences between CN/CL and HN/HL over 11-22°C, which showed a clear warm and cold acclimation temperature difference, although no difference in RP intercept or RP slope were found. In comparison HN/CL revealed very minor differences from CN/CL at 10, 21°C only, no differences in RP intercept or change with temperature were found, indicating the CNS temperature did not have a marked effect on walking leg acclimation in this case. Furthermore CN/HL revealed no significant differences from HN/HL RP intercepts, changes with temperature or individual RP values, which again indicates the CNS temperature did not effect walking leg acclimation. The acclimatory difference between the experimental groups shown in Figures 6.3A/B were smaller than expected, which may be due to the smaller acclimation temperature difference between warm and cold acclimated heterothermal walking legs.

C.pagurus.

All acclimatory groups were successfully fitted with a single straight line with a correlation coefficient greater than 0.92 (see Table 6.5). The RP change with temperature was calculated and is shown in Table 6.5, the RP change being significantly greater than that predicted by Nernst for all acclimatory groups.

Table 6.5: Mean RP (±S.E.mean) change with temperature (mV/°C) and mean (±S.E.mean) RP intercepts for heterothermally acclimated *C.pagurus*.

Species and acclimation type.	Correlation coefficient.	Intercept (mV).	RP change with Temp mV/°C.	n
Free *C.maenas* 8 +Ouabain (1mM).	0.9962	-75.2±0.1	-0.4065±0.08	5
C.pagurus CN/CL	0.97623	-70.7±1.2	-0.980±0.07	17
C.pagurus HN/CL	0.98435	-68.5±1.8	-1.014±0.07	14
C.pagurus HN/HL	0.9815	-63.7±1.3	-0.919±0.04	16
C.pagurus CN/HL	0.93	-62.4±1.9	-0.920±0.08	16

Figure 6.4A: Resting potential change in heterothermal *Cancer pagurus* acclimated with a cold CNS Mean data is presented for all groups and error bars are shown for all groups. See statistical matrix below (one way ANOVA) for full analysis of acclimatory group comparisons. The acclimatory shift was determined between temperature pairs, where CN/CL (n=17) to CN/HL (n=17) was 12.6°C= 100%.

Cancer Pagurus Resting Potential statistical matrix.

	Free *C.pagurus* 22°C	IM *C.pagurus* 8°C	IM *C.pagurus* 22°C	CN/CL	HN/CL	HN/HL	CN/HL
Free *C.pagurus* 22°C	6-25						
IM *C.pagurus* 8°C		7-26					
IM *C.pagurus* 22°C	6-25	19-25	6-26				
CN/CL	17-21	8-25		8-26			
HN/CL	6-7, 9-25	9, 11-23	9-25	10-26			
HN/HL	6-25		6-26	6, 23-25	6-25	6-23	
CN/HL	6-25	15-25	6-26		8-26	8-26	6, 17-20, 22, 24

Figure 6.4B: Resting potential change in heterothermal *Cancer pagurus* acclimated with a hot CNS. Mean data is presented for heterothermal acclimatory groups and error bars are shown for both acclimatory groups. See statistical matrix for full acclimatory group comparisons. The acclimatory shifts were calculated; HL-Hcns (n=16) to CL-Hcns (n=14) was 6.72°C= 58%.

Figure 6.4A: Resting potential change in heterothermal Cancer pagurus acclimated with a cold CNS.

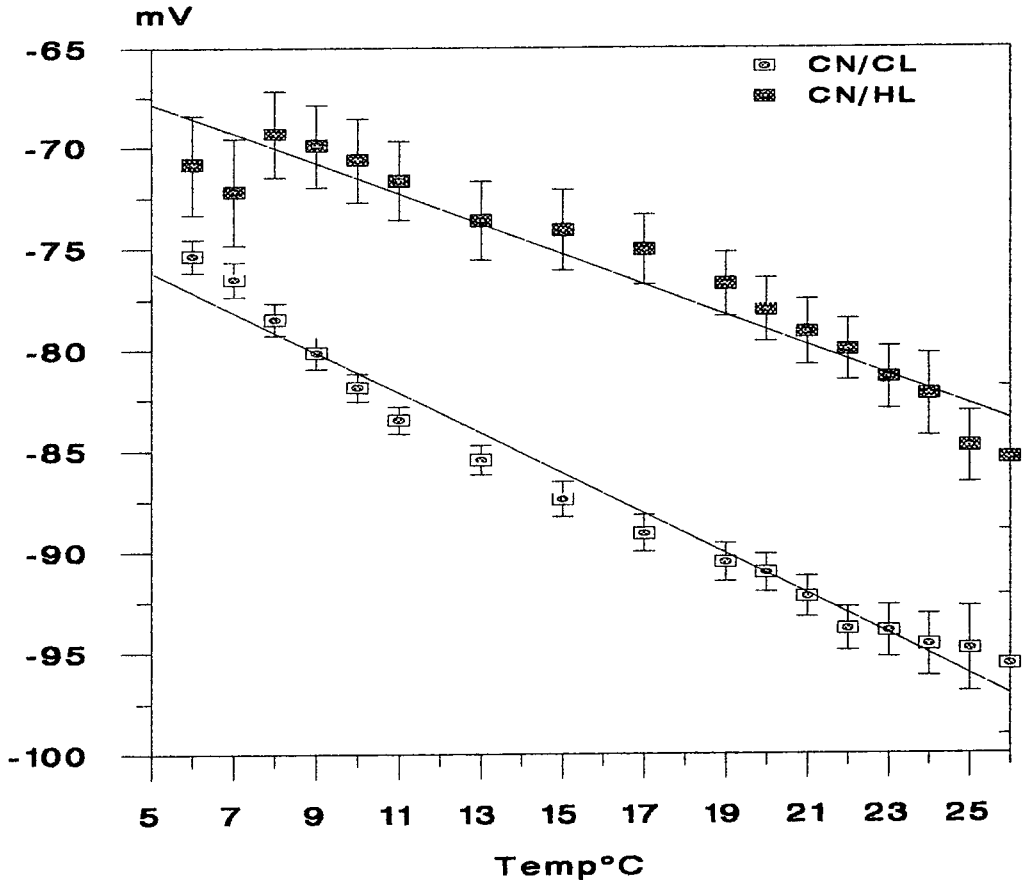


Figure 6.4B: Resting potential change in heterothermal Cancer pagurus acclimated with a hot CNS.

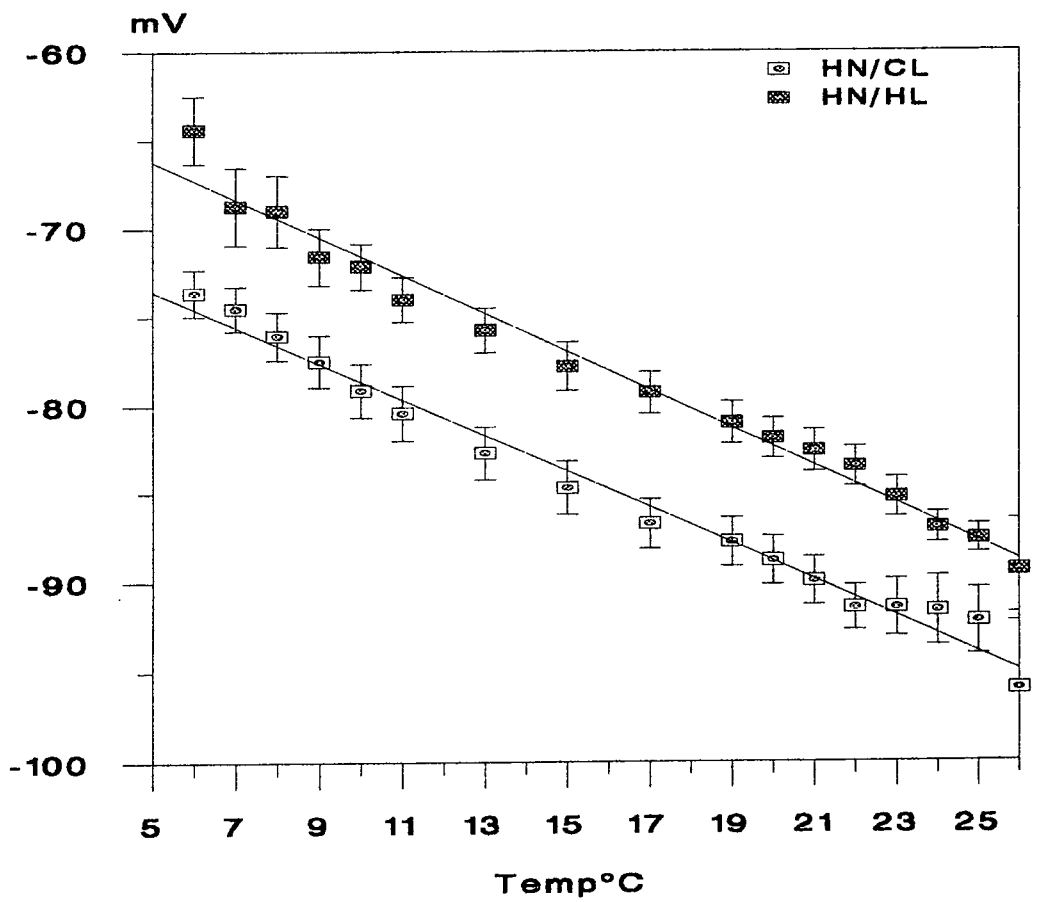


Figure 6.4A shows RP changes with temperature for warm and cold acclimated walking legs which were acclimated with a cold CNS. The CN/CL acclimated walking leg RP values were clearly more hyperpolarised than CN/HL acclimated walking leg RP values over the whole experimental temperature range, the estimated acclimatory shift (Table 6.6) for CN/HL acclimated walking leg RP values with respect to CN/CL acclimated walking legs was 100%, indicating complete acclimation (Type II after Precht, 1958). There were no significant differences in the RP change with temperature (see Table 6.5) when comparing CN/CL and CN/HL, although CN/CL RP intercept was significantly different from the CN/HL RP intercept (one way ANOVA). Comparisons of the individual RP values revealed; CN/CL acclimated walking leg RP values were significantly (one way ANOVA) hyperpolarised over 8-26°C when compared to CN/HL acclimated walking leg, indicating no CNS effect.

Figure 6.4B shows RP changes with temperature for warm and cold acclimated walking legs which were acclimated with a hot CNS. The HN/HL acclimated walking leg RP values were again clearly more depolarised than HN/CL acclimated walking leg RP values over the whole experimental temperature range, the estimated acclimatory shift (Table 6.6) of HN/HL acclimated walking leg RP values was 58% (partial acclimation or Type III after Precht, 1958) with respect to HN/CL acclimated walking legs. There were no significant differences (one way ANOVA) in the RP intercepts or RP changes with temperature when comparing HN/HL and HN/CL walking leg acclimatory groups. Comparisons of the individual RP values revealed; HN/HL acclimated walking leg RP values were significantly depolarised over 6-23°C when compared to HN/CL acclimated walking legs, indicating no CNS effect.

Table 6.6: Estimated acclimatory shift for cold to warm homothermally and heterothermally acclimated walking legs of *C.pagurus*.

	Percentage acclimatory shift (%).
Acclimation type.	*Cancer pagurus.*
Free homothermal 8°C→ 22°C	64.3
Homothermal immobilised 8°C→ 22°C	104.3
CN/CL → CN/HL	100
HN/HL → HN/CL	58

Comparison of the RP values shown on Figures 6.4A and B revealed significant differences (one way ANOVA) between CN/CL and HN/HL walking leg acclimatory groups over 6-25°C. Significant differences (one way ANOVA) between HN/HL and

CN/CL acclimated walking leg RP intercepts were also found, but no differences in their RP slope could be identified. Comparisons of HN/CL and CN/CL acclimated walking leg RP results revealed no differences in their RP intercept, slope, or individual RP values (one way ANOVA) over the whole experimental temperature range, confirming no CNS effect. Comparisons of HN/HL and CN/HL acclimated walking leg RP values revealed no significant differences (one way ANOVA) in their RP intercepts or RP changes with temperature. Comparisons of individual RP values revealed CN/HL acclimated walking leg RP values were significantly depolarised over 17-22°C when compared to HN/HL acclimated walking leg RPs. However, the CN/HL acclimated walking leg acclimatory shift was in the correct direction and more complete than that found for HN/HL acclimated walking legs.

Latency.

C.maenas

Figure 6.5A/B shows the latency values for heterothermally acclimated *C.maenas*, it can be seen that latency decreased with increasing experimental temperature. All results were successfully fitted with exponential curves with correlation coefficients greater than 0.96 and plotted on a log/linear scale.

Figure 6.5A shows latency changes with experimental temperature of warm and cold acclimated walking legs which were acclimated with a cold CNS. The cold acclimated walking leg (CN/CL) latent periods were almost identical to the CN/HL acclimated walking leg latent periods, no significant differences (one way ANOVA) were found between the CN/CL and CN/HL acclimated walking leg latent periods when compared at the same temperatures.

Figure 6.5B shows latency changes with experimental temperature of warm and cold acclimated walking legs which were acclimated with a hot CNS. The cold acclimated walking leg (HN/CL) latent periods were generally longer than the HN/HL acclimated walking leg latent periods, being significantly (one way ANOVA) longer over 10-21°C, indicating a warm and cold walking leg acclimation temperature difference.

Comparisons of the latent periods shown on Figures 6.5A and B did reveal some significant differences between CN/CL and HN/HL acclimated walking leg latent

Figure 6.5A: Latent period to first EJP in heterothermal *Carcinus maenas* acclimated with a cold CNS. All data shown is mean data, where standard errors of mean are shown for all groups. See statistical matrix below (one way ANOVA) for acclimatory group comparisons. Experimental numbers were HN/HL n=17 and HN/CL n=20. All data were fitted with an exponential curve using a least squares fit.

Carcinus maenas Latency statistical matrix.

	Free *C.maenas* 8°C	Free *C.maenas* 22°C	IM *C.maenas* 8°C	IM *C.maenas* 22°C	CN/CL	HN/CL	HN/HL
Free *C.maenas* 22°C	10, 13-20						
IM *C.maenas* 8°C	11-23, 25	8-26					
IM *C.maenas* 22°C	6-24	6-15, 20-22, 25	6-26				
CN/CL	21-22	10-22		6-23, 25			
HN/CL	7-8, 11	8-24		6-26			
HN/HL			8-25	6-24	13-15, 19-24	10-21, 24	
CN/HL		10-24	7-8, 11, 17-21, 25	6-25		7-8	8, 15, 20, 24

Figure 6.5B: Latent period to first EJP in heterothermal *Carcinus maenas* acclimated with a hot CNS. All data presented is mean data, error bars are shown for both acclimatory group. Experimental numbers were CN/HL n=17 and CN/CL n=17. All data sets were fitted with an exponential curve using a least squares fit.

Figure 6.5A: Latent period to first EJP in heterothermal Carcinus maenas acclimated with a cold CNS.

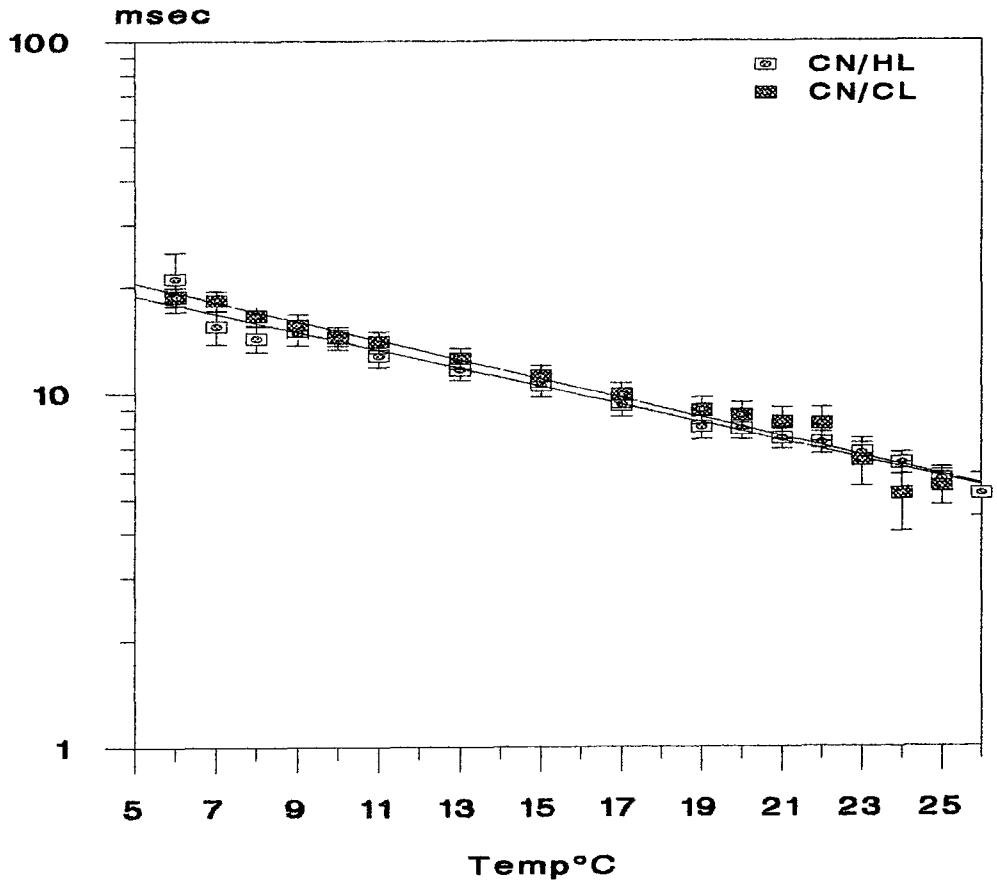
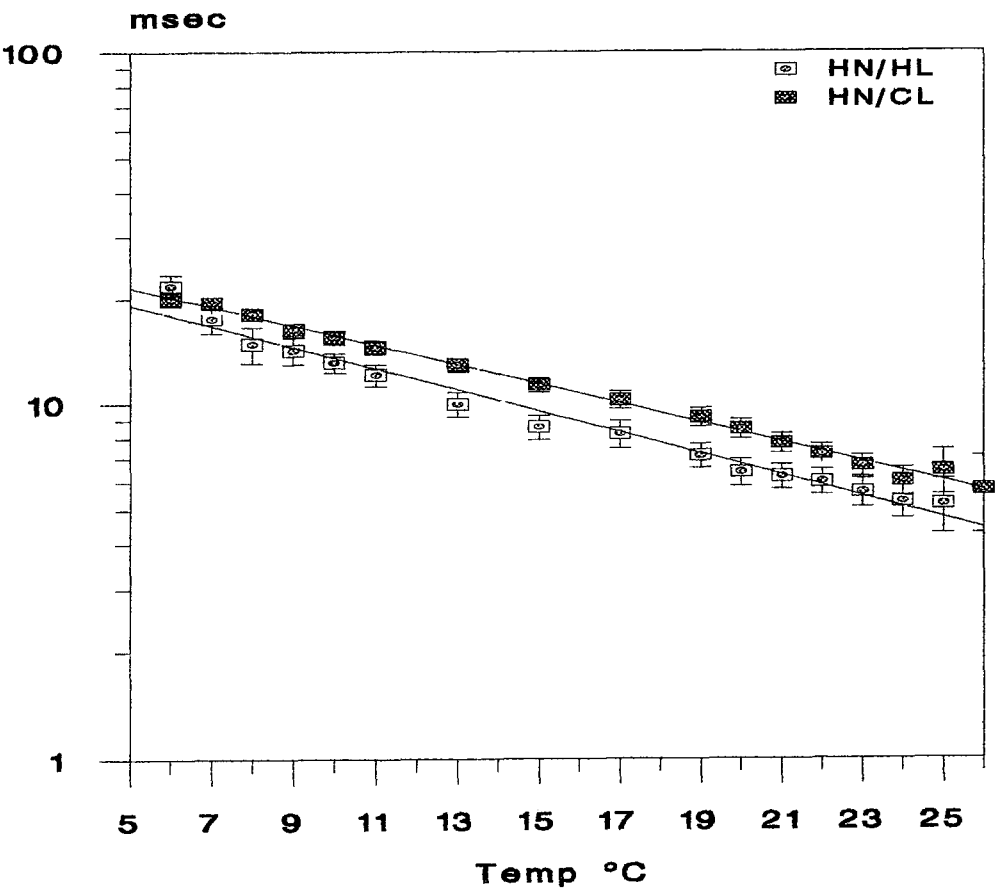


Figure 6.5B: Latent period to first EJP in heterothermal Carcinus maenas acclimated with a hot CNS.



periods over 13-15, 19-24°C, which indicated a warm and cold acclimation temperature difference supporting the results presented in Figure 6.5B. Comparisons between HN/HL and CN/HL acclimated walking leg latent periods revealed significant differences (one way ANOVA) at 8, 15, 20, 24°C only, these differences were minor and therefore indicate walking leg acclimation as being dependent on the local temperature, indicating no CNS effect. Comparisons between CN/CL and HN/CL latent periods revealed no significant differences (one way ANOVA) again indicating no CNS effect on temperature acclimation. The evidence indicating a CNS role in latency acclimation shown in Figure 6.5A may be due to the reduced acclimation temperature difference of 10.71°C between the CN/CL and CN/HL acclimated walking legs (see Figure 6.2A).

C.pagurus

Figure 6.6A/B show heterothermally acclimated *C.pagurus* latent periods, all latent periods decreased with increasing experimental temperature and all data sets were fitted with exponential curves with correlation coefficients greater than 0.98.

Figure 6.6A shows latent period changes of warm and cold acclimated walking legs which were acclimated with a cold CNS. The cold acclimated walking leg (CN/CL) latent periods were longer than the CN/HL acclimated walking leg latent periods over the whole experimental temperature range, being significantly longer (one way ANOVA) over 6-7, 11-13°C, which indicates an acclimatory shift between the warm and cold acclimated walking legs.

Figure 6.6B shows latent period changes for warm and cold acclimated walking legs which were acclimated with a hot CNS. The cold acclimated walking leg (HN/CL) latent periods were generally longer than the warm acclimated walking leg (HN/HL) latent period over 17-26°C temperature range. However, there were no significant differences (one way ANOVA) between the HN/HL and HN/CL individual latent periods when compared at the same experimental temperatures where some may be expected.

Comparison of the results presented on Figures 6.6A and 6.6B revealed no significant differences (one way ANOVA) between the HN/HL and CN/CL acclimatory groups, indicating no acclimation. Comparisons of CN/CL and HN/CL latent periods revealed no significant differences, and comparison of CN/HL and HN/HL revealed significant differences (one way ANOVA) at 9-13°C, the HN/CL acclimated walking

Figure 6.6A: Latent period to first EJP in heterothermal *Cancer pagurus* acclimated with a cold CNS. All data shown is mean data and error bars are shown for both acclimatory groups. Full statistical analysis between acclimatory groups was done and the results are shown in the matrix below (one way ANOVA). All acclimatory group data sets were fitted with an exponential curve using a least squares fit. Experimental numbers were CN/CL n=17 and CN/HL n=17.

