THE UNIVERSITY OF HULL

STUDIES ON SOME RESPONSES OF *CARCINUS MAENAS* (L.) AND OTHER BRACHYURANS TO HYPOXIA AND AERIAL EXPOSURE.

being a Thesis submitted for the Degree of

Doctor of Philosophy

in the University of Hull

by

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TO MY PARENTS

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ABSTRACT

Abstract

This work comprises a series of investigations of the effects of changes in environmental oxygen levels, which occur with hypoxia or aerial exposure, on the osmo-regulatory capacity, acid-base balance and general respiratory physiology of brachyuran crabs. The principal species studied was Carcinus maenas, although Cancer pagurus and Liocarcinus puber were also included in the aerial exposure experiments.

The effects of exposure to 4hr of moderate (P_wO_2 = 40 torr) or severe (P_wO_2 = 20 torr) hypoxia at 15°C on various blood solutes, acid-base balance and general respiratory physiology were assessed for C.maenas in low salinity (10.0, 13.5 and 19.0‰ S) and fully marine (30.0‰ S) media. Haemolymph pH increased at all test salinities and to an extent which was salinity/P_wO_2 dependent. During hypoxia the heart rate (f_H) was not significantly affected at any salinity but the scaphognathite rate (f_{sc}) increased significantly ($P<0.05$) at all the media tested. Scaphognathite rate changes were greater in moderate rather than severe hypoxia. In hypotonic media hypoxia produced no changes to circulating Na^+ or Mg^{2+} levels or to the Δ^oC values but circulating Cl^- levels fell and Ca^{2+} and lactate levels increased at salinity/P_wO_2 dependent rates and extents. In the 30.0‰S medium, no changes to any of the above blood ions occurred during moderate hypoxia but Ca^{2+} and lactate levels increased at P_wO_2 = 20 torr. At all test salinities original levels of blood Cl^-, Ca^{2+} and lactate were regained within 4hr of re-oxygenation (P_wO_2 = 130 torr). The implications of these findings have been considered in relation to the distribution of regulating species of crustaceans generally.

The effects of 24hr severe (P_wO_2 = 20 torr) hypoxia on haemolymph protein synthesis in fed and starved C.maenas in 13.5‰ S and 30.0‰ S media were studied. Blood total protein levels were higher in animals acclimated to 13.5‰ S whereas haemocyanin levels were higher in the 30.0‰ S group. A negative linear relationship between relative haemocyanin and blood total protein was observed in animals at both salinities. In fed animals small, but non-significant ($P>0.05$) increases in haemocyanin and total blood protein levels were recorded at both salinities (cf. normoxic animals). In starved groups at both salinities blood protein levels were maintained under hypoxia but control animals showed a fall in protein levels.

The responses of the crabs C.maenas, C.pagurus and L.puber to short (4hr) and longer (24hr) term aerial exposure were investigated. On exposure to air oxygen consumption ($\dot{M}O_2$) was reduced significantly ($P<0.05$) in C.pagurus and L.puber (cf. aquatic respiration rates), but the $\dot{M}O_2$ of C.maenas in air was comparable to that in water. No marked change in f_H or f_{sc} were recorded in C.maenas during re-immersion following aerial exposure but in L.puber tachycardia and hyperventilation were observed. In air (4hr) there were no changes to blood lactate levels in C.maenas but pronounced changes were found in C.pagurus and L.puber. Long-term aerial exposure resulted in mortalities and significant ($P<0.05$) increases in blood glucose and lactate levels in C.pagurus and L.puber but no mortalities or marked changes to these metabolites in C.maenas. The findings have been considered in relation to the structure and resistance to collapse of the gill lamellae in air.