Cancer pagurus Latency statistical matrix.

	Free *C.pagurus* 22°C	IM *C.pagurus* 8°C	IM *C.pagurus* 22°C	CN/CL	HN/CL	HN/HL	CN/HL
Free *C.pagurus* 8°C		6-25	6-25	6-25	6-26	6-26	6-10
Free *C.pagurus* 22°C			7-25	6, 26	6-25	6-26	6-26
IM *C.pagurus* 8°C				6-26	6-25	6-25	6-26
IM *C.pagurus* 22°C							
CN/CL					6-7, 11-13	6-7, 10, 13, 22	9-10, 13
HN/CL							
HN/HL							
CN/HL							

Figure 6.6B: Latent period to first EJP in heterothermal *Cancer pagurus* acclimated with a hot CNS. Mean data is presented and error bars are shown for both acclimatory groups. Experimental numbers were HN/HL n=17 and HN/CL n=14. Acclimatory group data sets were fitted with an exponential curve using a least squares fit.

Figure 6.6A: Latent period to first EJP in heterothermal Cancer pagurus acclimated with a cold CNS.

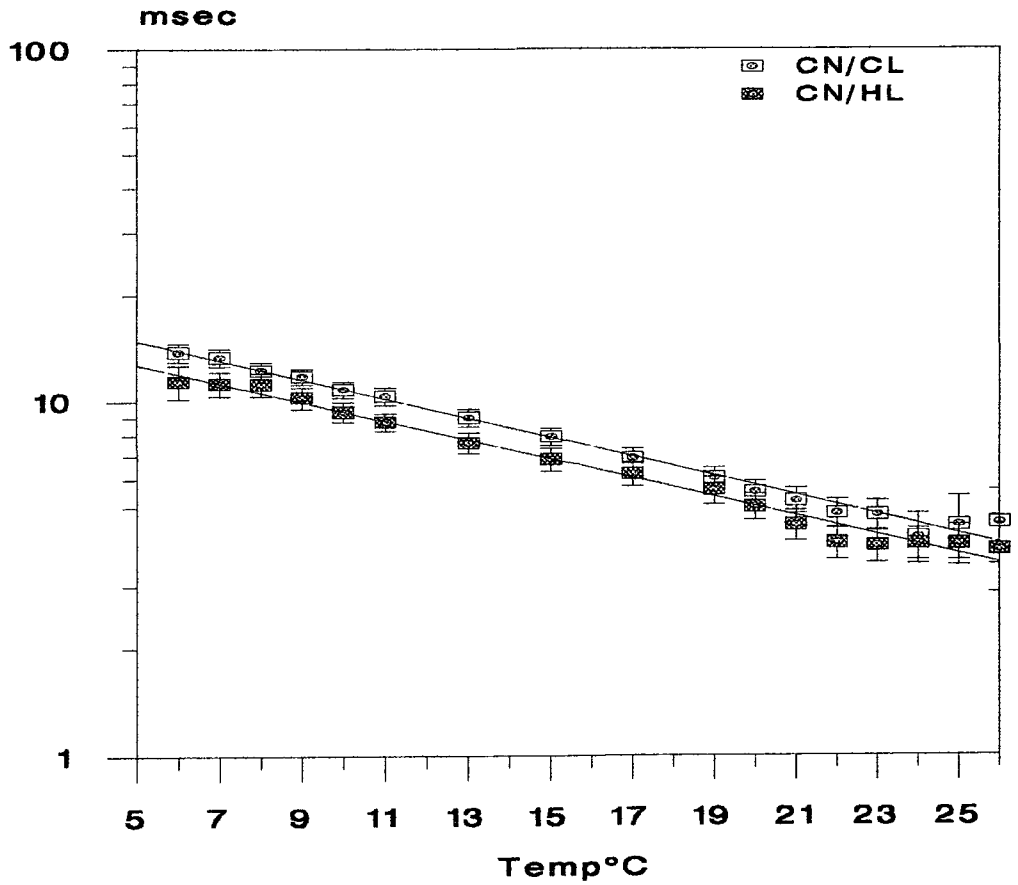
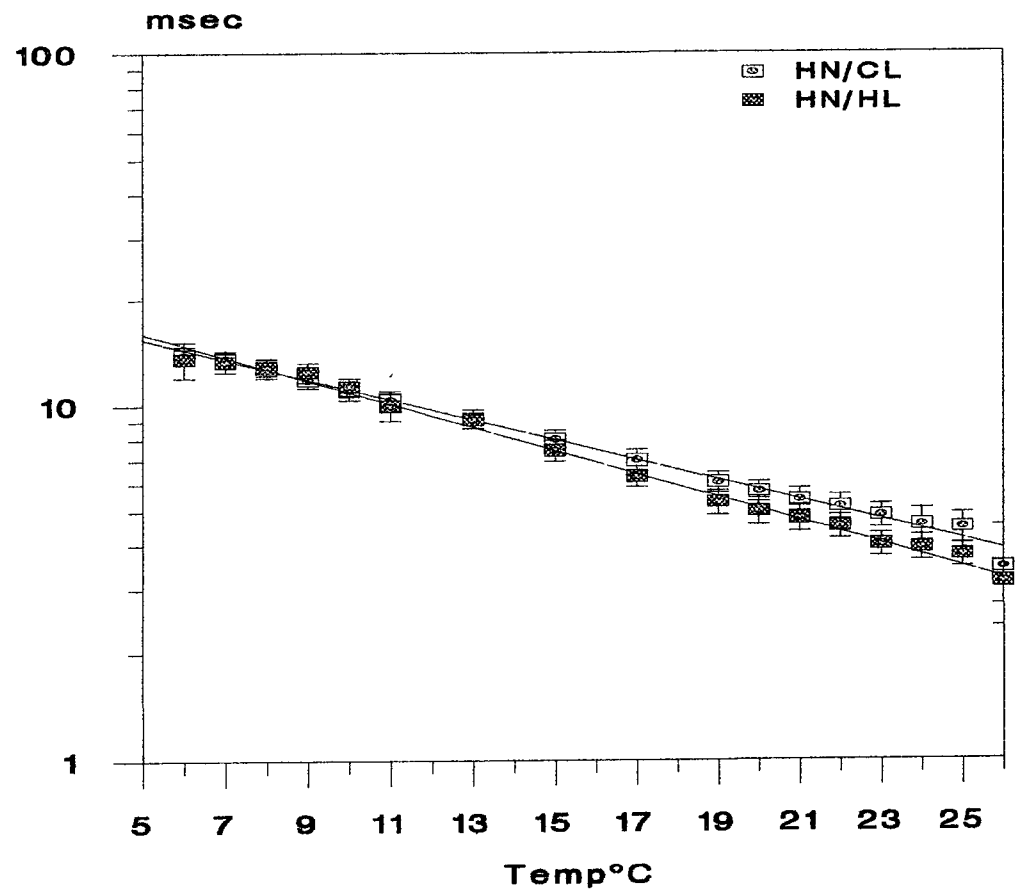


Figure 6.6B: Latent period to first EJP in heterothermal Cancer pagurus acclimated with a hot CNS.



leg latent periods being longer. The comparisons of *C.pagurus* heterothermally acclimated walking leg latent periods revealed a minor number of significant differences, which principally due to the lack of acclimation differences between the heterothermal control walking leg results (i.e. CN/CL and HN/HL), indicates the latent period results cannot be used to investigate a potential CNS influence on acclimation.

Single EJP amplitude.

C.maenas

Figure 6.7A shows single EJP amplitudes from warm and cold acclimated walking legs which were acclimated with a cold CNS. Amplitudes were averaged from responding crabs only. It can be seen that the CN/CL walking leg amplitudes were maintained up to approximately 15°C, thereafter they decreased rapidly with increasing experimental temperature. In comparison the CN/HL walking leg EJP amplitudes were relatively constant over 8-21°C, the amplitudes increased at experimental temperatures colder than 8°C although this was probably due to result variability. To reduce variability the amplitude at 6°C from CN/HL crabs were omitted as only two of the seventeen crabs were responsive. Single EJP amplitudes from CN/HL acclimated walking legs decreased at experimental temperatures warmer than 21°C, however, the CN/HL acclimated walking legs maintained their EJP amplitudes to 21°C some 6°C warmer than CN/CL. Statistical analysis (one way ANOVA) revealed no significant differences between the CN/CL and CN/HL acclimated walking leg EJP amplitudes when compared at the same temperatures. There was however a clear qualitative difference between the CN/CL and CN/HL acclimatory groups, which indicated that warm acclimated walking legs maintained their EJP amplitudes to warmer experimental temperature irrespective of the CNS acclimation temperature.

Figure 6.7B shows single EJP amplitudes of warm and cold acclimated walking legs which were acclimated with a hot CNS. The HN/CL acclimated walking leg EJP amplitudes were maintained over 6-15°C, at experimental temperatures warmer than 15°C the HN/CL acclimated walking leg EJP amplitudes decreased rapidly. The HN/HL acclimated walking leg single EJP amplitudes were smaller than HN/CL EJP amplitudes over the whole experimental temperature range, but the HN/HL acclimated EJP amplitudes were generally maintained over 10-21°C, and increased in amplitude at experimental temperatures colder than 10°C. Amplitudes from HN/HL crabs at 6°C were omitted as only three of the seventeen crabs were responsive. The HN/HL

Figure 6.7A: Single EJP amplitude in heterothermal *Carcinus maenas* acclimated with a cold CNS. All data presented is mean data, no error bars are shown so as to keep the figure uncluttered. Experimental numbers were; CN/CL n=17, CN/HL n=17. Statistical analysis was done on EJP amplitudes (one way ANOVA) and is shown in the statistical matrix below.

Carcinus maenas Single EJP Amplitude statistical matrix.

	Free *C.maenas* 8°C	Free *C.maenas* 22°C	IM *C.maenas* 8°C	IM *C.maenas* 22°C	CN/CL	HN/CL	HN/HL
Free *C.maenas* 22°C							
IM *C.maenas* 8°C	6-8, 10-15	7-17					
IM *C.maenas* 22°C	23-24	24, 26	6-15, 23-26				
CN/CL			6-17, 21	19-25			
HN/CL		8-9, 13, 17	6, 10-11	23-24	7, 17,25		
HN/HL			9-17	21-24		10-17	
CN/HL			7-17	23-24, 26		9-17	

Figure 6.7B: Single EJP amplitude in heterothermal *Carcinus maenas* acclimated with a hot CNS. Mean data is presented and no error bars are shown. Experimental numbers were; HN/CL n=20 and HN/HL n=17.

Figure 6.7A: Single EJP amplitude in heterothermal Carcinus maenas acclimated with a cold CNS.

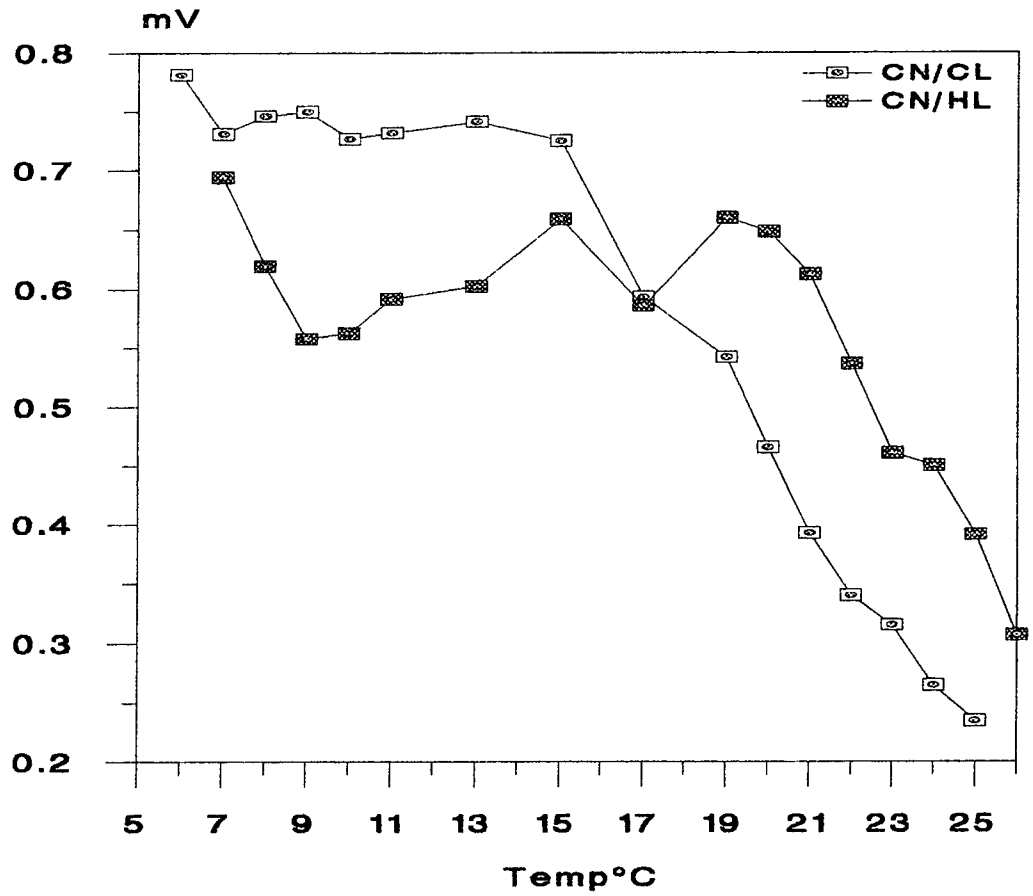
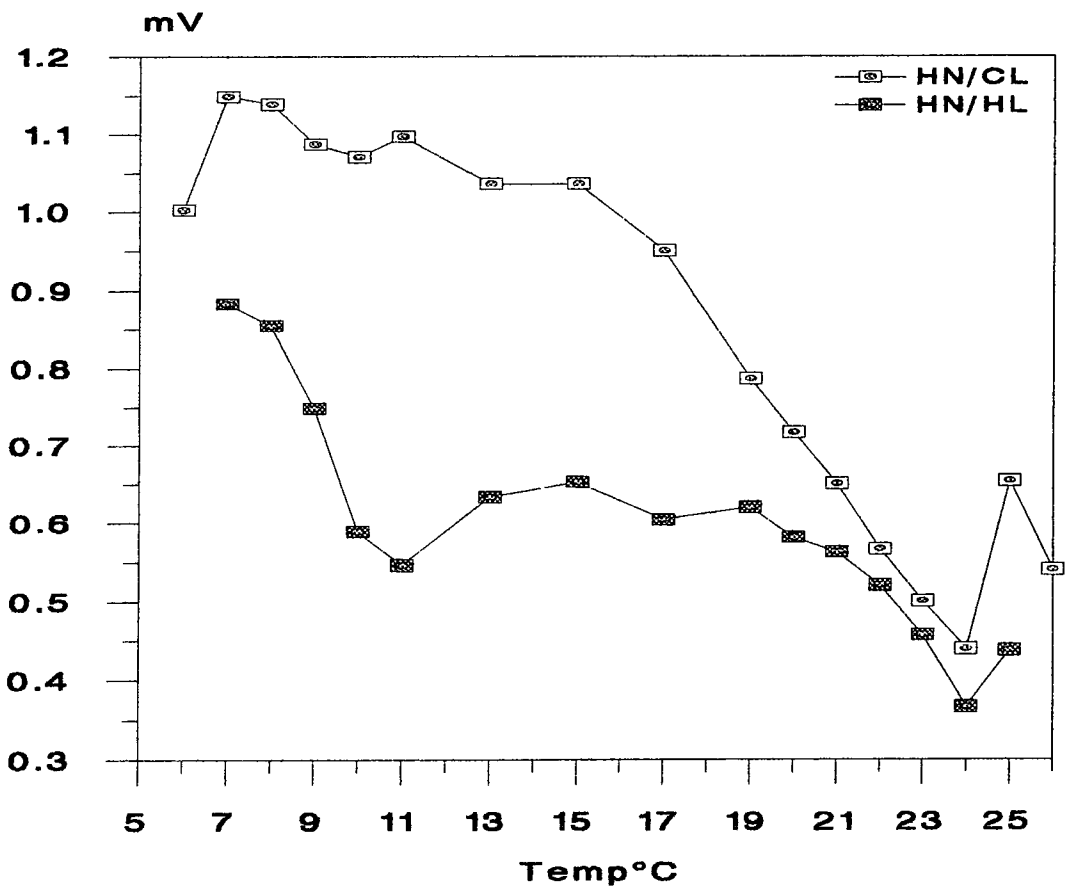


Figure 6.7B: Single EJP amplitude in heterothermal Carcinus maenas acclimated with a hot CNS.



acclimated walking leg EJP amplitudes decreased at experimental temperatures warmer than 21°C. However, the HN/HL acclimated walking legs maintained their EJP amplitudes up to 21°C before the onset of decreasing EJP amplitudes, some 6°C warmer than that found for HN/CL acclimated walking legs. Statistical analysis revealed HN/CL acclimated walking legs generated significantly larger EJP amplitudes than HN/HL over 10-17°C, indicating a warm and cold acclimated walking leg difference. The difference here was again more qualitative in that warm acclimated walking legs maintained their EJP amplitudes to warmer experimental temperatures near to their warm acclimation temperature before the onset of temperature induced decreases in EJP amplitude, irrespective of the CNS acclimation temperature.

Comparison of the amplitudes shown on Figures 6.7A and B revealed clear qualitative differences between the HN/HL and CN/CL acclimated walking leg EJP amplitudes. The warm acclimated walking legs maintained their amplitudes to warmer experimental temperatures, indicating a warm and cold acclimation temperature difference, although there were no significant differences (one way ANOVA) between the individual HN/HL and CN/CL acclimated walking leg EJP amplitudes when compared at the same experimental temperatures. Comparison of HN/HL and CN/HL acclimated walking leg EJP amplitudes revealed no significant differences (one way ANOVA) of either a qualitative or quantitative nature. Where comparison of CN/CL and HN/CL acclimated walking leg EJP amplitudes revealed a minor number of significant differences (i.e. at 7, 17, 25°C only), there was however no qualitative difference which again indicates walking leg acclimation was dependent on the walking leg local acclimation temperature.

C.pagurus

Figure 6.8A shows single EJP amplitudes of warm and cold acclimated walking legs which were acclimated with a cold CNS. It can be seen that CN/CL single EJP amplitudes were maintained up to 15°C, at experimental temperatures warmer than 15°C the EJP amplitudes decreased rapidly. In comparison CN/HL single EJP amplitudes were generally maintained over 6-22°C, the EJP amplitudes decreased at experimental temperatures warmer than 22°C. The CN/HL acclimated walking legs maintained their EJP amplitudes to 22°C before the onset of temperature induced decreases in EJP amplitude, some 7°C warmer than that of CN/CL acclimated walking legs. Statistical analysis (one way ANOVA) of the CN/CL and CN/HL acclimated walking leg EJP amplitudes revealed no significant differences when compared at the

Figure 6.8A: Single EJP amplitude in heterothermal *Cancer pagurus* acclimated with a cold CNS. All acclimatory group amplitudes presented were of mean data (no error bars are shown). Experimental numbers were; CN/CL n=17 and CN/HL n=17. Full statistical analysis (one way ANOVA) was done and is presented in the matrix below.

Cancer pagurus Single EJP Amplitude statistical matrix.

	Free *C.pagurus* 8°C	Free *C.pagurus* 22°C	IM *C.pagurus* 8°C	IM *C.pagurus* 22°C	CN/CL	HN/CL	HN/HL
Free *C.pagurus* 22°C	7-9, 23-24						
IM *C.pagurus* 8°C	6-10, 13-17	6, 8-17, 25					
IM *C.pagurus* 22°C	6-11, 21-25		6-17, 23-26				
CN/CL	8-10, 11		6-20	20-25			
HN/CL	8, 10		6-17	21-25			
HN/HL	6-13	6, 11, 26	7-17, 26		9, 11, 26	10-11	
CN/HL	7-13		6-17	23-25			

Figure 6.8B: Single EJP amplitude in heterothermal *Cancer pagurus* acclimated with a hot CNS. Mean data is presented for all acclimatory groups, no error bars are shown. Experimental numbers were; HN/CL n=17 and HN/HL n=16 EJP, see statistical matrix above for full analysis.

Figure 6.8A: Single EJP amplitude in heterothermal Cancer pagurus acclimated with a cold CNS.