CHAPTER 1

GENERAL INTRODUCTION

Introduction

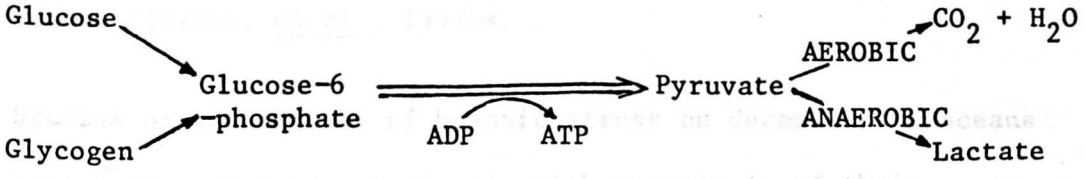
By virtue of its large number of constituent species, the range of adaptability and habit of those species, their economic importance and other criteria, the class Crustacea has been a popular subject for physiological studies comparable with those made on vertebrates. Numerous aspects of crustacean physiology have been extensively studied and a number of comprehensive reviews exist (e.g. Waterman, 1960, 1961; Florkin and Scheer, 1970, 1971 and the recent volume edited by Mantel, 1983).

In their natural habitat aquatic crustaceans, particularly intertidal species, may be subjected to changes of such environmental variables as temperature, salinity and medium oxygen saturation (e.g. during hypoxia or aerial exposure). The particular responses of any given species to an environmental stress will depend largely upon the extent to which its metabolism is affected. This, in turn will be a specific response but modulated by the magnitude of the stress and whether or not the animal is acclimated within the intensity range encompassing the environmental change.

Amongst studies made of crustacean responses to altered environmental conditions, that of oxygen consumption (uptake) has frequently been taken as an index of metabolic stress. Consequently, much information is available on the oxygen consumption ($\dot{M}O_2$) of a diverse range of crustacean species, exposed to a variety of environmental conditions (Wolverkamp and Waterman, 1960; McMahon and Wilkens, 1983 for reviews). Because many species show a wide range of metabolic rates, from low 'basal' values to high 'active' values, there are considerable difficulties inherent in the interpretation of oxygen

consumption data. The range of metabolic levels; which is an interspecific variable has been termed the 'scope for activity' or 'aerobic metabolic scope' (Bennett, 1978). Such comprehensive information is not yet available for many species and the majority of data come within the category 'routine metabolism' and refer to measurements made under minimal, but uncontrolled locomotor activity.

The classic Embden-Meyerhof glycolytic pathway has been shown to operate in crustaceans (Hu, 1958; Hochachka et.al., 1962; Huggins, 1966; Boulton and Huggins, 1970). Other studies have demonstrated that the principal metabolic routes by which crustaceans derive cellular ATP (i.e. a mitochondrial tricarboxylic acid cycle, coupled to an electron-driven oxidative phosphorylation of adenosine diphosphate, ADP) are apparently similar to those established in vertebrates (Burrin and Beechey, 1966; Huggins and Munday, 1968; Chen and Lehninger, 1973). The pathways of carbohydrate metabolism operating in crustaceans are summarised in the diagram below:



Consequently, in crustaceans as in vertebrates, an accumulation of lactate in the blood as a response to an external stress can be taken as an index of a shift from aerobic to anaerobic metabolism and, therefore, used to monitor changes in metabolism. Aquatic environmental hypoxia is brought about by a reduction in the

oxygen tension of the external medium and results in decreased oxygen availability in that medium. The extent of the external hypoxic stress experienced by an aquatic animal is dependent on both the medium salinity and temperature, since both these factors effect the solubility of oxygen in water (Murray and Riley, 1969).

The effect of environmental hypoxia on the internal physiology of a given species can be considered as dependent on a critical oxygen tension P_{cr} (Taylor, 1976). The P_{cr} is the external oxygen tension (P_wO_2) at which oxygen consumption becomes dependent upon the P_wO_2, although there is rarely a marked transition point but generally a changeover range (Taylor et. al., 1977b). At a given salinity and temperature the P_{cr} will vary between species. However, the P_{cr} has been shown to vary with environmental conditions, such as temperature and salinity, and the physiological status of a given animal, such as moult stage and haemocyanin concentration (Herreid, 198o for review). Furthermore, for an animal under given experimental conditions, the P_{cr} for oxygen consumption may have a different value to that for other physiological indices, such as heart rate or ventilation volume (Taylor, et. al., 1977b).

Studies of the effects of hypoxic stress on decapod crustaceans have been concerned principally with appraisals of their respiratory circulatory and ventilatory responses and have shown hyperventilation, a reduction in the oxygen tension of pre-branchial blood (P_{Vo_2}) and a concommitant depletion of the venous reserve as general responses to hypoxia (Taylor, et. al., 1973; McMahon et. al., 1974; McMahon and Wilkens, 1975; Taylor, 1976; Butler et. al., 1978;

Wilkes and McMahon, 1982; McMahon and Wilkens, 1983 for review). Most of the studies carriedout have usually been at some given salinity, although some information is available on the effects of salinity/P_wO_2 combinations on respiratory and circulatory responses (Taylor et.al., 1977b)

The concentration of solutes in the blood (i.e. inorganic ions and organic molecules) is accepted as having an important effect on ion/osmo-regulation. Blood solute concentrations also effect the blood acid-base balance, or S.I.D., which is the difference between strong-acid anions and strong-base cations and is one of the determinant variables of blood acid-base status (Stewart, 1978).

Changes in blood solute concentrations have been found in many species in response to variations in environmental conditions (e.g. salinity and temperature) and these changes have been shown to produce effects on ion/osmo-regulation (Robertson, 1960; Mantel and Farmer, 1983 for reviews) and acid-base balance (Henry and Cameron, 1982). However, there is only limited information available on the effects of hypoxic stress on blood solute concentrations. Natural incidences of environmental hypoxia have been increasing recently, particularly in low salinity waters (Jørgensen, 1980). In this thesis Chapter 3 describes an investigation of the effects of various salinity/P_wO_2 combinations on blood solute concentrations in Carcinus maenas and, through these, ion/osmo-regulation and acid-base balance in this species.

In decapod crustaceans, the oxygen carrying protein, haemocyanin occurs dissolved in the blood and thus behaves as a single-phase system. Consequently, haemocyanin has physiological functions other than

that of gas transport and is known to enhance the buffer capacity of the blood, influence the temperature sensitivity of blood acid-base balance and be almost totally responsible for blood colloid pressure (Mangum, 1983 for review).

The primary role of an oxygen carrier in a circulating body fluid is presumed to be the transport of oxygen from the site of uptake to a site within the limiting diffusion distance of the mitochondria. A pre-requisite property of a respiratory pigment is the ability to combine reversibly with molecular oxygen, i.e. by loading oxygen at the respiratory surface (a region of high P_{O_2}) and unloading the oxygen at the tissues (a region of low P_{O_2}). Haemocyanin containing bloods have been shown to be highly oxygenated at the gills, although the extent to which oxygen is transported by haemocyanin, as compared to that dissolved in the blood, varies between species and with environmental conditions (Redmond, 1955; McMahon and Wilkens 1975; Mangum, 1983 for review).

The concentration of haemocyanin in the blood, and hence the oxygen carrying capacity of most crustacean species, is low. Values range from approximately 1.0-3.5 ml $O_2 . 100ml^{-1}$ haemolymph (Wolverkamp and Waterman, 1960; Mangum, 1983 for reviews). The oxygen carrying capacity is limited by the colloid osmotic pressure of the haemocyanin molecule which has to be balanced with the low hydrostatic pressure of the blood in the large sinuses (Mangum and Johnansen, 1975; Mangum, 1979).

The affinity of crustacean haemocyanin for oxygen, which is expressed as the P_{50} value, i.e. the partial pressure of oxygen (P_{O_2}) at which the haemocyanin molecule is half-saturated with oxygen, is generally

low in comparison with other invertebrate respiratory pigments. The oxygen affinity is modulated by a number of internal factors, principal of which are blood pH, as shown in Figure 1.1 (Truchot, 1975a) and divalent cations, especially Ca^{2+} (Miller and Van Holde, 1974; Truchot, 1975b). The pH effect is a normal Bohr shift at alkaline pH, with an inversion in the acid range such as has been demonstrated for most crustacean haemocyanins (Redfield, 1934).