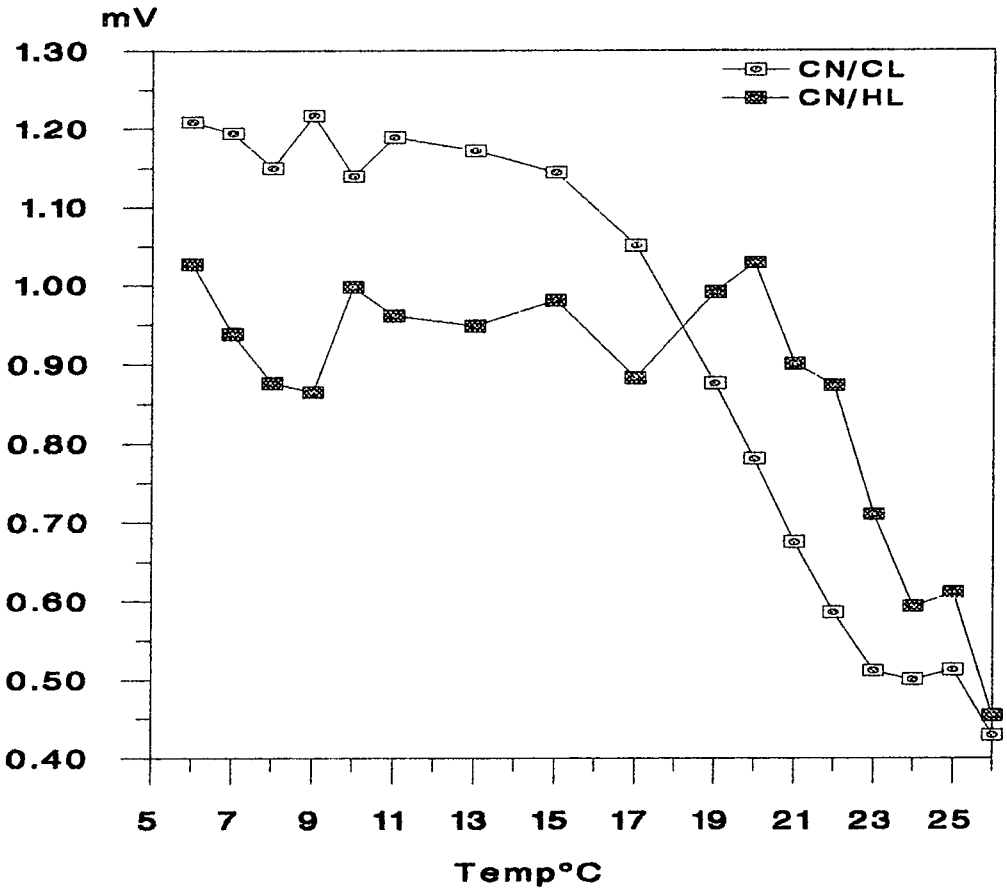
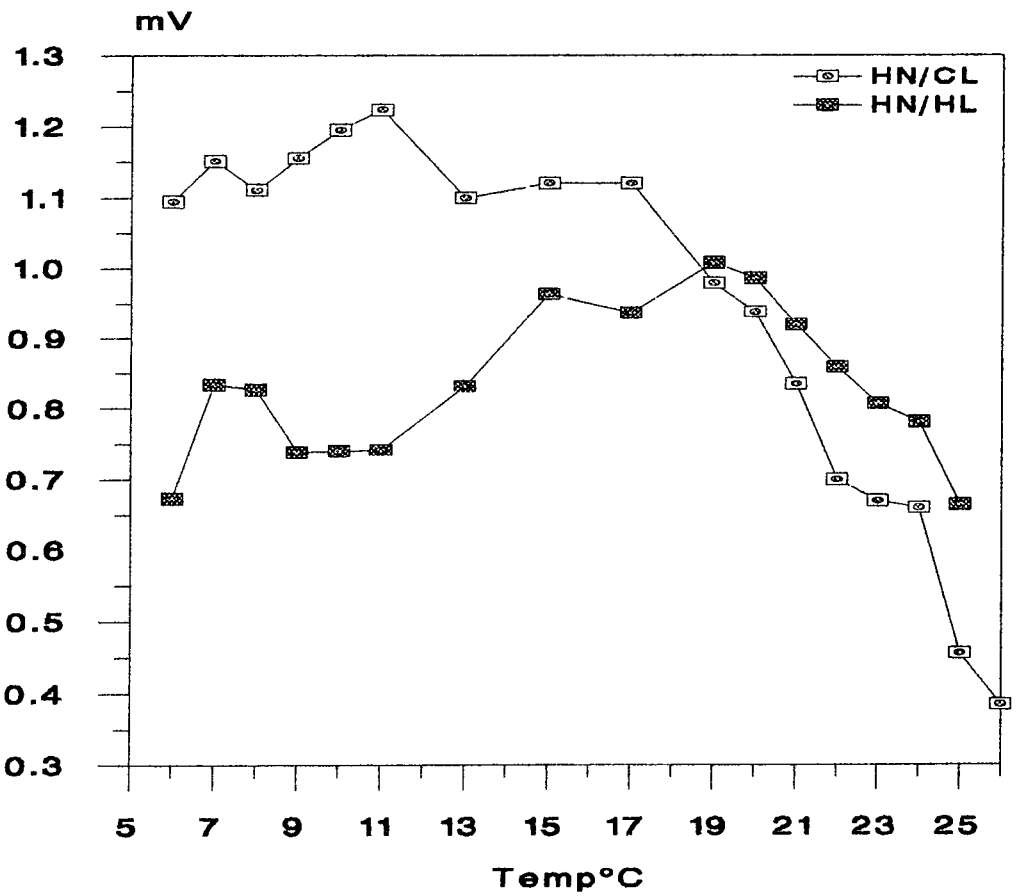


Figure 6.8B: Single EJP amplitude in heterothermal Cancer pagurus acclimated with a hot CNS.



same experimental temperatures, the differences between the two acclimatory groups were more qualitative.

Figure 6.8B shows single EJP amplitude changes of warm and cold acclimated walking legs which were acclimated with a hot CNS. The HN/CL acclimated walking legs maintained their EJP amplitudes up to 17°C, their EJP amplitudes decreased rapidly at experimental temperatures warmer than 17°C. In comparison, HN/HL acclimated walking leg EJP amplitude temperature dependency curve was generally n-shaped, and showed maintained EJP amplitudes over 9-25°C. The EJP amplitudes decreased at experimental temperatures warmer than 20°C or colder than 14°C. The HN/HL acclimated walking legs maintained their EJP amplitudes up to 20°C, some 3°C warmer than that of HN/CL acclimated walking legs. Amplitudes from HN/HL crabs at 26°C were omitted as only two of the sixteen crabs were responsive. Statistical analysis (one way ANOVA) revealed a minor number of significant differences between the EJP amplitudes of HN/HL and HN/CL acclimatory groups (i.e. at 10-11°C).

Comparison of the EJP amplitudes shown in Figures 6.8A and B revealed clear qualitative differences between CN/CL and HN/HL acclimated walking leg EJP amplitudes. The CN/CL acclimated crabs exhibited decreasing EJP amplitudes with increasing experimental temperatures, whereas the HN/HL acclimated crabs exhibited near constant EJP amplitudes over most of the experimental temperature range. Comparison of CN/CL and HN/CL acclimated walking leg amplitudes revealed no qualitative or quantitative differences. Additionally comparison of HN/HL and CN/HL acclimated walking leg EJP amplitudes revealed no significant qualitative or quantitative differences when compared at the same experimental temperatures. The results shown in Figures 6.8A and B were qualitatively identical in that a walking legs EJP amplitude change in response to direct temperature was dependent on the local acclimation temperature, and independent of the CNS acclimation temperature.

Facilitation.

C.maenas

Figure 6.9A shows facilitation changes over the experimental temperature range for warm and cold acclimated walking legs which were acclimated with a cold CNS. Both CN/HL and CN/CL acclimatory groups showed u-shaped facilitation versus temperature curves, minimal facilitation was recorded over approximately 10-17°C, positive facilitation values were recorded for both acclimatory groups over the whole

Figure 6.9A: Facilitation change in heterothermal *Carcinus maenas* with a cold acclimated CNS. Facilitation is the ratio between double pulse EJP amplitudes (see equation 1.4). Mean facilitation values are shown and no error bars are shown. Experimental numbers were; CN/CL n=17 and CN/HL n=17. Statistical analysis (one way ANOVA) is shown in the matrix below.

Figure 6.9B: Facilitation change in heterothermal *Carcinus maenas* acclimated with a hot CNS. All facilitation values presented are mean values and no error bars are shown. Where experimental numbers were; HN/HL n=17 and HN/CL n=20. Statistical analysis is shown below (one way ANOVA).

Carcinus maenas Facilitation statistical matrix.

	Free *C.maenas* 8°C	Free *C.maenas* 22°C	IM *C.maenas* 8°C	IM *C.maenas* 22°C	CN/CL	HN/CL	HN/HL
Free *C.maenas* 22°C	7-25						
IM *C.maenas* 8°C		6-26					
IM *C.maenas* 22°C		7-26					
CN/CL		7-25					
HN/CL	11, 21-23	7-20, 22-26	6, 9, 11, 21	7, 9, 10, 26			
HN/HL	11, 22	7-9, 11-25					
CN/HL		7-9, 11-13, 17-26					

Figure 6.9A: Facilitation change in heterothermal Carcinus maenas acclimated with a cold CNS.

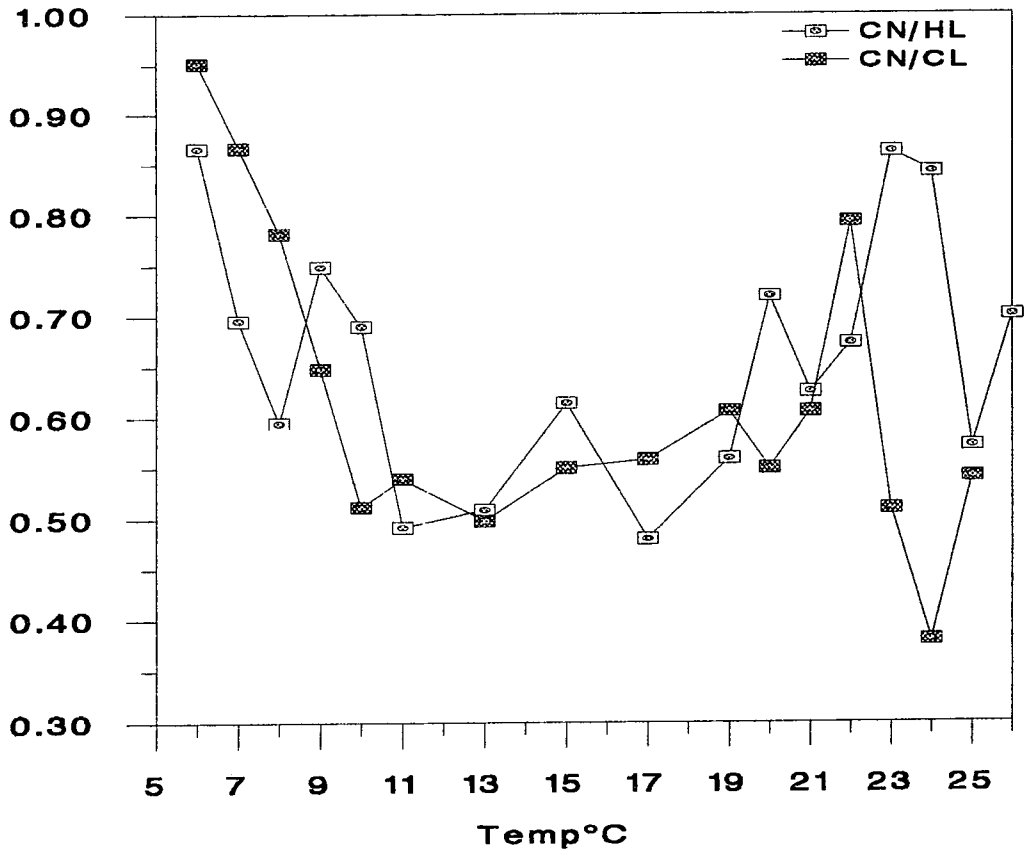
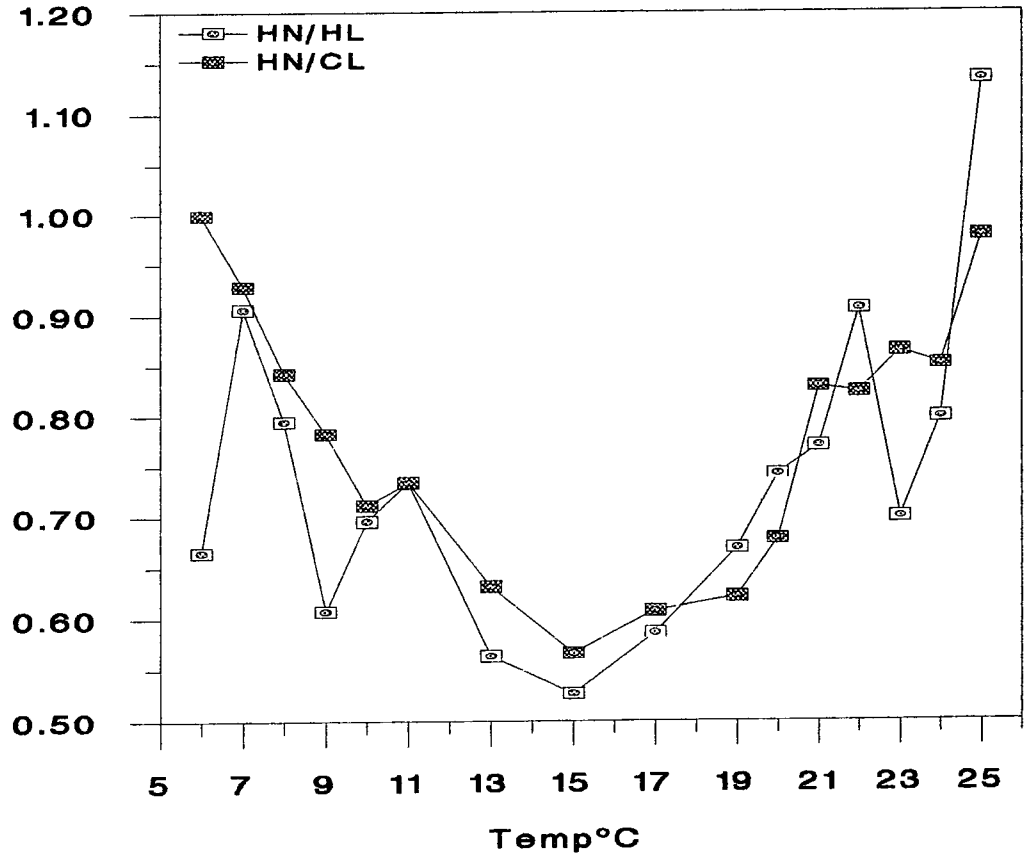


Figure 6.9B: Facilitation change in heterothermal Carcinus maenas acclimated with a hot CNS.



experimental temperature range. Facilitation increased for both acclimatory groups at experimental temperatures colder than 10°C or warmer than 17°C. Statistical analysis of CN/CL and CN/HL facilitation values revealed no significant differences (one way ANOVA) when compared at the same temperatures.

Figure 6.9B shows facilitation changes for warm and cold acclimated walking legs which were acclimated with a hot CNS. The HN/HL and HN/CL acclimatory groups both exhibited u-shaped facilitation versus temperature curve composed of positive facilitation values, minimal facilitation was recorded over 13-17°C. Facilitation again increased for both HN/HL and HN/CL acclimatory groups at experimental temperatures colder than 13°C or warmer than 17°C. Statistical analysis of facilitation values recorded from HN/HL and HN/CL acclimatory groups revealed no significant differences (one way ANOVA) when compared at the same experimental temperatures.

Comparison of the facilitation values shown in Figures 6.9A and B revealed no qualitative or quantitative differences between any heterothermal acclimatory group, irrespective of walking leg or CNS acclimation temperature. These results indicate facilitation cannot be used to identify any potential CNS influence acclimation as no cold to warm acclimation temperature changes were revealed.

C.pagurus

Figure 6.10A shows facilitation changes over the 6-26°C experimental temperature range of warm and cold acclimated walking legs which were acclimated with a cold CNS. Both CN/CL and CN/HL revealed u-shaped facilitation versus temperature curves, minimal facilitation was recorded over 13-17°C, and positive facilitation values were recorded over the whole experimental temperature range. The facilitation values increased for both CN/CL and CN/HL acclimatory groups at experimental temperatures colder than 13°C or warmer than 17°C. Statistical analysis of the facilitation values recorded from CN/CL and CN/HL walking legs revealed no significant differences (one way ANOVA) when compared at the same experimental temperatures.

Figure 6.10B shows facilitation changes for warm and cold acclimated walking legs which were acclimated with a hot CNS. The HN/CL acclimatory group exhibited a u-shaped facilitation versus temperature curve, with minimal facilitation being recorded around 12-15°C, facilitation increased at experimental temperatures colder than 12°C or warmer than 15°C. In comparison HN/HL acclimated walking leg facilitation values

Figure 6.10A: Facilitation change in heterothermal *Cancer pagurus* acclimated with a cold CNS. All facilitation data are mean and no error bars are shown, where experimental numbers were; CN/CL n=17 and CN/HL n=17. All data was analysed using one way ANOVA, comparisons are presented in the matrix below. The facilitation versus temperatures plots were u-shaped.

Figure 6.10B: Facilitation change in heterothermal *Cancer pagurus* acclimated with a hot CNS. All data shown was mean data, error bars are not shown. Statistical analysis (one way ANOVA) were carried out and results are presented in the matrix below. Experimental numbers were; HN/HL n=16 and HN/CL n=14.

Cancer pagurus Facilitation statistical matrix.

	Free *C.pagurus* 8°C	Free *C.pagurus* 22°C	IM *C.pagurus* 8°C	IM *C.pagurus* 22°C	CN/CL	HN/CL	HN/HL
Free *C.pagurus* 22°C							
IM *C.pagurus* 8°C		7, 25					
IM *C.pagurus* 22°C		7-8					
CN/CL				8, 13			
HN/CL			6, 9	8, 25			
HN/HL	8-15, 21	9-10, 13-15	8-10	8-10, 13		8, 10	
CN/HL	9-10, 13	9, 25	8-10	8-10, 13, 25			

Figure 6.10A: Facilitation change in heterothermal Cancer pagurus acclimated with a cold CNS.

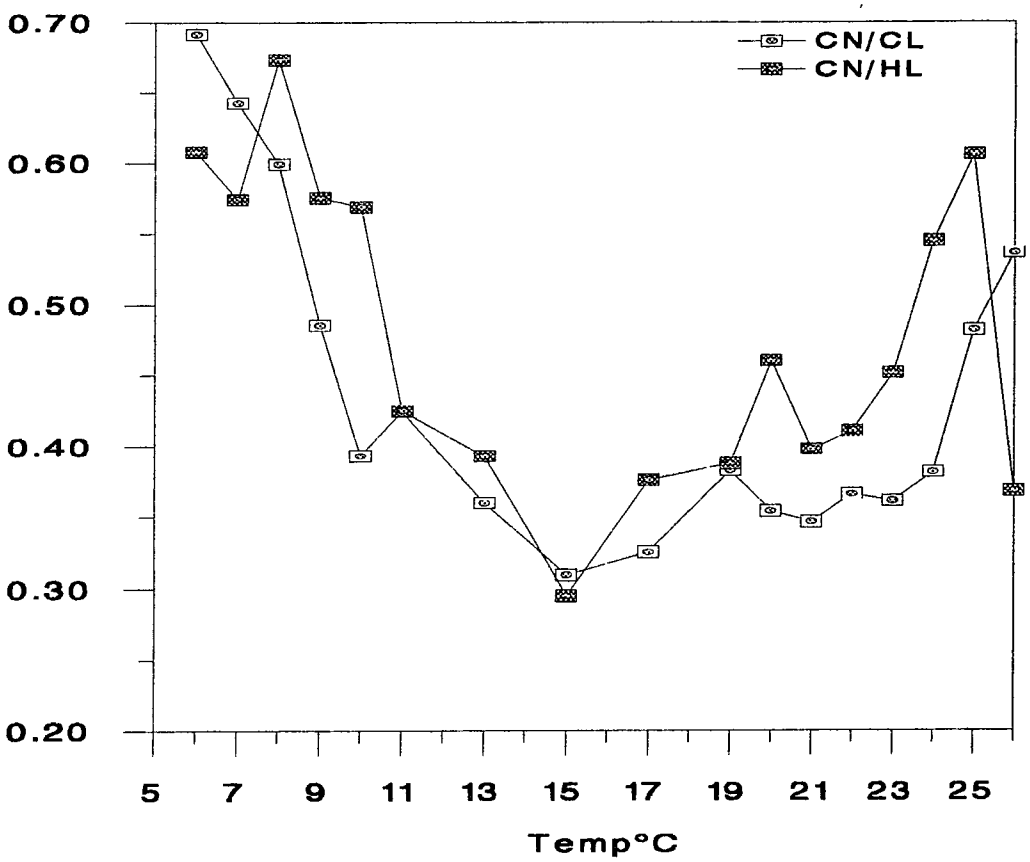
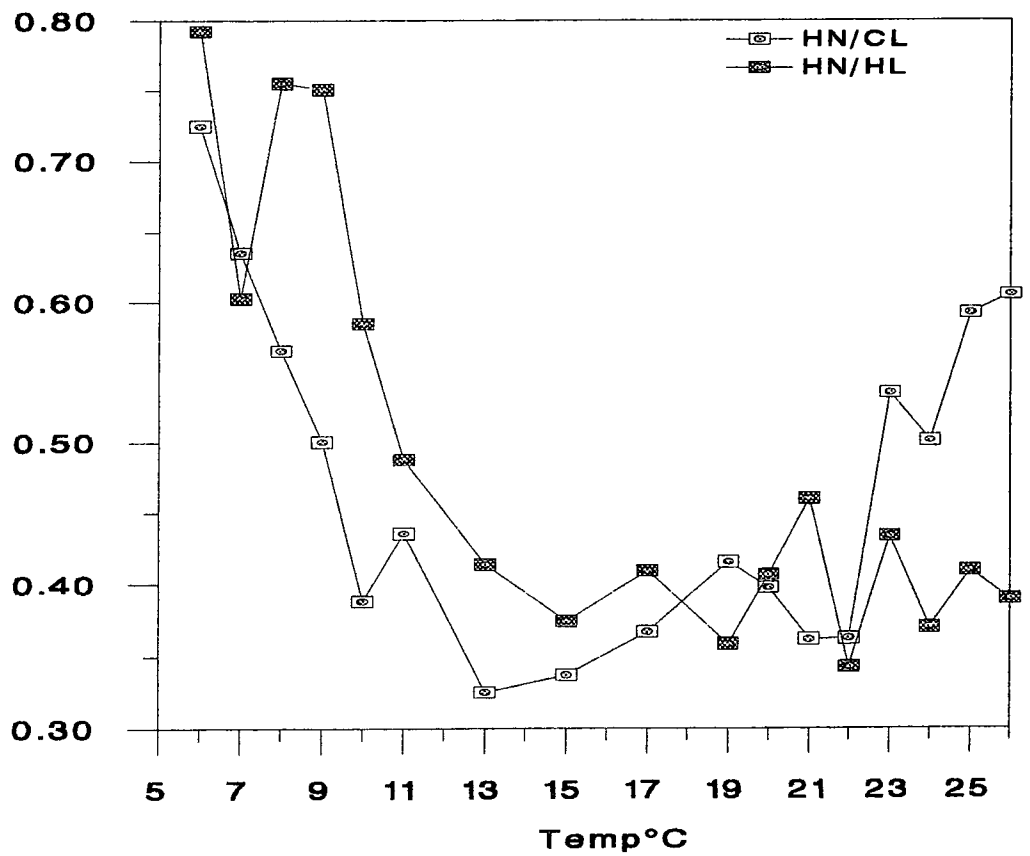


Figure 6.10B: Facilitation change in heterothermal Cancer pagurus acclimated with a hot CNS.



were also generally u-shaped, although the expected increase in facilitation at experimental temperatures warmer than 17°C was not found. Minimal facilitation was recorded over approximately 15-22°C from HN/HL acclimated walking legs, facilitation increased at experimental temperatures colder than 15°C. Statistical analysis revealed almost no significant differences (one way ANOVA) between facilitation values recorded from HN/HL and HN/CL acclimatory groups, except at 8, 10°C only.

Comparison of the facilitation values presented in Figures 6.10A and B revealed effectively no qualitative or quantitative differences between the different acclimatory groups (except for HN/HL acclimated walking legs maintained facilitation over 15-22°C, their facilitation was smaller than expected at experimental temperatures warmer than 17°C). The lack of any significant differences between the heterothermal acclimatory groups facilitation values, irrespective of the walking leg or CNS acclimation temperatures, indicates facilitation did not reveal any acclimation or potential CNS influence on walking leg acclimation.

Double EJP amplitude.

C.maenas

Double pulse EJP amplitudes contain a facilitative component that contributes to larger EJP amplitudes and increased variability. Figure 6.11A shows double pulse stimulated EJP amplitude changes for warm and cold acclimated walking legs which were acclimated with a cold CNS. The CN/CL acclimated walking leg EJP amplitudes decreased steadily with increasing experimental temperature. Whereas CN/HL acclimated walking leg EJP amplitudes were relatively constant over 9-21°C, the EJP amplitudes decreased at experimental temperatures warmer than 21°C. Additionally CN/HL amplitudes increased at experimental temperatures colder than 9°C, the increase at cold temperatures was probably due to increasing membrane resistance. The amplitudes from CN/HL crabs at 6°C were omitted as only two of the seventeen crabs were responsive. The warm acclimated walking legs clearly maintained their EJP amplitudes to warmer experimental temperatures near to their warm acclimation temperature. Statistical comparisons revealed no significant differences (one way ANOVA) between CN/CL and CN/HL acclimated walking leg EJP amplitudes, the difference between the acclimatory groups was qualitative in that CN/HL acclimated walking legs were warm tolerant and therefore indicated no CNS effect to walking leg acclimation.

Figure 6.11A: Double pulse EJP amplitude in heterothermal *Carcinus maenas* acclimated with a cold CNS. All data presented is mean data (no error bars shown). Statistical analysis results are shown in the statistical matrix below (one way ANOVA). Experimental numbers were; CN/CL n=17 and CN/HL n=17.

Carcinus maenas Double Pulse EJP Amplitude statistical matrix.

	Free *C.maenas* 8°C	Free *C.maenas* 22°C	IM *C.maenas* 8°C	IM *C.maenas* 22°C	CN/CL	HN/CL	HN/HL
Free *C.maenas* 22°C	22-23, 25						
IM *C.maenas* 8°C		23, 25					
IM *C.maenas* 22°C			6-11				
CN/CL		19-23	6-9, 11	21-23			
HN/CL				6-10	8, 11, 17-20		
HN/HL			9-15			9-15	
CN/HL		11-13	7-15			8-13, 17	

Figure 6.11B: Double pulse EJP amplitude change in heterothermal *Carcinus maenas* acclimated with a hot CNS. Mean data is presented, no error bars are shown. Experimental numbers were; HN/CL n=20 and HN/HL n=17.

Figure 6.11A: Double EJP amplitude change in heterothermal Carcinus maenas acclimated with a cold CNS.

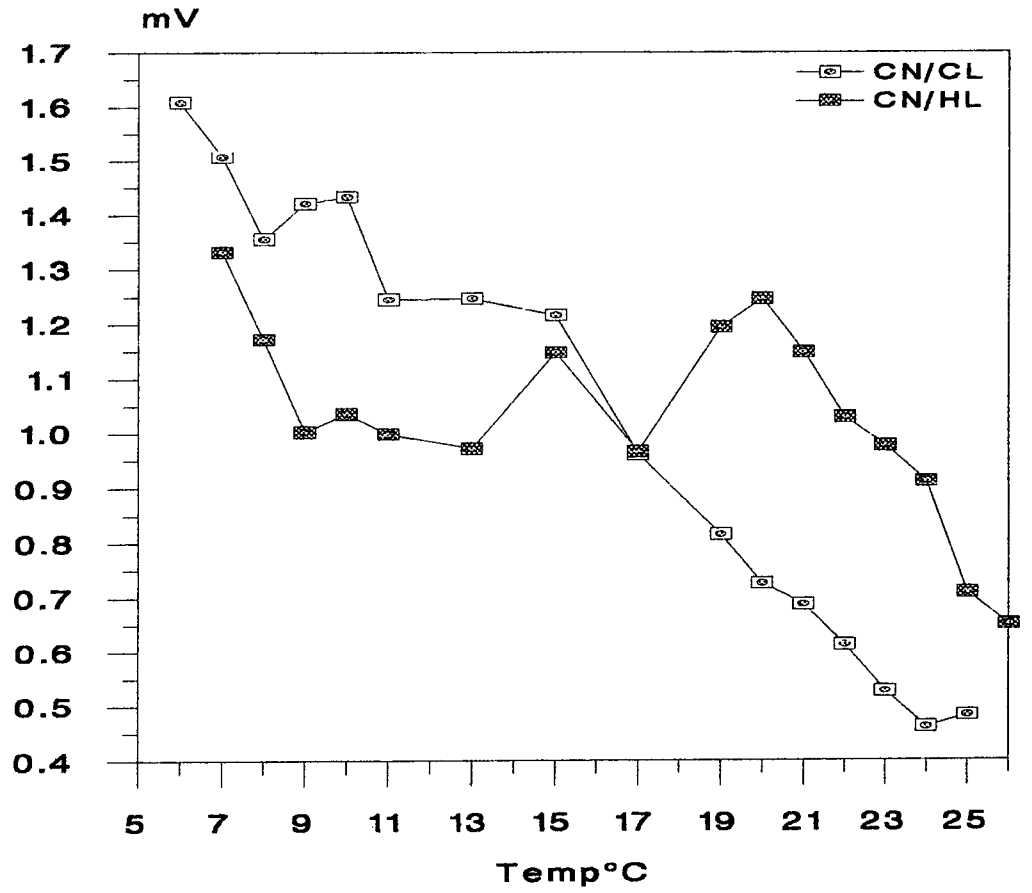


Figure 6.11B: Double EJP amplitude change in heterothermal Carcinus maenas acclimated with a hot CNS.

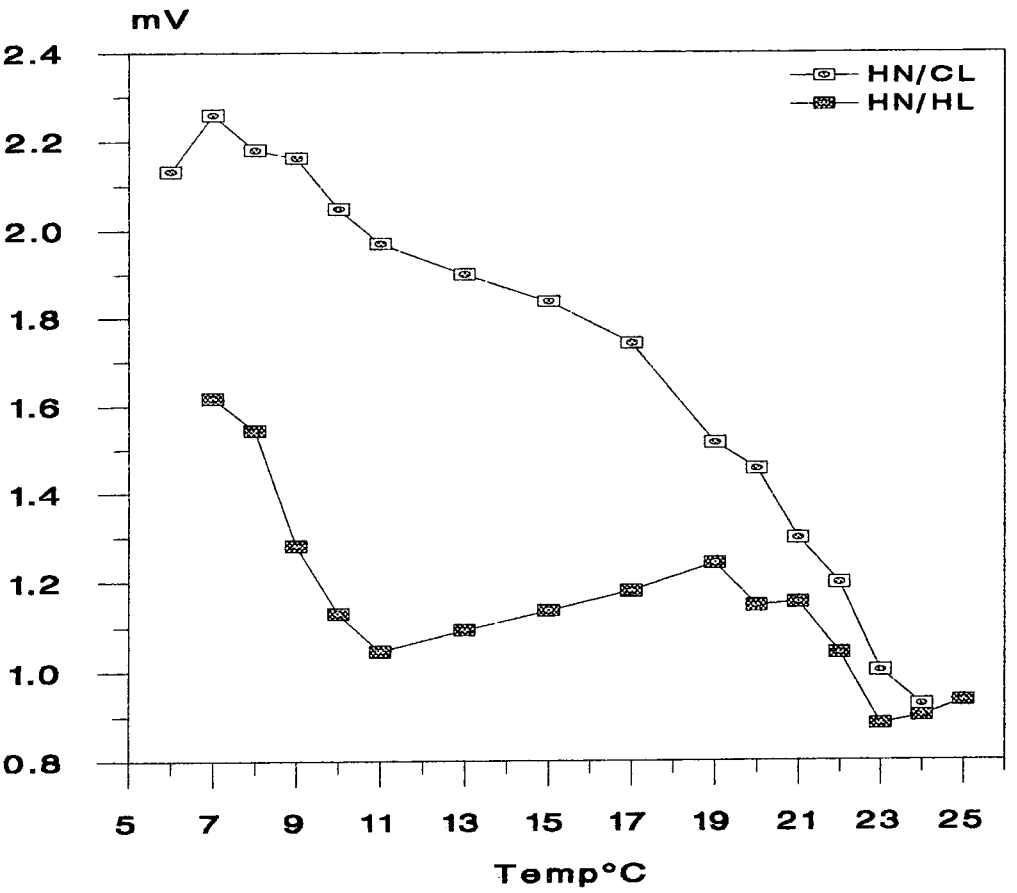


Figure 6.11B shows double EJP amplitudes of warm and cold acclimated walking legs which were acclimated with a hot CNS. The HN/CL acclimated walking leg EJPs decreased with increasing experimental temperature, typical of other cold acclimated walking legs and independent of their warm CNS acclimation temperature. Amplitudes from HN/CL crabs at temperatures warmer than 24°C were omitted as only four of the twenty crabs were responsive. HN/HL acclimated walking legs generated EJP amplitudes which were smaller over the whole experimental temperature range than HN/CL acclimated walking leg amplitudes. The HN/HL acclimated walking leg EJP amplitudes were relatively constant over 10-22°C, the EJP's decreased slightly at experimental temperatures warmer than 22°C, but increased in amplitude at experimental temperatures colder than 9°C. Despite their smaller EJP amplitudes the HN/HL acclimated walking legs maintained constant EJP amplitudes to warmer experimental temperatures than that shown by HN/CL acclimated walking legs. Amplitudes from HN/HL crabs at 6°C were omitted as only three of the seventeen crabs were still responsive. Statistical comparisons revealed HN/CL generated significantly larger (one way ANOVA) EJP amplitudes over 9-15°C than HN/HL acclimated walking legs, which again indicates no CNS influence to walking leg acclimation.

Comparison of the amplitudes shown in Figures 6.11A and B revealed no significant quantitative differences (one way ANOVA) between CN/CL and HN/HL acclimated walking legs when compared at the same temperatures, although the qualitative differences between their EJP amplitude versus temperature curves were quite clear. Comparison of CN/CL and HN/CL acclimated walking legs revealed some significant differences, i.e. HN/CL acclimated walking legs generated significantly larger EJP amplitudes over 17-20°C than CN/CL crabs. This difference did not effect the qualitatively, and for the most part quantitatively, identical decreases in EJP amplitude with increasing experimental temperature shown by cold acclimated walking legs, irrespective of the CNS acclimation temperature. Furthermore, comparisons between HN/HL and CN/HL acclimated walking leg EJP amplitudes revealed no significant differences when compared at the same experimental temperatures, which again supports the premise that the CNS acclimation temperature did not effect walking leg acclimation.

C.pagurus

Figure 6.12A shows double EJP amplitudes from warm and cold acclimated walking legs which were acclimated with a cold CNS. The CN/CL acclimated walking leg EJP amplitudes decreased with increasing experimental temperature. Whereas

Figure 6.12A: Double pulse EJP amplitude change in heterothermal *Cancer pagurus* acclimated with a cold CNS. All data presented is mean data (no error bars shown). Statistical comparisons are presented in the matrix below (one way ANOVA). Experimental numbers were; CN/CL n=17 and CN/HL n=17

Cancer pagurus Double Pulse EJP Amplitude statistical matrix.

	Free *C.pagurus* 8°C	Free *C.pagurus* 22°C	IM *C.pagurus* 8°C	IM *C.pagurus* 22°C	CN/CL	HN/CL	HN/HL
Free *C.pagurus* 22°C	6-7, 9, 23-24						
IM *C.pagurus* 8°C	6-9, 11-15, 20	6-17					
IM *C.pagurus* 22°C	6-8, 21, 23-25		6-17, 23-26				
CN/CL			6-21	20-25			
HN/CL	6-8		6-19	21-23, 25			
HN/HL	6-15		6-19, 26		11, 20		
CN/HL	6-9, 11-15		6-17, 24				

Figure 6.12B: Double pulse EJP amplitude change in heterothermal *Cancer pagurus* acclimated with a hot CNS. Mean EJP amplitudes are shown, no error bars are presented. Experimental numbers were; HN/HL n=16 and HN/CL n=14.

Figure 6.12A: Double EJP amplitude change in heterothermal Cancer pagurus acclimated with a cold CNS.

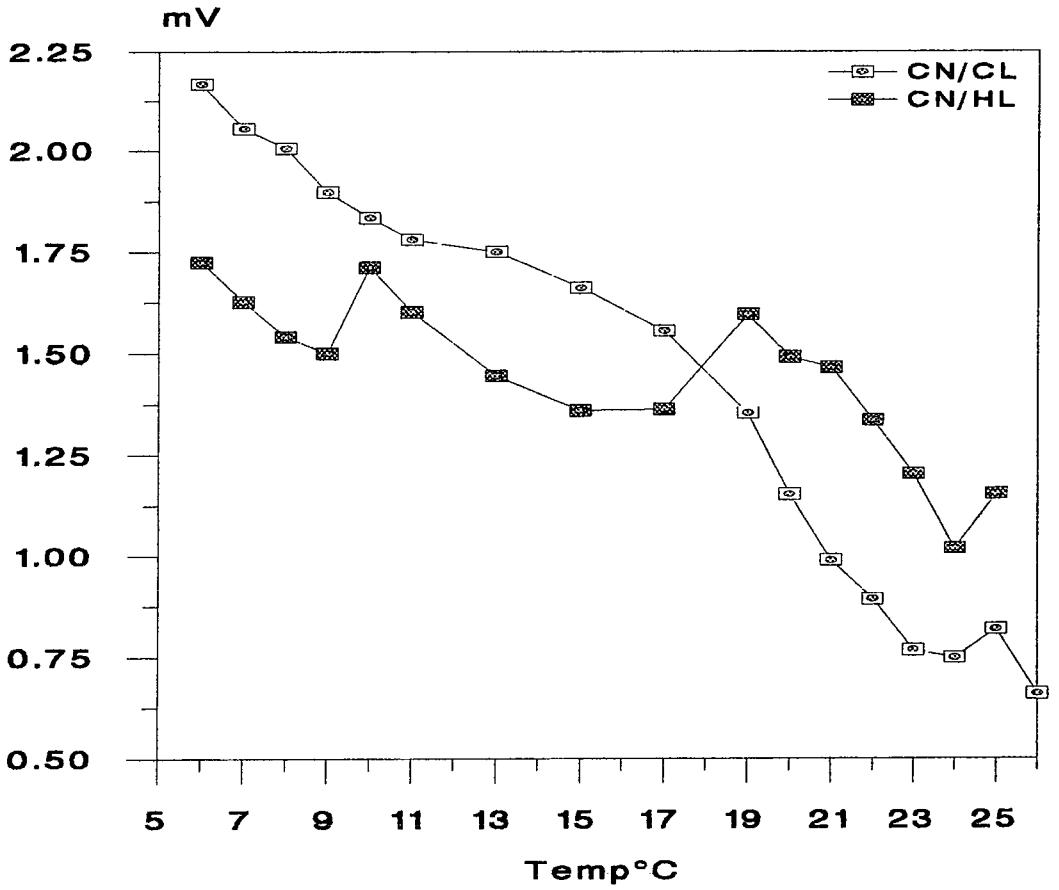
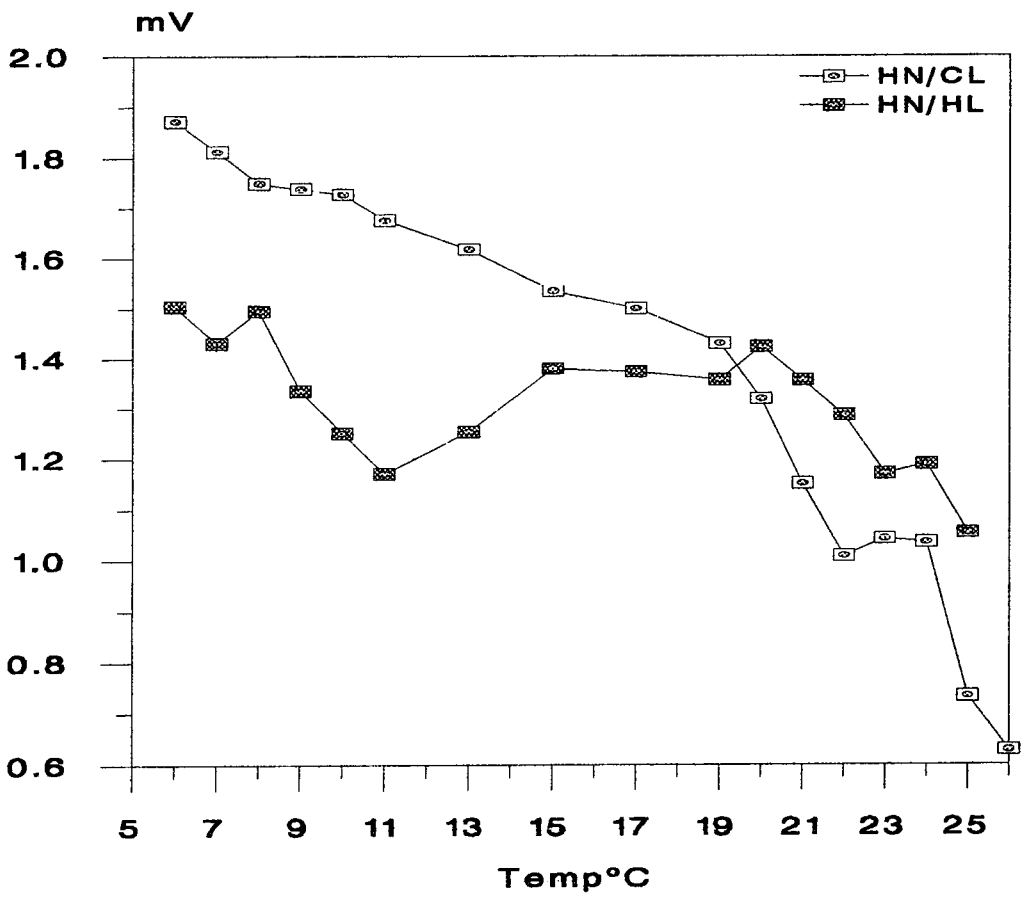


Figure 6.12B: Double EJP amplitude change in heterothermal Cancer pagurus acclimated with a hot CNS.



CN/HL acclimated walking legs exhibited relatively constant EJP amplitude over 6-21°C, their EJP amplitudes decreased at experimental temperatures warmer than 21°C. The CN/HL acclimated walking legs maintained their EJP amplitudes to warmer experimental temperatures near to their walking leg acclimation temperature. Amplitudes from CN/HL crabs at 26°C were omitted as only five of the seventeen crabs were still responsive. Statistical comparisons revealed no quantitative differences (one way ANOVA) between the CN/CL and CN/HL acclimated walking leg EJP amplitudes when compared at the same temperatures, although the qualitative differences between the two acclimatory groups was clear.

Figure 6.12B shows double EJP amplitude changes with temperature for warm and cold acclimated walking legs which were acclimated with a hot CNS. HN/CL acclimated walking leg EJP amplitudes decreased steadily over 6-19°C, but then decreased rapidly over the 19-26°C of the experimental temperature range. In comparison HN/HL acclimated walking leg EJP amplitudes revealed an n-shaped EJP amplitude versus temperature curve over a 11-25°C temperature range. Amplitudes from HN/HL crabs at 26°C were omitted as only four of the sixteen crabs were responsive. The HN/HL acclimated walking leg EJP amplitudes were maximal over 15-22°C temperature range, the EJP amplitudes increased at experimental temperatures colder than 11°C, which may again be related to result variability through increasing cold block. Statistical comparisons revealed no significant differences (one way ANOVA) between HN/HL and HN/CL acclimated walking leg amplitudes when compared at the same experimental temperatures. It was however clear that HN/HL acclimated walking legs maintained their EJP amplitudes to warmer experimental temperatures than the cold acclimated walking legs, irrespective of the CNS acclimation temperature.

Comparisons of the amplitudes shown on Figures 6.12A and B revealed almost no significant quantitative differences (one way ANOVA) between HN/HL and CN/CL acclimated walking leg EJP amplitudes, when compared at the same temperatures. The qualitative differences between the HN/HL and CN/CL acclimated walking legs was clear, the cold acclimated walking legs being clearly sensitive to increasing temperature. Comparisons of HN/HL and CN/HL acclimated walking leg EJP amplitudes again revealed no qualitative or quantitative differences, indicating no CNS influence. Furthermore comparisons of CN/CL and HN/CL acclimated walking leg EJP amplitudes revealed no qualitative or quantitative differences again indicating no CNS influence to walking leg double EJP amplitude acclimation.

Analysis of temperatures at which maximal EJP amplitudes were recorded.

To analyse further the effect of thermal acclimation and experimental temperature on EJP amplitude, the temperature at which a maximal EJP amplitude was initiated in an individual walking leg preparation was noted. These temperatures were pooled (see Table 6.7) for each acclimatory group and comparisons made between the acclimatory groups using one way ANOVA.

It can be seen that there were clear differences between cold and warm acclimated walking legs, irrespective of the species and type of acclimation. Statistically cold acclimated *C.maenas* single EJP amplitudes were generated at significantly (one way ANOVA) colder temperatures than found for all warm acclimated walking legs. This was also the case for double pulse stimulated EJP amplitude temperature measurements, i.e. cold acclimated walking legs initiated maximal EJP amplitudes at significantly colder temperatures than found for warm acclimated walking legs.

Identical results were determined for *C.pagurus*, all cold acclimated walking legs generated maximal single and double EJP amplitudes at significantly colder temperatures than in warm acclimated walking legs. Where, CN/HL acclimated *C.pagurus* also initiated maximal single EJP amplitudes at significantly (one way ANOVA) colder temperatures than HN/HL acclimated walking legs. The results clearly show an acclimatory shift for all warm acclimated walking legs which was independent of the CNS acclimation temperature.

Table 6.7: Mean (±S.E.mean) temperatures at which maximal EJP amplitude's were initiated in individual experiments for different acclimatory groups (n= number of preparations).

	Temperature at which maximal EJP amplitude were recorded (°C).							
	Carcinus maenas.				*Cancer pagurus.*			
Acclimation Type	**Single Amplitude**	**n**	**Double Amplitude**	**n**	**Single Amplitude**	**n**	**Double Amplitude**	**n**
Free homothermal 8°C	10.6±1.0	26	8.4±0.9	26	9.5±0.62	18	6.9±0.3	17
Free homothermal 22°C	14.1±1.1	22	14.3±1.2	22	15.8±0.99	17	15.8±0.98	19
Homothermal IM 8°C	10.8±0.9	15	8.7±1.3	15	10.3±1.1	16	6.8±0.3	16
Homothermal IM 22°C	18.1±1	23	18.8±1.1	23	18.8±1.1	13	18.2±1.9	13
CN/CL	9.3±0.98	16	7.8±0.63	15	9.5±0.9	16	6.9±0.5	17
HN/CL	9.4±1.0	21	9.2±1.2	20	11.1±1.4	15	9.6±1.3	14
HN/HL	15.4±1.1	16	16.1±1.4	16	17.8±0.7	15	16.6±1.	16
CN/HL	16.4±0.9	15	14.5±1.4	15	14.6±1.2	16	15±1.3	16

Figure 6.13A: Single EJP decay time constant in heterothermal *Carcinus maenas* acclimated with a cold CNS. The EJP decay time constant is the time in milliseconds it took an EJP to decay to 63% of its maximal value. Mean data is presented for all acclimatory groups, error bars are shown for CN/CL only. Experimental numbers were; CN/CL n=16 and CN/HL n=14. Statistical analysis (one way ANOVA) was done comparing all acclimatory groups and is shown in the matrix below.