Haemolymph oxygen affinity has been shown to be affected by changes to external (medium) factors such as temperature (Truchot, 1973a; Rutledge, 1981a) and salinity (Truchot, 1973a, Weiland and Magnum, 1975), the nature of such changes being shown in Figure 1.1.

During hypoxic stress when the ambient oxygen supply is decreased, the maintenance of the oxygen supply to the tissues could be assisted by a) alterations of the haemocyanin oxygen affinity and/or b) increases in haemolymph haemocyanin concentration and hence oxygen carrying capacity of the blood. The changes in blood pH and ionic solutes occurring in response to salinity/P_wO_2 combinations have been investigated in the third chapter of this study and may provide an indication of the changes in blood oxygen affinity during hypoxic stress.

In Chapter 4 the effects of various salinity/P_wO_2 combinations on circulating levels of haemocyanin and total protein were investigated using fed and starved groups of C.maenas. The principal aim of such studies was to determine whether hypoxia-induced protein synthesis occurred in this species, as has been shown to occur in Homarus americanus (Senkbiel and Wriston, 1981a) and Nephrops norvegicus

(Hagerman and Uglow, in press).

In addition to hypoxic stress in water, a change in environmental oxygen level for aquatic decapod crustacean species accompanies emersion into air. Air contains approximately 40x more oxygen than does air equilibrated normal (34‰ S) seawater at 15°C and is ca 1000x less dense than seawater. Although both these factors theoretically should considerably reduce the level and the energetic cost of ventilation, aerial gas exchange of aquatic crustaceans is complicated by the fact that the gills are designed primarily to function in water.

On exposure to air aquatic decapod crustaceans may respond with either

i) maintained active respiration in air with no reduction in oxygen uptake

ii) a shift from aerobic to anaerobic metabolism with a consequent accumulation of metabolites such as succinate or lactate.

The extent to which either of these strategies operates appears from the available evidence to be related to the normal habitat of the species (McMahon and Wilkens, 1983 for review) and brachyurans inhabit a wide range of environments from the sub-littoral (e.g. Callinectes sapidus) to terrestrial (e.g. Birgus latro).

Studies have been made of respiratory and circulatory responses of decapod crustaceans during exposure to air and these have shown that the magnitude of the changes in oxygen uptake when in air, compared to that in aerated seawater, is generally dependent on the natural habitat of the species (McMahon and Wilkens, 1983 for review).

However, few studies have been made of the effects of aerial exposure on blood solute concentrations of decapods. Consequently, the effects of aerial exposure on the levels of certain blood solutes, including glucose and lactate from three brachyuran species with differing preferentia for normal habitat, were studied and comprise Chapter 5 of the present work. From these investigations on haemolymph solute levels along with studies in haemocyte glycogen levels, gill strucutre and aerial oxygen uptake an attempt has been made to correlate metabolic status and ultimately survival in air with the normal habitat of each species.

On the basis of the general findings of the results section of this thesis, an overall discussion has been made in Chapter 6.

Figure 1.1. The typical relationships between haemocyanin oxygen affinity and A) pH in cold water species at 15°C (Truchot, 1983 B) temperature for C.maenas (Truchot, 1973a) and C) total salinity for C.maenas (Truchot, 1973a).