Figure 6.13B: Single EJP decay time constant in heterothermal *Carcinus maenas* acclimated with a hot CNS. Mean EJP decay values are presented, error bars are shown for HN/CL only. All data sets were fitted with exponential curves using a least squares fit. Experimental numbers were; HN/HL n=15 and HN/CL n=14. All EJP decay values were analysed using one way ANOVA and result comparisons are presented in the matrix below.

Carcinus maenas EJP decay (Tau) statistical matrix.

	Free *C.maenas* 8°C	Free *C.maenas* 22°C	IM *C.maenas* 8°C	IM *C.maenas* 22°C	CN/CL	HN/CL	HN/HL
Free *C.maenas* 22°C	9-17, 21-23						
IM *C.maenas* 8°C		6, 8-17, 21-23, 25					
IM *C.maenas* 22°C		6, 8-26					
CN/CL	22-23	6, 8, 10-15		9-23			
HN/CL		6, 8, 10-17		8-11, 17-24			
HN/HL		8, 10-15		6-7, 11-22, 25			
CN/HL	8-11	8, 10-15, 22		11, 15-24			

Figure 6.13A: Single EJP decay time constant in heterothermal Carcinus maenas acclimated with a cold CNS.

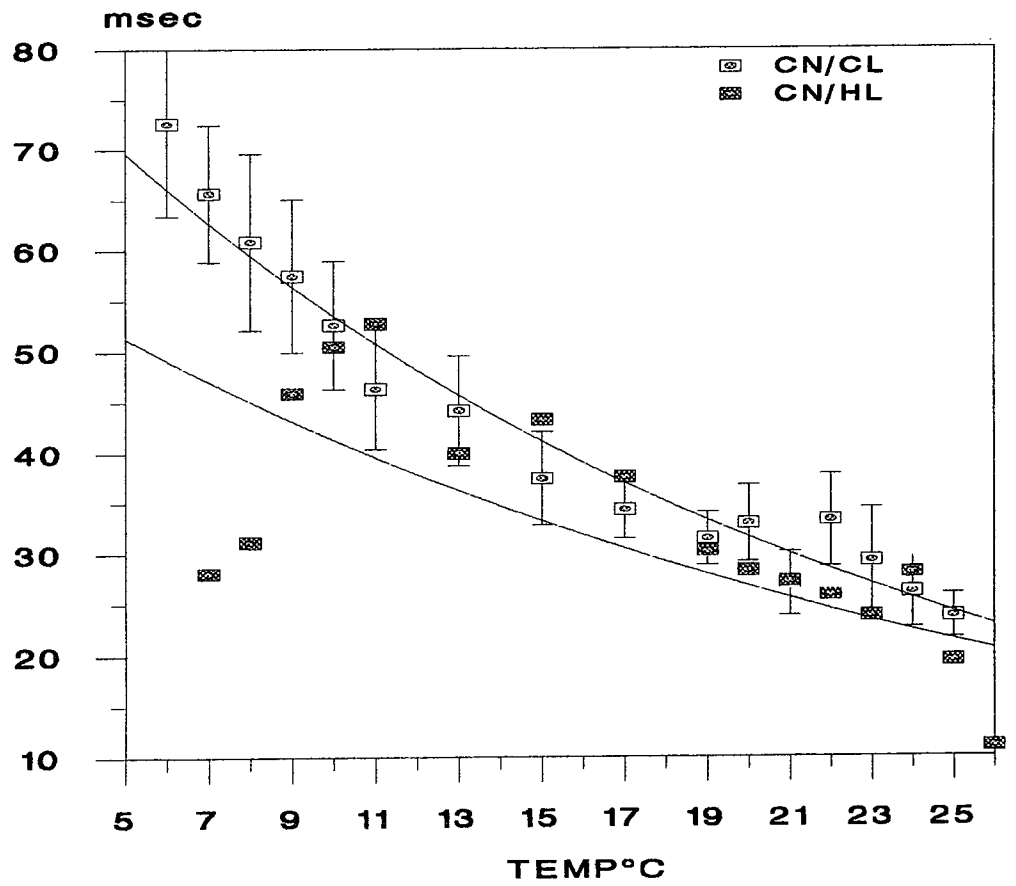
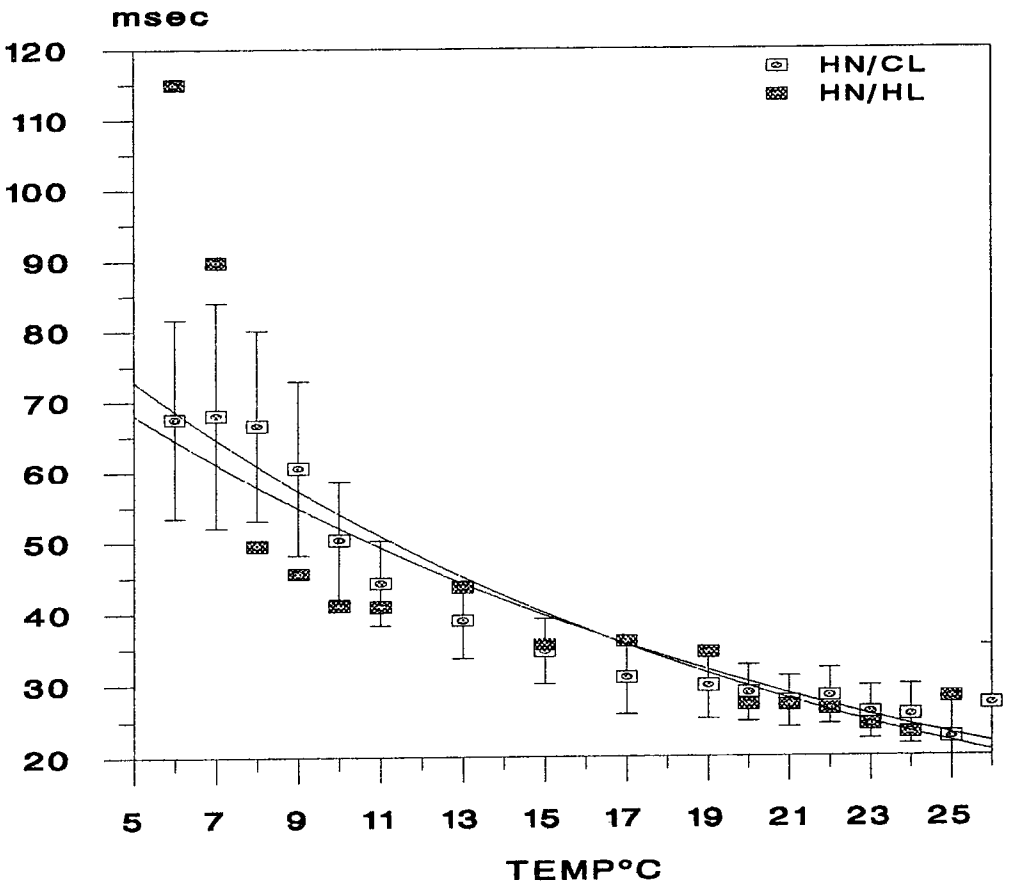


Figure 6.13B: Single EJP decay time constant in heterothermal Carcinus maenas acclimated with a hot CNS.



Cold acclimated walking legs from *C.pagurus* generated maximal double EJP amplitudes at colder experimental temperatures than *C.maenas*, although not significantly colder. Warm acclimated *C.pagurus* walking legs generated maximal EJP amplitudes at warmer (not significantly) experimental temperatures than *C.maenas* which was unexpected.

Single EJP decay time constants (Tau).

C.maenas

Tau is determined as the time (msec) it takes for an EJP to decay to 63% of its maximal amplitude, this being related to membrane resistance (R_m), as such it gives an indication of the number of open channels. The longer an EJP takes to decay, the larger the Tau value and the higher the R_m, this gives a potential for increased summation, therefore maintaining EJP amplitudes. Figure 6.13A shows changes in Tau, or the single EJP amplitude decay time constant for warm and cold acclimated walking legs which were acclimated with a cold CNS. The Tau values of CN/CL acclimated walking legs decreased with increasing experimental temperature, indicating decreasing R_m or the number of open channels increased with increasing experimental temperature. The CN/HL acclimated walking leg Tau values also decreased with increasing experimental temperature, although the Tau values did undergo a change between 7-9°C. The decreased Tau values over 7-9°C for CN/HL acclimated walking legs was clearly different from the Tau values of CN/CL acclimated walking legs. No significant differences (one way ANOVA) were found when comparing Tau values from CN/CL and CN/HL acclimated walking legs when compared at the same temperatures, the CN/CL and CN/HL acclimatory group Tau versus temperature curves were overlapping over 10-25°C temperature range.

Figure 6.13B shows Tau from warm and cold acclimated walking legs which were acclimated with a hot CNS. Tau values from both HN/HL and HN/CL acclimated walking legs decreased with increasing experimental temperatures. Their Tau versus temperature curves were identical over 8-26°C temperature range, the HN/HL acclimated walking leg Tau values over 6-7°C were much larger than expected. There were no significant differences (one way ANOVA) between HN/HL and HN/CL acclimated walking leg Tau values when compared at the same experimental temperatures.

Figure 6.14A: Single EJP decay time constant in heterothermal *Cancer pagurus* acclimated with a cold CNS. Mean data is presented, error bars are shown for CN/CL only. All data sets were fitted with a exponential curve using a least squares fit. Statistical analysis of decay values are presented in the matrix below (one way ANOVA), experimental numbers were; CN/CL n=17 and CN/HL n=14.

Figure 6.14B: Single EJP decay time constant in heterothermal *Cancer pagurus* acclimated with a hot CNS. Mean data is presented, error bars are shown for HN/CL only. All data sets were fitted with an exponential curve using a least squares fit. Where experimental numbers were; HN/HL n=13 and HN/CL n=14. Statistical analysis was performed (one way ANOVA) on the EJP decay values, the comparisons are shown in the matrix below.

Cancer Pagurus EJP decay (Tau) statistical matrix.

	Free *C.pagurus* 8°C	Free *C.pagurus* 22°C	IM *C.pagurus* 8°C	IM *C.pagurus* 22°C	CN/CL	HN/CL	HN/HL
Free *C.pagurus* 22°C							
IM *C.pagurus* 8°C							
IM *C.pagurus* 22°C		7-9					
CN/CL		7,10		6-9			
HN/CL		24-25	23-25	24-25	6-7, 24		
HN/HL			7-9		6-9		
CN/HL	9-13, 17-19	7, 10, 17		7, 9-13			7, 9-13, 17, 21

Figure 6.14A: Single EJP decay time constant in heterothermal Cancer pagurus acclimated with a cold CNS.

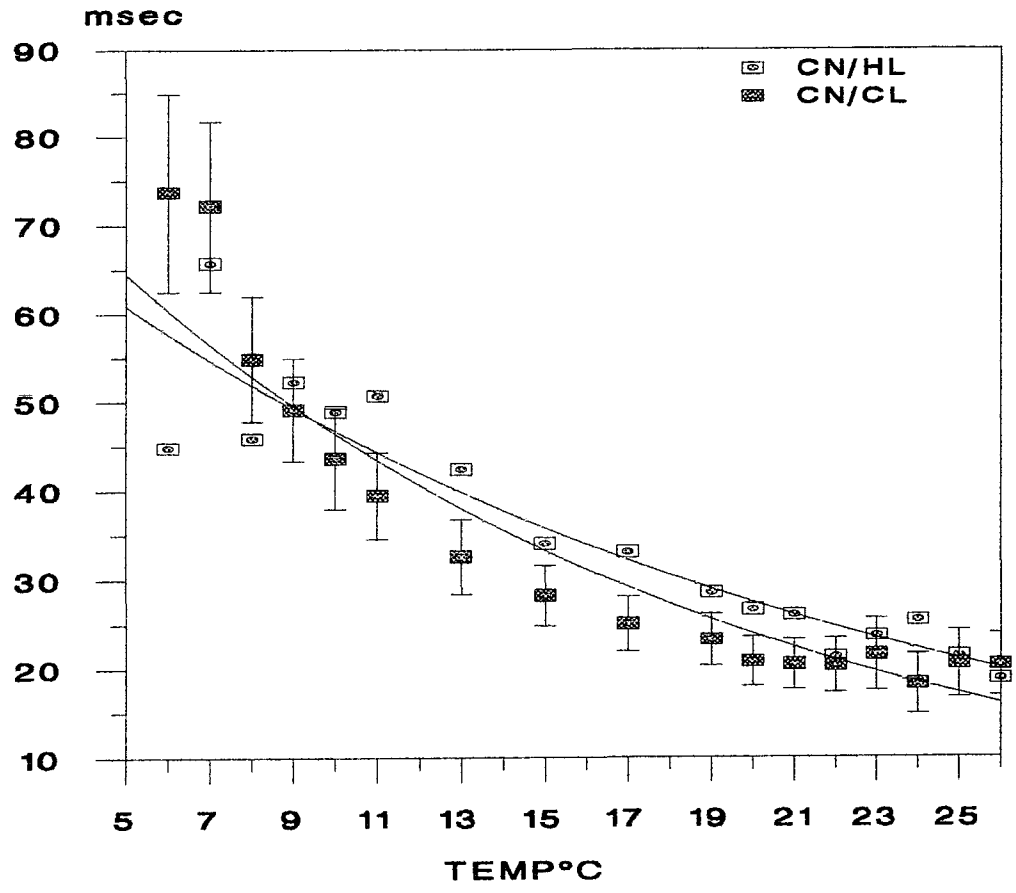
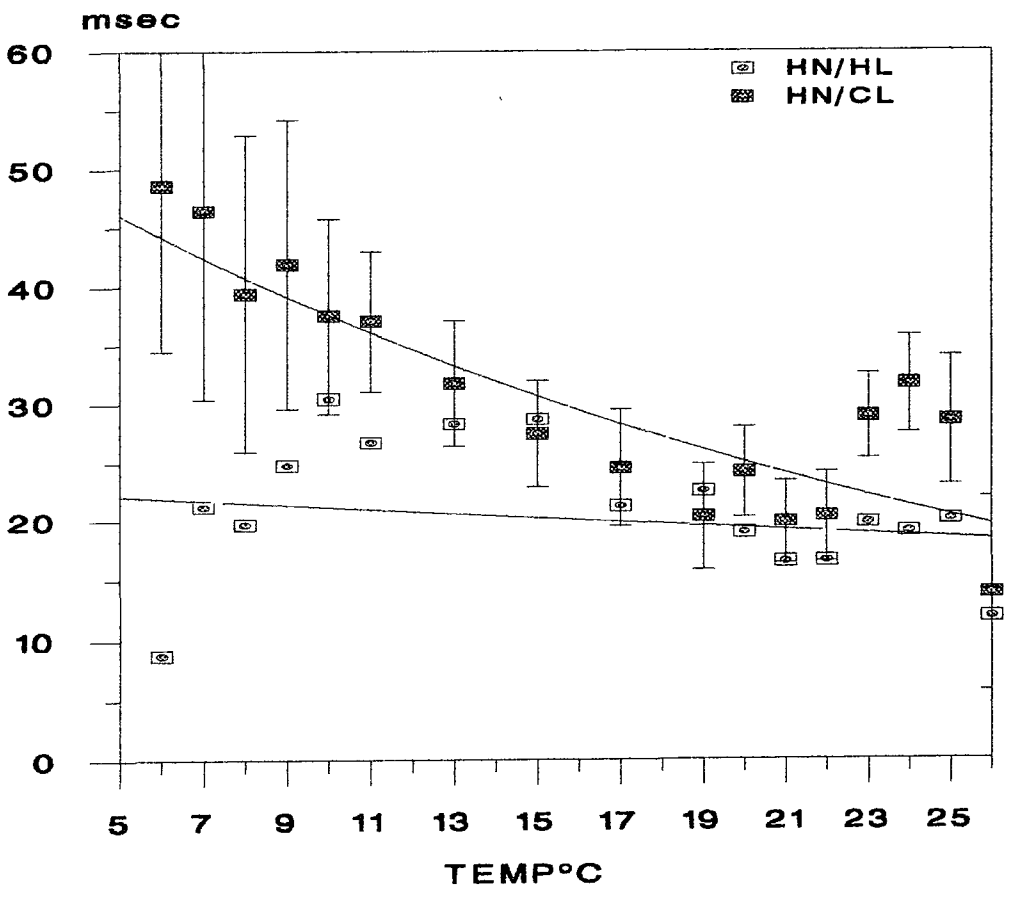


Figure 6.14B: Single EJP decay time constant in heterothermal Cancer pagurus acclimated with a hot CNS.



Comparison of the Tau values shown in Figures 6.13A and B revealed no significant differences (one way ANOVA) between any heterothermally acclimated walking leg Tau value when compared at the same temperatures.

C.pagurus

Figure 6.14A shows Tau changes with experimental temperature for warm and cold acclimated walking legs which were acclimated with a cold CNS. The CN/CL acclimated walking leg Tau values decreased with increasing experimental temperature indicating lower R_m and a lower potential for summation. CN/HL acclimated walking leg Tau values also decreased with increasing experimental temperature. There were no significant (one way ANOVA) quantitative or qualitative differences between CN/CL and CN/HL acclimated walking leg Tau values when compared at the same temperatures.

Figure 6.14B shows single EJP decay time constants from warm and cold acclimated walking legs which were acclimated with a hot CNS. The HN/CL acclimated walking leg Tau values decreased with increasing experimental temperature. Where HN/HL acclimated walking legs exhibited decreasing Tau with increasing experimental temperatures (9-26°C), although with a change in Tau over 6-9°C. The Tau values over 6-9°C were lower than expected, but the Tau values over 10-26°C from HN/HL and HN/CL acclimated walking legs were qualitatively identical. There were no significant differences (one way ANOVA) between HN/HL and HN/CL acclimated walking leg Tau values when compared at the same temperatures.

Comparison of the Tau values presented in Figures 6.14A and B revealed a small number of significant differences (one way ANOVA) between HN/HL and CN/CL acclimated walking legs, the CN/CL acclimated walking leg Tau values were significantly larger than HN/HL over 6-9°C, indicating higher R_m over that temperature range. Comparison of CN/CL and HN/CL acclimated walking leg Tau values revealed almost no significant differences (one way ANOVA) except at 6-7°C, where CN/CL acclimated walking leg Tau values were significantly larger than those of HN/CL acclimated walking legs. Furthermore comparison of HN/HL and CN/HL acclimated walking leg Tau values revealed a larger number of significant differences (one way ANOVA) over 7-13, 17°C, where HN/HL acclimated walking leg Tau values were significantly shorter over that temperature range. The number of significant differences between all *C.pagurus* heterothermal acclimatory group Tau values was small, this indicated that Tau could not be used to investigate a potential CNS influence on walking

Figure 6.15: Muscle fibre diameters of free and heterothermally acclimated *Carcinus maenas*. All data is presented as mean±S.E mean, experimental numbers are shown above each result column (i.e. number of legs measured). Statistical analysis (one way ANOVA) determined that warm acclimated legs exhibited a degree of muscle fibre hypertrophy, but with varied significance as walking leg length differences between the acclimatory groups accounted for most of the muscle fibre differences.

Figure 6.16: Muscle fibre diameters of free and heterothermally acclimated *Cancer pagurus*. All data is presented as mean±S.E mean, experimental numbers are shown above each result column where the surface muscle fibre diameters were measured. The warm acclimatory groups had significantly larger (one way ANOVA) diameter muscle fibres than the cold acclimatory groups, as no differences in leg length were found between any acclimatory group.

Figure 6.15: Muscle fibre diameters of free and heterothermally acclimated Carcinus maenas.

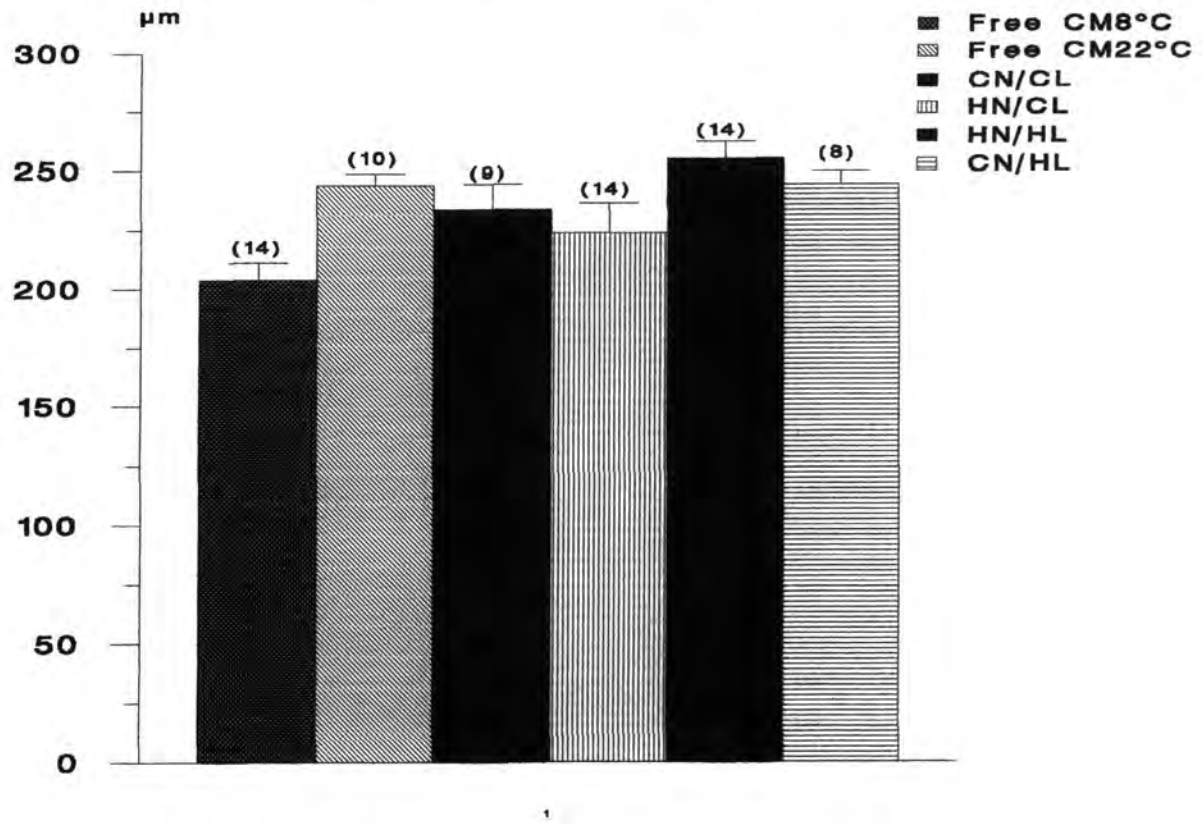
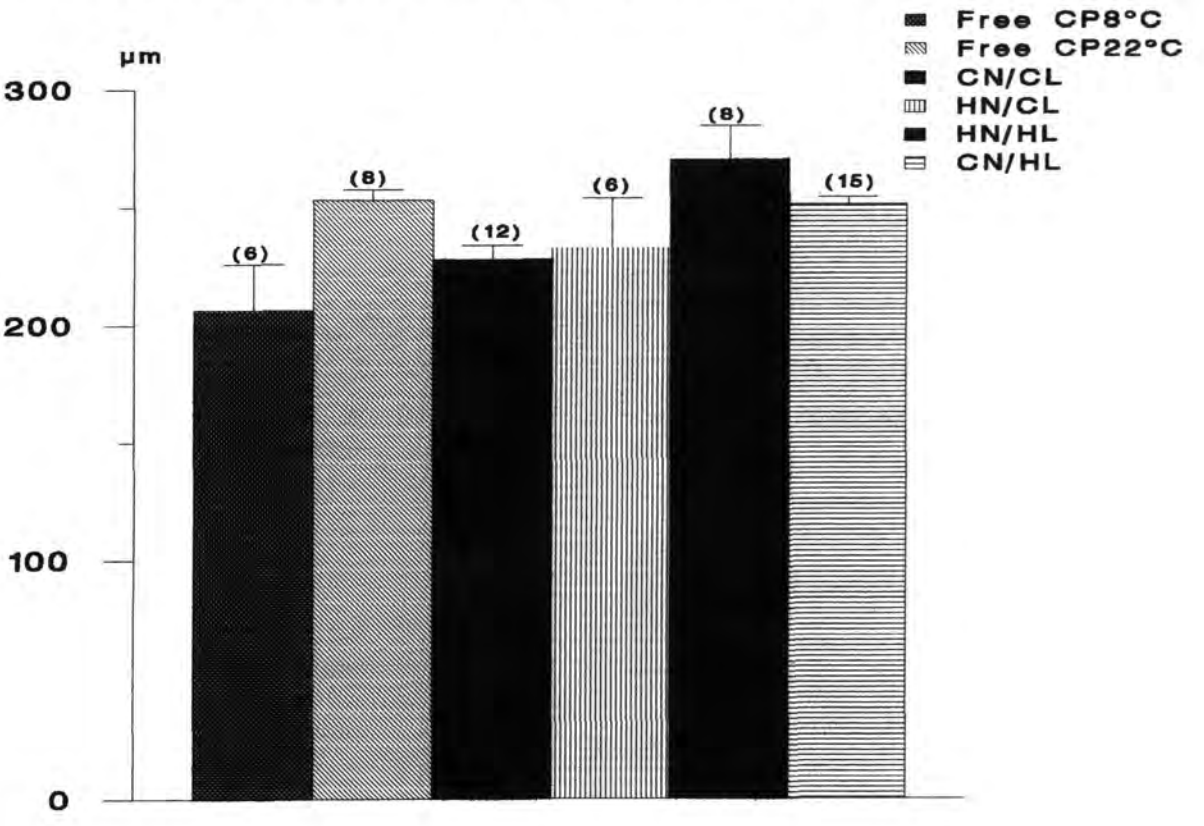


Figure 6.16: Muscle fibre diameters of free and heterothermally acclimated Cancer pagurus.



leg Tau acclimation. It was however interesting that the warm acclimated walking legs of HN/HL and CN/HL acclimatory groups exhibited irregular Tau over a 6-9°C temperature range, indicating their cold intolerance which was qualitatively different from CN/CL and HN/CL acclimatory groups.

Muscle fibre diameter and leg length.

Figure 6.15 shows mean muscle fibre diameters for all *C.maenas* experimental groups, the numbers above result columns indicate the number of preparations where surface muscle fibre diameters were measured. There were some significant differences (one way ANOVA) between *C.maenas* acclimatory groups, specifically, free *C.maenas* 8 had significantly smaller diameter muscle fibres than CN/CL, HN/HL, CN/HL and free *C.maenas* 22. HN/CL had significantly smaller diameter muscle fibres than HN/HL. The results were justified by analysing mean leg length shown in Table 6.8 below, where some significant differences (one way ANOVA) were found i.e. free *C.maenas* 8 crabs had significantly smaller length legs than all other experimental groups. The leg length significant differences effectively accounted for most of the muscle fibre diameter differences between *C.maenas* acclimatory groups, indicating muscle hypertrophy had not occurred, except in the case of HN/HL and HN/CL acclimated walking leg muscle fibre diameter comparisons.

Table 6.8: Mean (±S.E.mean) leg length measured in *C.maenas* and *C.pagurus* acclimatory groups (n= number of different preparations).

	Mean leg length (cm).			
Acclimation Type.	*C.maenas*	n	*C.pagurus*	n
Free homothermal 8°C	7.46±0.38	5	8.15±0.17	6
Free homothermal 22°C	8.32±0.13	9	7.7±0.15	8
Homothermal immobilised 8°C	-	-	7.88±0.08	13
Homothermal immobilised 22°C	8.9±0.09	16	8.12±0.06	12
CN/CL	8.7±0.14	8	7.8±0.26	9
HN/CL	8.4±0.19	11	8.21±0.19	6
HN/HL	8.6±0.14	12	7.9±0.33	8
CN/HL	8.2±0.25	8	7.93±0.14	12

Figure 6.16 shows mean muscle fibre diameters for homothermal and heterothermally acclimated *C.pagurus*. Significant differences (one way ANOVA) were

found between; free *C.pagurus* 8 which had significantly smaller muscle fibre diameters than CN/HL, HN/HL and free *C.pagurus* 22. CN/CL muscle fibre diameters were significantly smaller than CN/HL and HN/HL. HN/CL had significantly smaller diameter muscle fibres than HN/HL. There were no significant differences in measured mean leg length (see Table 6.8) to account for the muscle fibre diameter changes with acclimation temperature. The muscle fibre diameter differences can be wholly attributed to the different walking leg acclimation temperatures. Warm acclimation induced walking leg muscle fibre hypertrophy even in un-fed preparations. The leg acclimation temperature (see Figure 6.2B) to muscle fibre diameter correlation was good, i.e. free *C.pagurus* 8 experienced colder acclimation temperatures which therefore maintained its smaller diameter muscle fibres. The acclimation temperature to muscle fibre diameter relationship was also good for CN/CL and HN/CL acclimatory groups, their slightly warmer acclimation temperatures resulted in slightly increased diameter muscle fibres.

Discussion

No changes were found in sea water conductivity and so no serious persistent evaporation occurred during the course of crab acclimation. In consequence, the crabs would not have experienced significant changes in haemolymph ionic or osmotic concentrations, thus this possibility can be dismissed from consideration as a factor complicating acclimation.

Within the heterothermal apparatus, compartment temperatures were maintained at 21.25±0.12°C and 8.9±0.41°C. This temperature difference was 12.35°C, which was significantly lower than the difference in acclimation condition for homothermally acclimated crabs, which was 14°C. The difference of 1.65°C in acclimation temperatures between the two protocols is not considered to invalidate the heterothermal protocol.

Consideration however, must be given to the comparisons of the acclimation temperature experienced by the tissue. The temperature experienced by walking leg muscle was assumed to be the same as for chela muscle in the same animal (see Figure 6.2A/B). In both species, chela muscle temperatures were not different from the bath temperature to which they were exposed. Thus, it is possible to maintain peripheral walking legs at the external water bath temperature to which they are directly exposed. The determination of CNS temperature in heterothermally acclimating crabs also shows the CNS can be maintained close to the temperature of the water bath, notwithstanding the other side of the crab is experiencing a markedly different temperature. However,

CNS temperatures were always displaced by 1-2°C towards the other water bath temperature. For example when the water bath temperature was 21.84±0.06°C or 8.3±0.15°C, the CNS temperature was found to be either 19.0±0.2°C or 9.9±0.1°C respectively, depending on where the membrane was placed separating the bath temperatures. In effect the difference in CNS temperatures achieved between CN and HN was 9.1°C (from Figure 6.1A/B), which was a smaller difference than was found between the water baths, which was 13.54±0.17°C in that experiment. These acclimation temperature differences were also smaller than that set for homothermal acclimation, which was 14°C. This is a factor that must be considered in comparisons between homothermal and heterothermally acclimated crabs. The heterothermal CNS acclimation temperature differences were smaller than the heterothermal walking leg acclimation temperature differences. It is likely that the mixing of haemolymph from the cold and warm exposed gills is the main factor in narrowing the temperature difference between CNS and contralateral limb temperatures. However, the sluggishness of the circulatory system clearly allowed the direct effect of bath temperature to be the primary determinant of CNS temperature. Fahmy (1972) heterothermally acclimated carp to both 10°C and 20°C, she reported mean whole fish body temperatures to be 13.2°C and 16.2°C for head sections acclimated at 10°C and 20°C respectively. Unfortunately Fahmy did not measure CNS, head or tail temperatures *in situ*, it is clear that our heterothermal protocol is more thorough.

The voltage thresholds to initiate an EJP in heterothermally acclimated walking leg closer muscles are shown in Table 6.2, the results were comparable to those reported for control homothermal free crabs, see Table 4.0. Successful identification of the tonic motor axon in all experimental preparations identified all results as being recorded from muscle fibre types I and II only. Rathmayer and Hammelsbeck (1985) reported tonic axons innervated muscle fibre types I and II only, whereas phasic axons innervated muscle fibre types III and IV only. It was observed from the data in Table 6.2 that tonic axon thresholds from heterothermally acclimated crabs were generally (significantly in some cases) higher than those from equivalent free homothermal control walking legs, shown in Table 4.0, irrespective of species and temperature of acclimation. Differences in motor axon voltage threshold are dependent on axon diameter (Castillo and Machne 1953; Adams 1987; Fatt and Katz 1953). What is important is that heterothermally acclimated walking leg tonic motor axons had voltage thresholds which were always significantly smaller than those of phasic motor axons, irrespective of the type and temperature of acclimation. Atwood *et al.*, (1994) reported that phasic synaptic terminals were very slender and threadlike, this may account for the higher voltage

thresholds of phasic axons due to their increased input resistance, a view also supported by Nicholls *et al.*, (1992).

There were no significant differences between any phasic motor axon voltage threshold irrespective of the acclimation protocol. King *et al.*, (1996) reported that *Procambarus clarkii* had phasic synapses which had a higher proportion (50%) of multiple active zones than tonic synapses. The phasic synapses were associated with a greater capacity for neurotransmitter release, which may also account for the reduced variation in voltage thresholds shown here between phasic axons (King *et al.*, 1996) over the experimental temperature range. In contrast there were some significant differences between the tonic motor axon voltage thresholds of heterothermally acclimated walking legs when compared to free homothermal tonic axon voltage thresholds. The synaptic endings of crayfish tonic axons are reported to be less profuse than phasic axons and may indicate a phenotypic reason for the variation in thresholds seen in tonic axons (Atwood *et al.*, 1994; Atwood and Nguyen 1995). That is, a decreased number of synaptic terminals may increase the probability of branch point or axon terminal heat block, requiring an increase in the stimulation voltage (White 1983) with increasing experimental temperature, this could account for the differences between differently acclimated tonic motor axon voltage thresholds. Differences between crab walking leg tonic axon stimulation voltage thresholds cannot be attributed to axon damage during dissection, as no equivalent voltage threshold changes were found for phasic axons. The tonic motor axon stimulation voltage differences observed must therefore be attributed to the acclimation temperature affecting the synaptic terminals and diameter of the motor axons (or size of crab).

Both species of crab when heterothermally acclimated had walking legs which exhibited hyperpolarising RP with increasing experimental temperature, whether the walking legs were cold or warm acclimated. Similar hyperpolarising RPs have been reported by many groups, such as for crab by Stephens and Atwood (1982), for crayfish by White (1983) and Kivivuori *et al.*, (1990). The RP changes with temperature (or slope) of all acclimatory groups are significantly greater than that predicted by Nernst (0.3163mV/°C). White (1983) reported the temperature dependency of RP change was due to changes in Na^+/K^+ ATPase activity, a view supported by Prosser and Nelson (1981) who also identified temperature induced changes in Na^+/K^+ permeability ratio. To account for temperature induced changes in Na^+/K^+ ATPase activity, muscle fibres from free cold acclimated *C.maenas* were incubated with ouabain, this reduced the temperature dependency of the RP to 0.4065mV/°C. This clearly supports the view that all but 22% of the RP change with temperature can be accounted for by changes in

Na^+/K^+ ATPase activity described by White (1983), the 22% discrepancy can be accounted for by ATP independent processes such as changes in Na^+/K^+ permeability reported by Prosser and Nelson (1981). The question now arises, are there differences in the RP sensitivity to temperature between the different species and acclimatory groups?

Specifically, for *C.maenas* there were no significant differences between the RP slopes or intercepts of CN/CL and CN/HL acclimated walking legs, indicating no acclimation induced changes in RP sensitivity to temperature which was unexpected. This along with the small number of significant differences found between CN/CL and CN/HL acclimated walking leg RPs when compared at the same temperatures may indicate some CNS influence. However, it is important to point out that CN/HL acclimated walking legs did show some acclimatory compensation to the warm acclimation temperature. The CN/HL acclimated walking legs exhibited a 37.5% acclimatory shift along the temperature axis (partial acclimation, or Type III after Precht, 1958), indicating compensation in an adaptive manner against the prevailing CNS acclimation temperature. The acclimatory shift of CN/HL acclimated walking legs (Table 6.4) was smaller than that calculated for homothermal free warm acclimated *C.maenas* walking legs, which may itself be due to the lower acclimation temperature of 20.7±0.25°C experienced by CN/HL acclimated walking legs (see Figure 6.2A).

Furthermore, it should be noted that statistical comparisons (one way ANOVA) of individual RPs, slopes and intercepts from CN/HL acclimated walking legs to equivalent RP results from free *C.maenas* 22 and HN/HL acclimated walking legs revealed no significant differences. This indicates that CN/HL acclimated walking legs were not affected by a potential cold acclimated CNS hormonal influence. Further comparisons of CN/HL acclimated walking leg RPs to free *C.maenas* 8 acclimated walking leg RPs revealed a high number of significant differences when compared at the same experimental temperatures. The CN/HL RP slope was significantly smaller than that of free *C.maenas* 8 indicating a change in RP sensitivity to temperature, although no differences in RP intercept were found. This further indicates that CN/HL acclimated walking leg RPs were affected by the local acclimation temperature only. The rather small number of significant differences found between CN/HL and CN/CL acclimated walking legs is probably due to the warmer acclimation temperature of CN/CL acclimated walking legs, some 2.1°C warmer than the acclimation temperature of cold homothermal control crabs.

C.maenas CN/CL acclimated walking legs showed no significant differences from free *C.maenas* 8 acclimated walking leg RP values or RP intercepts, although free *C.maenas* 8 RP slope was significantly greater than that of CN/CL acclimated crabs. Furthermore, HN/HL acclimated walking legs showed no significant differences

between RP values or RP slope when compared to free *C.maenas* 22 acclimated crabs, although HN/HL acclimated crab RP intercept (at -70.1mV) was significantly different from free *C.maenas* 22 (at -63.76mV). The differences between free warm and cold acclimated walking leg RP compared to heterothermal control walking leg (i.e. CN/CL, HN/HL) RP responses to direct temperature respectively were minor, indicating immobilisation had no affect on the walking leg RP acclimation. However, warm acclimation did decrease the RP sensitivity to experimental temperature.

RP changes with experimental temperature for *C.maenas* walking legs acclimated as HN/CL and HN/HL revealed a number of significant differences at experimental temperatures warmer than 20°C. However, no significant differences in the RP intercepts were found for HN/HL and HN/CL acclimated walking legs, whereas a significant difference between their RP slopes (see Table 6.3) was found indicating an acclimation temperature induced change in RP sensitivity to experimental temperature. The shift in RP slope is due to acclimatory changes in membrane associated pumps, as it has been previously reported that warm acclimation decreases the density/activity of enzymes and pumps (Gelman *et al.*, 1992; Schwarzbaum *et al.*, 1991), possibly as a method of counteracting the temperature induced increases in activity at warmer temperatures. The differences in RP sensitivity to temperature probably account for the estimated acclimatory shift of 25.3% (partial acclimation, or Type III after Precht, 1958) calculated for HN/HL acclimated walking legs compared to the contralateral HN/CL acclimated walking legs. This acclimatory shift was again in a compensatory direction so as to maintain relatively constant RP values at the warm compared to cold walking leg acclimation temperatures, which indicates no CNS influence to RP acclimation. Acclimatory shifts in RP have also been reported for warm and cold acclimated crayfish (Kivivuori *et al.*, 1990; Stephens and Atwood 1982). The comparatively small number of significant differences between HN/CL and HN/HL acclimated walking leg RP values and intercepts was unexpected, and may be due to the warmer acclimation temperature of 11.3±0.71°C measured from HN/CL acclimated chela.

It is also notable that HN/CL acclimated walking leg RPs revealed only a minor number of significant differences from free *C.maenas* 8 and CN/CL acclimated walking legs, supporting the conclusion that no CNS influence on walking leg RP acclimation was evident. Furthermore HN/CL acclimated walking legs showed no significant differences in RP intercept or RP slope when compared to free *C.maenas* 8 and CN/CL acclimated crabs, further indicating no CNS influence on walking leg RP acclimation. In contrast HN/CL acclimated walking leg RPs revealed an increased number of significant differences from free *C.maenas* 22, which generally indicated that the cold acclimated walking legs response to direct temperature was determined by the local acclimation temperature. However, HN/CL acclimated walking legs showed no

significant differences in RP intercept or slope when compared to free *C.maenas* 22 indicating no changes in Na^+/K^+ ATPase activity.

In heterothermally acclimated *C.pagurus* the differences between CN/CL and CN/HL acclimated walking leg RP values were more dramatic than those shown for *C.maenas*. There were no significant differences between CN/CL and CN/HL acclimated walking leg RP slopes (see Table 6.5), although there was a significant difference between the CN/CL and CN/HL acclimatory group RP intercepts. Furthermore the CN/HL acclimated walking leg RP values were more depolarised than the RP values of CN/CL acclimated walking legs, significantly so over 8-26°C which indicated an acclimatory shift. The CN/HL acclimatory shift with respect to CN/CL acclimated walking leg RPs was 100%, indicating complete acclimation (Type II after Precht, 1958) and in this case acclimation was more complete than that of free homothermal warm acclimated *C.pagurus*. As the RP slopes were not different no changes in the temperature sensitivity of Na^+/K^+ ATPase were evident, therefore it may be that there were changes in pump density only.

It is also noted that the CN/HL acclimated walking leg RPs exhibited an increased number of significant differences from free *C.pagurus* 8 RP values over 6-25°C when compared at the same experimental temperatures. CN/HL acclimated walking leg RP slope was significantly smaller than that of free *C.pagurus* 8, which indicated no CNS influence on walking leg RP acclimation although no differences in their RP intercepts were found. Furthermore, CN/HL acclimated walking leg RP slopes and intercepts were not significantly different from those of free *C.pagurus* 22 and HN/HL acclimated crabs. CN/HL acclimated walking leg RPs did reveal some significant differences from RP values of free *C.pagurus* 22 and HN/HL acclimated crabs at warmer experimental temperatures. The differences in RPs from CN/HL acclimated walking legs compared to other warm acclimated walking legs can be discounted, as the CN/HL acclimated walking leg RPs were shifted along the temperature axis showing complete compensation, i.e. CN/HL acclimated walking legs exhibited complete acclimation whereas other warm acclimated control walking legs only exhibited partial acclimation. These results indicate no CNS influence on CN/HL walking leg RP acclimation, all RP changes with temperature were affected by the local acclimation temperature.

The RPs of *C.pagurus* HN/CL and HN/HL acclimated walking legs were significantly different over most of the experimental temperature range. The HN/HL acclimated walking legs exhibited a 58% acclimatory shift (partial acclimation, or Type III after Precht, 1958). The HN/HL acclimatory shift was estimated with respect to HN/CL acclimated walking legs and was the smallest acclimatory shift calculated for all *C.pagurus* acclimatory groups (see Table 6.6), and may be due to the slightly lower

acclimation temperature of 21.2±0.41°C measured from HN/HL acclimated walking legs. There were no significant differences between the HN/HL and HN/CL acclimated walking leg RP slopes or intercepts (see Table 6.5), indicating no change in the membrane associated pump density or sensitivity to experimental temperature despite a clear acclimatory shift.

Comparison of HN/CL acclimated walking leg RPs to free control warm acclimated (i.e. free *C.pagurus* 22) walking leg RP values revealed a high number of significant differences over most of the experimental temperature range when compared at the same temperatures. HN/CL acclimated walking legs showed no significant differences in RP intercept when compared to free *C.pagurus* 22, but did show a significant difference between their RP slopes which confirmed a change in RP sensitivity to temperature and showed no CNS influence. Furthermore, HN/CL acclimated walking legs revealed no significant differences of any kind between the RPs, slopes or intercepts when compared to CN/CL acclimated walking legs, which further indicates no CNS influence to walking leg acclimation. Comparison of HN/CL and free *C.pagurus* 8 acclimated walking leg RP intercepts revealed no difference, whereas HN/CL acclimated walking leg RP slope was significantly smaller than the slope of free *C.pagurus* 8 acclimated crabs. Furthermore, HN/CL and free *C.pagurus* 8 individual RPs did reveal an increased number of significant differences, where few were expected, this may indicate some CNS influence. However, HN/CL crabs were acclimated at a temperature 1.6°C warmer than that of free *C.pagurus* 8 which may account for the differences i.e. a warmer walking leg acclimation temperature shifted the HN/CL RPs along the temperature axis in a somewhat warm adapted manner when compared to colder acclimating free *C.pagurus* 8 walking leg RP values.

Free *C.pagurus* 8 RPs showed a small number of significant differences at warmer experimental temperatures from CN/CL acclimated crabs when compared at the same temperatures, free *C.pagurus* 8 RP slope was also significantly greater than that of CN/CL acclimated walking legs although no significant difference in their RP intercept were found. In contrast no significant differences of any kind were found between free *C.pagurus* 22 and HN/HL acclimated walking leg RP results. The increased sensitivity of free *C.pagurus* 8 RP to experimental temperature when compared to CN/CL crabs indicates some immobilisation effect, although this was not found in warm acclimated *C.pagurus* and has no consequences on the investigation of the control site of thermal acclimation.

Both species of crab, whether warm or cold acclimated in the heterothermal apparatus showed decreasing latency with increasing experimental temperature.

Latency is the time taken for an AP to travel (i.e. conduction velocity) to an axon terminal and successfully initiate a depolarisation of the post-synaptic membrane (i.e. synaptic delay). Decreasing latency with increasing experimental temperature is due to thermal activation of Na^+ channel function and changes in synapse function (MacDonald 1990). Latent period changes through acclimation are dependent on the axon diameter and number of Na^+ channels, increases of both these factors shorten the latent period (Adams 1987; MacDonald 1990; Hille 1992). It was not unreasonable to estimate which parameter accounted for most of the latent period, i.e. conduction velocity or synaptic delay. This was done by comparing conduction times of warm and cold acclimated free homothermal *C.maenas* and *C.pagurus* (conduction velocity times were taken from Cuculescu 1996) to the latent periods shown in Figure 6.5 and 6.6 for warm and cold acclimated walking legs. It was determined that conduction time accounted for most of the latent period in walking legs from warm and cold acclimated *C.maenas* and *C.pagurus*, irrespective of the CNS acclimation temperature, the synaptic delay was proportionately small. However, latency changes in an intact walking leg may be different from conduction time changes in an isolated nerve, due to differences in synapse function when compared at the same experimental temperatures especially at temperature extremes (White 1983; Prosser and Nelson 1981). Some workers found conduction velocity curves with Arrhenius style break points of slope, such as in giant nerve of *Lumbricus terrestris* (Lagerspetz and Talo 1967), giant axon of squid (Eastern and Swenberg 1975) and lobster nerve from *Homarus gammarus* (Romey *et al.*, 1980). The correlation coefficients of all latency versus temperature curves here were high (for both *C.maneas* and *C.pagurus*) and indicated no discontinuities of slope.

It is worth noting here that control immobilised warm and cold acclimated latent periods exhibited a clear immobilisation effect (Chapter Four), this indicates heterothermally acclimated latency comparisons to free control latencies are equivocal. However, all heterothermally acclimated walking leg latencies were as different from the immobilised (warm and cold) latent periods as the homothermal free (warm and cold) acclimated walking leg latencies were. This might indicate some effect of anomalous immobilisation on heterothermally acclimated walking leg latencies, despite their period of restraint.

Latent periods from *C.maenas* HN/CL acclimated walking legs were significantly longer over most of the experimental temperature range (Figure 6.5B) than all warm acclimated walking legs whether free or heterothermally acclimated. The shorter latent periods of warm acclimated walking legs indicated inverse or Type V acclimation (after Precht, 1958), and so no compensatory changes occurred in latency to compensate for the direct effects of temperature. Cunningham (1995) showed latent period acclimatory shifts in carp horizontal cells acclimated to 8, 16 and 26°C, latency was displaced

towards the acclimation temperature to keep the latent period constant at those temperatures. Comparisons of HN/CL acclimated walking leg latent periods to cold acclimated control latent periods (i.e. free *C.maenas* 8 and CN/CL) revealed no significant differences, indicating no CNS influence to HN/CL walking leg latency acclimation.

Both *C.maenas* CN/HL and CN/CL acclimated walking leg latent periods decreased with increasing experimental temperature, the decreases in latency with experimental temperature were qualitatively and quantitatively identical which might indicate some CNS influence. Furthermore, CN/HL acclimated walking leg latent periods revealed no quantitative differences from the cold acclimated homothermal control walking leg (i.e. free *C.maenas* 8) latent periods, which may also indicate some CNS effect. CN/HL acclimated walking legs revealed an increased number of significant differences from free *C.maenas* 22 and HN/HL acclimated walking leg latent periods, which again supports a potential cold acclimated CNS effect on walking legs exposed to a warmer acclimation temperature. However, it is important to note that the actual tissue acclimation temperature of CN/HL acclimated walking legs was 20.7±0.23°C which was somewhat colder than the homothermal control (22°C) acclimation temperature. This lower acclimation temperature would shift the CN/HL acclimated walking leg latent periods slightly in a cold acclimated direction, which may account for the increased number of differences shown by CN/HL acclimated walking legs when compared to warm acclimated control walking legs. In Chapter Four an immobilisation effect was noted, which may further influence the latent period results here. However, all heterothermally acclimated walking leg latent periods shown in Figures 6.5A/B were closer to the respective free homothermal cold and warm acclimated control latent periods than to the equivalent immobilised walking leg latent periods. This may indicate the immobilisation effect on latency was more pronounced for immobilised homothermally acclimated than heterothermally acclimated crabs, but still doesn't change the conclusion of a possible CNS influence to CN/HL acclimation.

Comparison of free *C.maenas* 8 acclimated walking leg latent periods to those from CN/CL crabs revealed almost no significant differences. Furthermore, no significant differences were found between free *C.maenas* 22 and HN/HL acclimated walking leg latent periods confirming that the immobilisation effect was more prevalent in immobilised controls than heterothermally acclimated crabs. It also confirms that free and heterothermal control walking leg latent period changes in response to direct temperature were the same, and that no acclimation was evident.

C.pagurus acclimated as CN/HL showed latencies that were shorter than CN/CL acclimated walking legs over the whole experimental temperature range, being

significantly shorter at experimental temperatures colder than 13°C. This indicated little or no CNS effect. The CN/HL acclimated walking leg latent periods revealed no significant differences from cold acclimated homothermal control walking leg latent periods (i.e. free *C.pagurus* 8) which was unexpected and may indicate some CNS influence. However, CN/HL acclimated walking legs only showed a small number of significant differences form control warm acclimated walking legs (i.e. free *C.pagurus* 22, HN/HL) at experimental temperatures colder than 11°C. It cannot be identified whether a CNS influence was acting on *C.pagurus* CN/HL acclimated walking leg latencies here as the significant differences which indicated a CNS influence were small and may be due to the period of restraint or difference in acclimation temperatures. The immobilisation effect on latency was clearer for *C.pagurus* than *C.meanas*.

Latent periods from *C.pagurus* HN/CL acclimated walking legs compared to all other heterothermal and free homothermally acclimated walking legs revealed no significant differences, irrespective of the walking leg or CNS acclimation temperature. Which along with no significant differences being found between control homothermal free cold and warm acclimated walking leg latencies, or between latencies from CN/CL and HN/HL heterothermal controls indicated latency could not be used to investigate if a potential CNS influence was acting during latency acclimation. Comparison of latent periods from CN/CL and HN/HL to latent periods from free *C.pagurus* 8 and free *C.pagurus* 22 respectively showed no significant differences of any kind. Thus, confirming free and heterothermally acclimated walking leg latencies response to direct temperature was the same and that no significant acclimatory compensation occurred. MacDonald (1994) reviewed conduction velocity in tortoise, where the latent period did not show acclimation to temperature.

The changes of single and double pulse stimulated EJP amplitude with experimental temperature from both species when their walking legs were either warm or cold acclimated were qualitatively similar and so both species results will be discussed together. Double EJP amplitudes include a facilitative component which adds to their amplitudes, this facilitative component has almost no qualitative affect on the shape of double pulse stimulated EJP amplitude versus temperature curves when compared to similarly acclimated single EJP amplitude curves. The qualitative difference between single and double pulse stimulated EJP amplitude versus temperature curves was found for cold acclimated walking legs only, in that single EJP amplitudes from both species were generally constant over 6-15°C whereas double EJP amplitudes from both species were clearly decreasing over that temperature range. This small difference in curve shape between single and double EJP amplitudes over that 6-

15°C temperature range is accounted for by the facilitative component included in double EJP amplitudes. In contrast, single EJP amplitudes do not incorporate any facilitative component as the frequency of stimulation at 0.5Hz was too low (see Chapter Three), therefore single EJP amplitude changes over the experimental temperature range are dependent on direct temperature, which affects membrane resistance and neurotransmitter release but also successful AP conduction.

The important point to make is that both species' cold acclimated walking leg EJP amplitude versus temperature curves were a different shape from the warm acclimated walking leg EJP amplitude versus temperature curves. The cold acclimated walking leg EJP amplitudes decreased with increasing experimental temperature, whereas warm acclimated walking leg EJP amplitudes were relatively constant over the same experimental temperature range, these differences in curve shape in response to direct temperature were independent of the CNS acclimation temperature but were dependent on the local acclimation temperature. EJP amplitudes are dependent on many factors, such as, neurotransmitter release, membrane resistance, excitability of the post-synaptic membrane etc. (for a review on transmitter release see Matthews 1996).

Calcium sensitivity is reported to decrease with increasing experimental temperature (Stevens and Godt 1990), as neurotransmitter release is calcium dependent (Matthews 1996), this reduced calcium sensitivity may affect transmitter release at warmer experimental temperatures and explain the decreasing EJP amplitudes of cold acclimated walking legs. However, White (1983), Montgomery and MacDonald (1990) have reported increases in quantal content and release with increasing temperature which may reduce the effect of decreasing calcium sensitivity. In contrast Van der Goor *et al.* (1995) reported that *Drosophila* had a temperature sensitive allele of the *shibire* locus, *Drosophila* when moved to warm temperatures were paralysed. The paralysis was linked to depleted neurotransmitter vesicles in nerve terminals as seen by electron microscopy. Additionally, Ca^{2+} affects active zone organisation with respect to vesicle release as shown in 15°C acclimated *Rana pipiens* (Meriney *et al.*, 1996), where preparations bathed in low Ca^{2+} (0.1nM) exhibited disrupted active zones, which altered transmitter release (although to a small extent). It has been reported by White (1976) that extracellular calcium concentration is important in the function of frog neuromuscular junction. Frog neuromuscular heat block occurred at 36°C for preparations bathed in 1.0 or 0.5mM Ca^{2+}, and block occurred at 33°C for preparations bathed in 0.0mM Ca^{2+}. These workers results indicate $[Ca^{2+}]_i$ is important with respect to EJP amplitude. Peng (1996) reported that bullfrog isolated ganglia had Ca^{2+} stores in nerve terminal smooth endoplasmic reticulum deep in the nerve terminal some distance from the pre-synaptic voltage gated Ca^{2+} channels, Ca^{2+} release from internal stores was linked to neuropeptide release. In contrast calcium release from post-synaptic

internal muscle stores is linked to electromechanical coupling (Large and Wang 1996) and CICR (Györke and Palade 1994). Post-synaptic calcium release from sarcoplasmic reticulum (Hain *et al.*, 1994; Ikemoto and El-Hayek 1996; Meis *et al.*, 1996) is normally associated with muscle contraction, although contraction was not recorded here, some of the calcium contributing in muscle fibre depolarisation may be from internal stores. However decreases in vesicular calcium release are reported with increases in experimental temperature (Hidalgo and Donoso 1995) as are changes in Ca^{2+}-ATPase activity (Meis *et al.*, 1996). Changes in $[Ca^{2+}]_i$ may be profound over the experimental temperature range, which may affect transmitter release and calcium dependent channel function according to Meriney *et al.*, (1996) and White (1976) but also affect CICR.

Atwood (1976) reported decreasing muscle Tau (or R_m) with increasing experimental temperature had an important effect on EJP amplitude, especially during a train of EJPs, decreasing Tau reduces summation and therefore reduces muscle depolarisation. This view is supported by White (1983), who also reported decreasing R_m with increasing temperature, White also suggested that decreasing axon R_m could cause AP block at axonal branch points which may reduce the amount of transmitter released. Some of the above factors act to decrease transmitter release with increasing temperature therefore reducing the post-synaptic response of cold acclimated crabs. Furthermore walking leg muscle fibre RP hyperpolarisation decreases muscle fibre excitability (Hille 1992), this decreases the muscle fibres sensitivity to any transmitter that is released further contributing to the decrease in EJP amplitude with increasing experimental temperature shown by all cold acclimated walking legs, irrespective of species or the CNS acclimation temperature.

All heterothermally cold acclimated walking leg EJP amplitude decreases with increasing experimental temperature were significant, the decrease in all cases, whether single or double pulse, was between 65-70% over the experimental temperature range. Decreasing EJP amplitudes with increasing experimental temperature have also been reported by Fischer and Florey (1981) when working on *Procambarus clarkii* maintained at 10°C, and for 12°C acclimated *Astacus leptodactylus* when tonically innervated (Harri and Florey 1979), but also by Cuculescu (1996) who worked on 8°C acclimated *C.maenas* and *C.pagurus*.

In contrast all warm acclimated walking legs, whether free or heterothermally acclimated, showed near constant or n-shaped EJP amplitude versus temperature curves between 7-22°C of the experimental temperature range. In most cases warm acclimated walking legs generated larger EJP amplitudes at experimental temperatures between 15-17°C than amplitudes generated by cold acclimated walking legs at the same temperatures, indicating a clear acclimatory shift for warm acclimated walking legs of between 1-2.5°C. This acclimatory shift was independent of the CNS acclimation

temperature and was dependent on the local acclimation temperature only, the shift in EJP amplitude is probably significant with regard to warm acclimated walking legs ability to maintaining function at the warmer experimental temperatures. This is further supported by data shown in Table 6.7, which showed cold acclimated walking legs of both species generated maximal EJP amplitudes at significantly colder experimental temperatures than the maximal EJP amplitudes generated by warm acclimated walking legs, thus proving acclimation induced changes in EJP amplitudes in response to the local acclimation temperature. Warm acclimated animals larger EJP amplitudes at warmer experimental temperatures have also been reported in 25°C acclimated *Astacus leptodactylus* when phasically and tonically innervated (Harri and Florey 1979), 21°C acclimated *Pachygrapsus crassipes* (Stephens and Atwood 1982), and Hawaiian ghost crab *Ocypode ceratophthalma* which generated maximal EJP amplitudes over 22-28°C when acclimatized to 26-27.5°C (Florey and Hoyle 1976).

As reported above, the EJP amplitudes of warm acclimated walking legs were maximal or maintained over the mid temperature range, where cold acclimated walking leg EJP amplitudes were decreasing. This clear acclimation temperature induced difference in EJP amplitude sensitivity to direct temperature is not due to facilitation or Tau changes (see below), but can be attributed to acclimation induced changes in RP and probably neurotransmitter release. As previously reported warm acclimated walking legs successfully shifted their RPs so as to maintain equivalent RPs at both cold or warm acclimation temperatures (Kivivuori *et al.*, 1990; Stephens and Atwood 1982). The RP shift maintains muscle excitability (Hille 1992) at the warm acclimation temperature, indeed, enhances muscle excitability with decreasing experimental temperature such as over the 22-13°C temperature range. The RP contribution to muscle excitability probably synergises with increases in calcium sensitivity reported by Stevens and Godt (1990), thus maintaining or slightly increasing the EJP amplitude with experimental temperature decreases from 22°C to 13°C, despite decreases in quantal content and release with decreasing experimental temperature reported by White (1983).

Facilitation is intimately linked to muscle fibre depolarisation and is incorporated within the double pulse EJP amplitude results. Facilitation values were calculated as a ratio of the double pulse EJP amplitudes (see Figure 2.4), facilitation being principally Ca^{2+} dependent (Stephens and Atwood 1982; Van der Kloot 1994). The shape of the facilitation versus temperature curves may be expressed as Ca^{2+} sensitivity changes over the experimental temperature range, which may include factors such as $[Ca^{2+}]$ gradients, Ca^{2+} buffering or Ca^{2+} sequestration, and the contribution of Ca^{2+} to muscle fibre depolarisation.

Both species, when warm and cold acclimated showed u-shaped facilitation versus temperature curves, minimal facilitation was recorded over 10-17°C. No acclimatory shifts with warm or cold acclimation were evident, irrespective of the CNS acclimation temperature, indicating facilitation changes were in response to experimental temperature changes only.

U-shaped facilitation versus temperature curves were reported by Stephens and Atwood (1982) in the shore crab *Pachygrapsus crassipes* although they exhibited near minimal facilitation around their acclimation temperature. Facilitation in *Pachygrapsus crassipes* exhibited an acclimatory shift; 12°C acclimated crabs exhibited minimal facilitation around 12.6°C which shifted to 16.7°C for 21°C acclimated crabs. Cuculescu (1996) showed similar facilitation values to those presented here, greatest facilitation from warm and cold acclimated *C.maenas* and *C.pagurus* walking legs was recorded at temperatures near to their acclimation temperature. With minimal facilitation being recorded for both warm and cold acclimated *C.maenas* and *C.pagurus* over 12-17°C. Harri and Florey (1979) studied 12°C and 25°C acclimated *Astacus leptodactylus* and identified minor differences between facilitation from warm and cold acclimated crayfish when phasically innervated, but did note an acclimatory shift in facilitation when tonically innervated i.e. maximal facilitation was generated at an experimental temperature of 24°C and 16-18°C for 25°C and 12°C acclimated crayfish respectively. Their results show greatest facilitation being generated near to the acclimation temperature, being similar to the results presented here. The u-shaped facilitation versus temperature curve indicated that facilitations direct contribution to muscle depolarisation over 10-17°C was small, and therefore some other factor(s) must have acted to maintain and in some cases potentiate muscle fibre depolarisation in warm acclimated walking legs over that temperature range

Statistical analysis of all *C.maenas* heterothermally acclimated walking leg facilitation results revealed no significant differences between the acclimatory groups. This absence of qualitative or quantitative differences between heterothermally acclimated crab walking leg results indicates facilitation cannot be used to investigate a potential central influence to walking leg facilitation acclimation.

Comparison of facilitation from free *C.maenas* 8 to *C.maenas* CN/CL revealed no significant differences of any kind, although free *C.maenas* 8 facilitation versus temperature curve did not increase as expected at temperatures warmer than 19°C indicating a qualitative difference (see Chapter Four). In contrast free *C.maenas* 22 crabs generated facilitation which was significantly greater than *C.maenas* HN/HL acclimated walking legs over most of the experimental temperature range, indicating a quantitative difference. However, free *C.maenas* 22 walking leg facilitation versus temperature curve was u-shaped and indicated no qualitative differences from any other

acclimatory groups facilitation change with experimental temperature. Facilitation is very variable, although it is not changed through acclimation here, facilitation only appears to change in response to direct temperature. Rathmayer and Hammelsbeck (1985) reported different levels of facilitation from different muscle fibres in *Eriphia spinifrons* depending on the fibre type, axon type and stimulation frequency of innervation.

All *C.pagurus* heterothermal acclimatory groups showed u-shaped facilitation versus temperature curves, minimal facilitation was recorded over approximately 12-17°C. However, the HN/HL acclimated walking leg facilitation results did not increase as expected at experimental temperatures warmer than 17°C, which was unexplainable. It is again clear from the lack of any significant differences either qualitative (except for HN/HL) or quantitative that facilitation cannot be used to investigate a potential CNS influence to walking leg facilitation acclimation.

Facilitation generated by free *C.pagurus* 8 and CN/CL were not different from each other, further indicating facilitation changed in response to direct temperature only. In contrast, free *C.pagurus* 22 and *C.pagurus* HN/HL acclimated walking leg facilitation values were significantly different over most of the experimental temperature range, being similar to the facilitation results reported for similarly acclimated *C.maenas*.

Because no acclimatory differences in facilitation were apparent, the contribution of facilitation to muscle function was most conspicuous over the 6-26°C experimental temperature range. Large facilitation was recorded from cold acclimated walking legs at colder experimental temperatures which may be due to optimum Ca^{2+} sensitivity, as Stevens and Godt (1990) reported that calcium sensitivity increases with decreasing experimental temperature. The potential increase or maintenance of calcium sensitivity at cold temperatures would maintain muscle depolarisation even though at low experimental temperatures the quantal release and quantal content are reduced (White 1983; Montgomery and MacDonald 1990).

Over the experimental temperature range of 12-17°C facilitation was minimal for all acclimatory groups (and both species). Decreased Tau may contribute slightly to minimal facilitation, although Tau is clearly not primarily involved here as EJP amplitudes were near maximal over this temperature range in warm acclimated walking legs. At warmer experimental temperatures facilitation values were increasing in cold acclimated walking legs, which may be linked to calcium buffering failure as Ushio and Watabe (1993) reported decreased Ca^{2+}-ATPase activity at increased perturbing experimental temperatures. At even warmer experimental temperatures, cold acclimated walking legs calcium sensitivity is further reduced, therefore synaptic and

axon terminal heat block may be a limiting factor (Prosser and Nelson 1981; Montgomery and MacDonald 1990) of polyterminal axons (White 1983). Axon or synapse block may also be effected by changes in the width or amplitude of action potentials, as crab action potentials are Na^+ and Ca^{2+} dependent (Stephens and Church 1988). At experimental temperatures warmer than 14°C EJP amplitudes were starting to decrease rapidly in cold acclimated walking legs, which correlates well with the facilitation minima. In warm acclimated walking legs Ca^{2+} sensitivity changes over a temperature range may alter calcium dependent potassium channel function ($K_{(Ca)}$), as reported by Johnson *et al.*, (1991) in a lobster ganglion. Increasing experimental temperature reduces Ca^{2+} sensitivity which may result in $K_{(Ca)}$ channels not being activated, therefore EJP amplitude would be maintained through an increase in R_m despite no contribution to amplitude from facilitation at mid-temperatures. Furthermore it has been shown that homeoviscous adaptation through addition of cholesterol to rabbit smooth muscle membranes decreased fluidity and caused a two fold reduction in the probability of $K_{(Ca)}$ opening (Bolotina *et al.*, 1989), which may affect warm acclimated walking leg muscle function at low experimental temperatures although this seems unlikely in crabs. Facilitation is not contributing significantly to warm acclimated walking leg EJP amplitudes over 12-17°C, as no differences in Tau were evident, the amount of transmitter released or receptor sensitivity to transmitter must be significant. It would be interesting to know if the neurotransmitter released at experimental temperatures warmer than 14°C was completely Ca^{2+} dependent?

Exponential functions were fitted to the decay phases of single EJPs, this identified Tau or the EJP decay time constant, which itself gave an indication of membrane resistance (R_m) (see equation 1.1). Directly related to muscle fibre function and the extent of muscle fibre depolarisation is R_m and other electrical parameters such as capacitance (C_m) (see Fatt and Katz 1953), the higher a muscle fibres R_m (or C_m) the longer an EJP will take to decay, therefore Tau gives an indication of the number of open channels (Hille 1992). Long decay times maintain EJP amplitudes through summation and to a lesser extent facilitation, especially during a train of stimulatory pulses.

Both species' walking legs when warm or cold acclimated, whether free or heterothermally acclimated, showed decreasing Tau with increasing experimental temperature which indicated the number of open channels increased with increasing experimental temperature (Nicholls *et al.*, 1992; Fatt and Katz 1953).

It has been reported that changes in membrane fluidity may effect channel function and therefore conductance; Bolotina *et al.*, (1989) found a twofold decrease in

$K_{(Ca)}$ channel open probability if cholesterol was added to rabbit muscle membranes, the cholesterol decreased muscle membrane fluidity, decreased membrane fluidity is found at warmer temperatures in saturated membranes or warm acclimated tissues when compared to unsaturated or cold acclimated tissues (Hazel 1995). Green and Andersen (1991) reported that the net charge on lipid head groups in a bilayer could alter $K_{(Ca)}$ and L-type Ca^{2+} channel function. Where Hazel (1995) reported that decreasing experimental temperatures generated a mixture of fluid and gel phases in the membrane which markedly increased the membranes permeability to cations, presumably due to packing defects that form at the boundaries between the lipid microdomains.

No significant differences were found between any *C.maenas* heterothermally acclimated crab walking leg Tau results, whether warm or cold acclimated and irrespective of the CNS temperature. However, CN/HL acclimated walking leg Tau values unexpectedly decreased at experimental temperatures colder than 9°C, which indicated an increase in the number of open channels. The change in Tau may indicate a change in lipid gel-fluid phase which affects channel function (MacDonald 1988; Hazel 1995) and contributes to the decreased Tau in CN/HL acclimated walking legs at low experimental temperatures. CN/CL exhibited a high number of significant differences from free *C.maenas* 22 acclimated walking leg Tau values, however, free *C.maenas* 22 acclimated walking leg Tau values showed almost identical differences from all other free and heterothermal acclimatory groups, irrespective of the acclimation temperature. Free *C.maenas* 8 acclimated walking leg Tau showed almost no significant differences from *C.maenas* CN/CL acclimated walking leg Tau results, indicating no immobilisation effect. Changes in Tau over the experimental range were in response to direct temperature only, no acclimatory compensation was evident.

The HN/CL and HN/HL acclimated walking leg Tau values were similar over most of the experimental temperature range, although the HN/HL acclimated walking leg Tau values were longer than expected at experimental temperatures colder than 8°C. Both HN/HL and HN/CL acclimated walking leg Tau values were not significantly different from free *C.maenas* 8. Free *C.maenas* 22 showed significant differences in Tau from *C.maenas* HN/HL over 10-15°C, as no acclimatory shift was evident this difference indicates a change in response to direct temperature or immobilisation. It is clear that there were few significant differences between the heterothermally acclimated walking leg Tau values, which may be due to the narrower acclimation temperature differences measured between the heterothermal acclimatory groups. The lack of any warm and cold acclimated heterothermal differences in Tau indicates Tau cannot be used to investigate a potential CNS influences to walking leg acclimation. However, it was interesting that both CN/HL and HN/HL acclimatory group Tau results exhibited a

change in trend at an experimental temperature of 8°C, irrespective of the CNS acclimation temperature and is therefore related to the local acclimation temperature.

Heterothermally acclimated *C.pagurus* Tau changes with experimental temperature were slightly more variable than those reported for *C.maenas*. Both CN/CL and CN/HL acclimated walking leg Tau values decreased with increasing experimental temperature in qualitatively identical manners, their Tau values were not significantly different from each other when compared at the same experimental temperatures where some differences may be expected. The CN/HL acclimated walking leg Tau values revealed an increased number of significant differences from HN/HL acclimated walking leg Tau values which was unexpected, but also revealed a large number of significant differences from free *C.pagurus* 8 walking leg Tau values. Some of the differences between HN/HL and CN/HL acclimated walking leg Tau appeared to be due to a change in trend of HN/HL (Figure 6.14B) acclimated walking leg Tau at experimental temperatures colder than 10°C. Furthermore HN/CL acclimated walking leg results were increasingly variable at experimental temperatures warmer than 22°C indicating increased temperature sensitivity typical of a cold acclimated walking leg, although HN/CL acclimated walking leg Tau revealed almost no significant differences from any other heterothermal acclimatory group Tau value. Free *C.pagurus* 22 and *C.pagurus* HN/HL acclimated walking leg Tau values were not significantly different, indicating no immobilisation changes or differences in response to direct temperature. And, CN/CL acclimated walking leg Tau values revealed almost no differences from any other free or heterothermal acclimatory group walking leg Tau values. This indicated that *C.pagurus* Tau results could not be used to identify a potential CNS influence to walking leg acclimation due to the lack of warm and cold acclimated walking leg Tau differences, in some cases Tau results were contradictory.

Tau is dependent on the muscle fibre size (i.e. diameter and surface area). Mean muscle fibre diameters are shown in Figure 6.15 for *C.maenas*. Throughout it can be seen that warm acclimated walking legs had larger diameter muscle fibres than the cold acclimated walking legs, although with varying degrees of significance, larger diameter muscle fibres are known to have shorter Tau times (Nicholls *et al.*, 1992; Fatt and Katz 1953). Triestman and Grant (1993) reported a general increase in cell size with warm acclimation. In some cases the larger diameter muscle fibres were linked to larger leg size, it cannot be determined if acclimatory hypertrophy had occurred in the different *C.maenas* acclimatory groups since the results were masked by significant differences in leg size (see Table 6.8). The results do not reveal any acclimatory effect where CNS

acclimation temperature was significant to muscle fibre diameter changes, but do reveal a warm to cold acclimatory difference in fibre diameter.

Equivalent muscle fibre diameters for *C.pagurus* acclimatory groups are shown in Figure 6.16. Because no significant differences in leg size were found for *C.pagurus*, any significant differences between muscle fibre diameters can be attributed to the acclimation temperature only as Chapter Four proved no significant immobilisation induced fibre hypertrophy. There were clear warm and cold acclimation temperature induced differences in muscle fibre diameter. For example, CN/CL acclimated walking leg fibre diameters were significantly smaller than all warm acclimated walking leg fibre diameters except free *C.pagurus* 22. Furthermore all warm acclimated crab walking legs in most cases had significantly larger diameter muscle fibres than cold acclimated crab walking legs, the larger diameter of warm acclimated walking legs did not seem to reduce their EJP decay time constants as may be expected (see Fatt and Katz 1953; Adams 1987) over the whole temperature range, but may influence the changes in trend of Tau seen between 8-9°C in some warm acclimated walking legs.

The muscle fibre diameters of *C.pagurus* heterothermally acclimated walking legs correlated to their actual acclimation temperature (see Figure 6.2B and 6.16), i.e. the colder the acclimation temperature the smaller the walking leg muscle fibre diameter. This indicated the local acclimation temperature was important and the CNS acclimation temperature was not.

Because significant fibre hypertrophy was shown for warm acclimated walking legs irrespective of the CNS acclimation temperature, compared to fibre diameters of cold acclimated walking legs (significantly so for *C.pagurus*), it is concluded that the un-fed and immobilised acclimation period had no affect on the different acclimatory group fibre diameters.

Summary.

The results presented in this chapter indicate that thermal acclimation was independent of a CNS influence. More correctly, it cannot be said if a CNS trophic factor was acting, but any that was could not overcome the effect of the local walking leg thermal environment, a view similarly reported by Fahmy (1972; 1973). It can also be said that eye-stalk hormones were also not important, as both eye-stalks were acclimated to the CNS acclimation temperature, thereby preventing cross-talk.

The clearest warm and cold acclimation temperature difference showing independence from the CNS acclimation temperature is shown in Table 6.7, where cold

acclimated walking leg muscle function was optimal at colder experimental temperatures for both *C.maenas* and *C.pagurus*. Additionally, muscle function was significantly improved at warmer experimental temperatures when warm acclimated, irrespective of the CNS acclimation temperature. Furthermore, acclimatory shifts in RP were also found which would maintain muscle excitability, appropriate for the walking leg acclimation temperature and independent of the CNS acclimation temperature. Acclimation would have to be locally controlled, if a walking leg exposed to a continuously cold environment adjusted its physiology toward that of a warm environment. Its muscle function at colder experimental temperatures would not be as efficient as found in other appropriately cold acclimated animals. For example, if *C.pagurus* HN/CL acclimated walking legs adjusted its muscle phenotype to the warm CNS acclimation temperature, it would be expected to have a somewhat depolarised RP, equivalent to other warm acclimated walking legs, this would result in inappropriate increased excitability and possibly uncoordinated movement at the lower temperatures of its local environment. It was also clear that no differences in acclimation, with respect to a CNS influence, were found between *C.maenas* and *C.pagurus*, and no significant differences in their warm or cold acclimated walking legs to direct temperature were evident.

Chapter Seven.

General discussion.

The aim of the work presented in this thesis was to compare the capacity of *Carcinus maenas* and *Cancer pagurus* to acclimate to 8°C and 22°C, and to determine if centrally derived hormones or trophic factors influenced thermal acclimation. A crustacean neurophysiological system was chosen for these investigations due to the large size and accessibility of their nerves and muscles (Atwood *et al.*, 1994).

C.maenas and *C.pagurus* thermal acclimation.

The effect of direct temperature on various neurophysiological parameters were determined in walking legs of crabs acclimated to 8°C and 22°C. In summary, both species of crab were acclimated to 8°C and 22°C and no profound acclimatory effects were evident on latency, facilitation or Tau, however, clear changes in RP and EJP amplitude were found with acclimation.

With respect to RP it was found that increasing the experimental temperature resulted in muscle fibre hyperpolarisation, this is a commonly observed phenomenon and has been suggested to result from an activation of the Na^+/K^+ ATPase and changes in membrane permeability to Na^+/K^+ (White 1982; Montgomery and MacDonald 1990; Harri and Florey 1979). The effect of acclimation on RP was to shift the RP/°C response curve to the right with acclimation from 8°C to 22°C, this is a compensatory effect and is demonstrated for both species. However, when the efficacy of acclimation is determined (Cossins and Bowler 1987) with respect to RP, it was found that the response in *C.pagurus* was greater than that observed for *C.maenas* (Table 4.1), this suggests that *C.pagurus* is able to make a more complete acclimatory response. The temperature dependent RP hyperpolarisation would be expected to decrease the excitability of the muscle fibre (Hille 1992; Adams 1987).

With respect to the effect of acclimation on EJP amplitude, there was a marked difference observed in the effect of experimental temperature on EJP amplitude between walking legs from cold and warm acclimated crabs. In general cold acclimation resulted in the single EJP amplitudes decreasing gradually with experimental temperatures above about 14-15°C. Whereas warm acclimated walking leg muscle fibres were able to maintain single EJP amplitudes with increasing experimental temperatures up to 21-24°C, above which the EJP amplitudes fell slightly. This pattern of response was seen for both *C.maenas* and *C.pagurus*, however, it was noted that single EJP amplitudes from free warm acclimated

C.pagurus walking legs although shifted along the temperature axis, decreased at experimental temperatures lower than 10°C. Decreasing EJP amplitudes with increasing experimental temperature have been reported by Harri and Florey (1979) who worked on tonically innervated closer muscles of cold acclimated *Astacus leptodactylus* and by Cuculescu (1996) who worked on cold acclimated *C.maenas* and *C.pagurus*. Whereas Stephens and Atwood (1982) reported that EJP amplitudes of 12°C acclimated *Pachygrapsus crassipes* increased from 8°C to 14°C before decreasing at experimental temperatures warmer than 14°C. Similar acclimatory shifts in EJP amplitude have been reported by Harri and Florey (1979) in phasically and tonically innervated warm acclimated *Astacus leptodactylus* walking leg closer muscles, but also by Stephens and Atwood (1982) who worked on 21°C acclimated *Pachygrapsus crassipes*, which showed decreased EJP amplitudes at colder experimental temperatures.

This raises the question of how acclimation responses in these neurophysiological parameters affect continued muscle function at different thermal conditions? In particular, RP hyperpolarisation with increasing experimental temperature would be expected to decrease muscle fibre excitability. Clearly acclimation compensates for the direct effect of temperature on RP, this compensation is more complete for *C.pagurus* than *C.maenas*. The acclimation effect on EJP amplitude versus temperature curves is more difficult to interpret. Other authors have variously suggested; i) increasing experimental temperature results in increased quantal content and release of neurotransmitter (White 1983; Montgomery and MacDonald 1990), ii) RP hyperpolarisation would be expected to reduce EJP amplitude (Hille 1992; Adams 1987), iii) decreasing calcium sensitivity with increasing experimental temperature (Meis *et al.*, 1996), iv) action potential block at polyterminal branch points with increasing experimental temperature (White 1983). Clearly one or more of these various factors (i-iv) could affect EJP amplitude with respect to temperature. An explanation of the acclimation effect on EJP amplitude temperature response curves must try to take these factors (i-iv) into account. Considering first cold acclimated crabs where EJP amplitudes decreased gradually with increasing experimental temperature, clearly this cannot be explained by an increase in neurotransmitter release. It is more likely that RP hyperpolarisation at higher experimental temperatures may be a dominant factor in cold acclimated muscles, however, the other factors of changing calcium sensitivity and axon branch block cannot be ignored. In the case of warm acclimated walking leg muscles, their EJP amplitudes were relatively constant over the 9-22°C of the experimental temperature range. Furthermore the shift in the RP/°C curve to the right in warm acclimated crabs means that RP hyperpolarisation will not be such an important factor in determining the EJP

amplitudes at higher measuring temperatures. However, in warm acclimated crab walking legs the muscle RP depolarisation that would occur at low measuring temperatures might be expected to result in an increase in muscle excitability, however, this could be compensated for, at lower experimental temperatures, by a decrease in neurotransmitter quantum release. Therefore cold temperature block probably results from an inhibition of neurotransmitter release and a decrease in EJP amplitude which is more dramatic in single EJP amplitudes of *C.pagurus* than *C.maenas*.

Although no acclimatory compensation was evident for Tau or facilitation, both Tau and facilitation changed in response to direct changes in temperature. Tau decreased with increasing experimental temperature in both species whether warm or cold acclimated, indicating a decrease in membrane resistance which would contribute to reducing EJP amplitudes with increasing temperature, a point which is most clearly seen for cold acclimated crab walking legs. Whereas facilitation generated u-shaped temperature dependency curves, again for both species whether warm or cold acclimated. The contribution of facilitation to EJP amplitude over the mid-temperature range was minimal, whereas at extremes of experimental temperature facilitation did contribute to muscle depolarisation. It is important to note for both species that, since both warm and cold acclimated walking leg facilitation and Tau changes with direct temperature were similar, therefore, other factors were influencing the EJP amplitude dependency on experimental temperature.

Force recorded from 8°C and 22°C homothermally acclimated *C.maenas* (Chapter Three) revealed a link between EJP amplitude and force over the experimental temperature range, commonly known as electromechanical coupling (Carl *et al.*, 1996). Cold acclimated crab walking legs revealed decreasing force with increasing experimental temperature (Figure 3.3A), showing a similar shaped curve to that of EJP amplitude changes with temperature (Figure 4.6A). Furthermore warm acclimated crab walking leg force versus temperature curves exhibited an acclimatory shift i.e. maintained force (although of decreased strength) over the whole 6-26°C experimental temperature range and a shift in peak force along the temperature axis (Figure 3.3B) similar to that shown by warm acclimated walking leg EJP amplitudes. Orkand (1962) reported increasing force with increasing muscle fibre depolarisation in crayfish *Orconectes virilis*, but went on to report that tension was dependent on the magnitude of RP change rather than the amount of depolarisation when using direct electrical stimulation. Similar changes in force with experimental temperature have been reported by Stephens and Atwood (1982) and Harri and Florey (1979). It is known that nerve-evoked force measurements are smaller than expected at warmer experimental temperatures despite excitation contraction coupling having a positive

temperature coefficient (Fischer and Florey 1981; Dudel and Rüdel 1968). No force measurements were recorded for *C.pagurus* because it would be expected that the changes would be similar to their EJP amplitude changes with temperature.

One of the aims of this study was to determine if a central control of the acclimatory phenomena was evident, therefore, it was decided to determine whether there were any seasonal differences in neuromuscular parameters that could be identified in the two species of crab. The same neuromuscular parameters were determined with respect to experimental temperature for walking leg muscles from animals caught in summer and winter and the crabs used without further acclimation. Considering *C.pagurus* first, it was noted that EJP amplitudes were generally greater in summer as compared with winter caught animals, this is likely to be explained by a seasonal difference in quantal content/release of neurotransmitter. It is interesting that Lnenicka (1993) reported a similar effect in fresh water crayfish a view supported by Atwood and Nguyen (1995). It is important to emphasise that the differences between summer and winter *C.pagurus* were non-compensatory. In contrast, *C.maenas* showed significant differences in Tau between summer and winter caught crabs, winter animals had significantly longer Tau values indicating a decrease in the number of open channels which would maintain the muscle depolarisation. Tau is dependent on membrane resistance and capacitance, changes in either affect Tau, as summer and winter caught *C.maenas* Tau values were different it indicated a seasonal change in membrane structure/composition. Cuculescu (1996) investigated muscle membrane phospholipid changes in seasonally acclimatized *C.maenas* and *C.pagurus*, in general she reported that autumn crabs had lower saturated-unsaturated fatty acid and lower cholesterol-phospholipid molar ratios than spring crabs. Remodelling of membrane lipid composition with respect to temperature is a widely reported phenomenon and is believed to account for compensatory changes in fluidity (Cossins 1994) in a process referred to as homeoviscous adaptation (Sinensky 1974). It remains to be established if the observed changes in Tau are a direct consequence of the reported seasonal remodelling in the crabs membrane lipids.

Investigation into the control site of thermal acclimation.

The technique of heterothermal acclimation allowed both species of crab to have one set of walking legs acclimated at the same temperature as it's CNS and eye-stalks, and the contralateral set of walking legs acclimated to a different acclimation temperature. This allowed an investigation into the control site of thermal acclimation through selective positioning of a temperature dividing membrane. In practice, it

proved difficult to maintain the water bath temperatures at 8°C and 22°C, the conditions for the homothermally acclimated animals, indeed the actual temperature difference was 12.47°C rather than 14°C. The decreased temperature difference experienced by heterothermally acclimated crabs was largely due to small leaks around the animals, this resulted in water mixing between the two compartments. It was considered essential that not only the CNS but both eye-stalks experienced the thermal conditions of one set of walking legs, this is because the eyestalks have been shown to be a principle source of hormones (Fingerman 1995; Crothers 1967). Indeed Silverthorn (1975) showed that thermal acclimation of metabolism in the fiddler crab was dependent on eye-stalk hormones and so eye-stalk hormones may be important in thermal acclimation. In common with homothermal acclimation, heterothermally acclimated *C.maenas* and *C.pagurus* showed marked acclimation effects of both RP and EJP amplitude, whereas no acclimatory changes were found for latency, facilitation or Tau.

On comparing the legs from the warm and cold sides of a heterothermally acclimated crab, its RP versus temperature plots were shifted to the right with warm acclimation. In common with the homothermal results for RP measurements the acclimatory shift in RP was greater for *C.pagurus* than *C.maenas*. It is important to emphasise that these differences in leg muscle RP versus temperature plots were the same irrespective of the temperature of the CNS. This argues that, RP acclimatory responses are locally determined and do not show a central influence.

Once again the pattern of response of EJP amplitude to measuring temperature was similar between species. Furthermore, the responses obtained from walking legs that had been cold acclimated were similar to results from homothermal cold acclimated walking legs in that the EJP amplitudes decreased with increasing experimental temperature. Warm acclimated walking legs also in common with walking legs from homothermally acclimated crabs maintained near constant EJP amplitudes over most of the experimental temperature range. Once again a clear distinction in the EJP amplitude response to temperature between the cold and warm exposed walking legs of heterothermally acclimated crabs, irrespective of the CNS temperature, is seen. In this parameter too acclimatory responses are locally determined with no evidence for a central effect. Relatively few studies investigating the control site of thermal acclimation have been attempted; Fahmy (1972; 1973) used heterothermal acclimation and drew similar conclusions about the control site of thermal acclimation from her heterothermal studies on fish as reported here. In contrast Prosser *et al.*, (1991) did identify a central hormone factor from catfish hepatocytes that overrode the local thermal effect.

Stenothermal and eurythermal comparisons.

The question that remains is whether the experimental results obtained contribute to our understanding of the biological basis of eurythermy. Generally speaking *C.maenas* is considered to be eurythermal, in the summer it is intertidal and is likely to be exposed to thermally fluctuating conditions (Taylor and Wheatly 1979). In the winter it is probable that *C.maenas* migrates off-shore where it experiences temperatures substantially below 10°C (Naylor 1985). *C.pagurus* is considered to be more stenothermal, in that it is sublittoral throughout the year and so would not experience the range of temperatures as *C.maenas* (Nicol 1964; Southward 1958; Taylor and Wheatly 1979). One might expect therefore that an animal that is subject to larger seasonal ranges in temperature would have the need for a greater acclimatory response than an animal that was subject to a relatively small seasonal temperature range.

In this respect the results are contradictory, *C.pagurus* gave a more complete (Precht 1958, type II) acclimatory response with respect to RP, whereas for *C.maenas* the acclimatory response was only partial (Precht 1958, type III). This may suggest that one acclimatory response in *C.pagurus* is to produce more membrane pump sites in cold acclimation. With respect to the temperature dependency of EJP amplitude, there were no marked differences between the two species. However, cold acclimated *C.pagurus* did show a more rapid decrease in experimental numbers than *C.maenas* at experimental temperatures warmer than 22°C. In contrast warm acclimated *C.pagurus* walking legs did show some neuromuscular block at temperatures below about 9°C, however similar block was also evident for walking legs from warm acclimated *C.maenas* which was unexpected. Furthermore, warm acclimated *C.pagurus* were able to maintain maximal EJP amplitudes to slightly warmer experimental temperatures than *C.maenas* which is again contradictory to their relative eurythermal status.

On the other hand, seasonally acclimatized winter *C.pagurus* showed decreased heat block temperatures when compared to winter *C.maenas*, drawing again a distinguishing feature between the seasonal and laboratory acclimatized animals. This warm sensitivity of EJP amplitudes in *C.pagurus* correlates with CTMax data obtained by Cuculescu (1997). *C.maenas* were found to have much higher CTMax temperatures than *C.pagurus*, indeed cold acclimated *C.maenas* had a CTMax value similar to warm acclimated *C.pagurus*. However, it is significant that the extent of the shift in CTMax by acclimation was much larger in *C.pagurus* than in *C.maenas*. Thus, there is some evidence that *C.maenas* has a wider thermotolerant range than *C.pagurus* and so would be consistent with its eurythermal status, however for a

number of parameters *C.pagurus* is able to make a greater compensation in acclimation than *C.maenas* which is not consistent with the generally held expectation of a stenothermal animal.

Future Work.

Future experiments should relate to further electrophysiological investigations. Significant differences in Tau were found for summer compared to winter acclimatized *C.maenas* only, it would be interesting to know what specific factor(s) were involved in the resistance changes.

It would be interesting to know if the Na^+ and Ca^{2+} channel conductance in crustacean nerves and muscles was effected by thermal acclimation i.e. channel number and kinetics (which would also facilitate investigations into depolarising and hyperpolarising after potentials). The closer muscle is composed of four different muscle fibre types, some muscle fibres are dually innervated and some phasically innervated only, are there any differences in a 'type' of muscle fibres ability to thermally acclimate? How might muscle fibre acclimation change with respect to the type of innervation? This would also allow investigations into quantum release and active zone morphology especially if investigated over the seasons. Are there any differences in membrane lipid composition between the different muscle fibre types? It is also particularly interesting that warm acclimated walking leg muscle function appeared to be maximal over mid-temperature ranges, where facilitation was minimal and Tau was decreasing, so what maintained muscle depolarisation over that temperature range?

Are there any differences between *C.maenas* and *C.pagurus*'s heat shock response? Do the different HSP's have different efficacies to different stresses and does that change with acclimation?

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