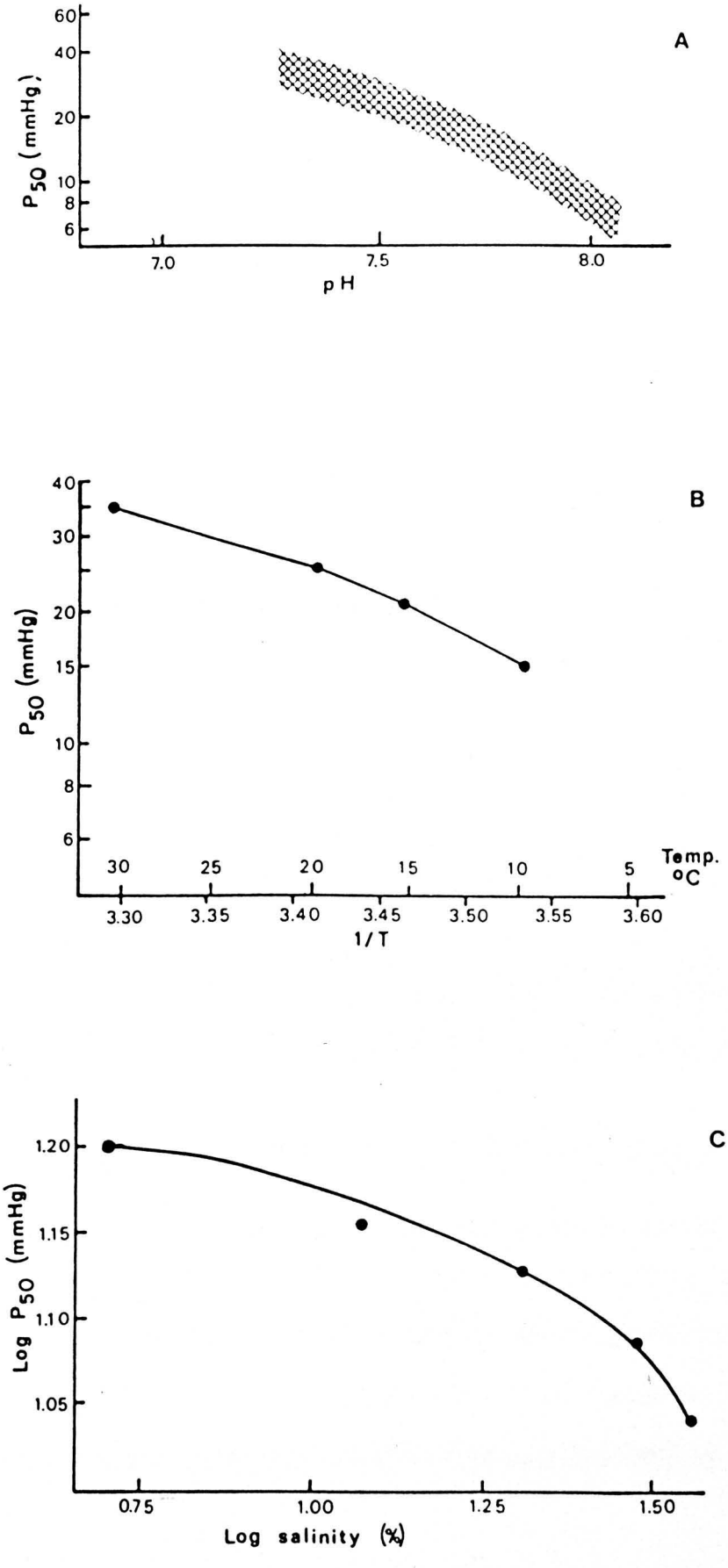

A
60
40
20
10
8
6
P50 (mmHg)
7.0
7.5
8.0
pH
B
40
30
20
15
10
8
6
P50 (mmHg)
30
25
20
15
10
5
Temp. °C
3.30
3.35
3.40
3.45
3.50
3.55
3.60
1/T
C
1.20
1.15
1.10
1.05
Log P50 (mmHg)
0.75
1.00
1.25
1.50
Log salinity (%)

Fig. 1.1

CHAPTER 2

GENERAL MATERIALS AND METHODS

GENERAL MATERIALS AND METHODS

2.1. Animal Husbandry

Male and non-ovigerous female specimens of Carcinus maenas (L.) and Cancer pagurus (L.), ranging from 30-200g wet weight were collected in the spring and summer months (April - September) from the rocky shores at either Filey Brigg or Burniston, North Yorkshire, England. In the winter months, or when assured supplies of large animals were required, the animals were obtained from suppliers at the Marine Station, Millport, Scotland. Male and non-ovigerous female specimens of Liocarcinus puber (L.) of wet weight 50-150g, were obtained from a shellfish exporter, Rodney George, Salen, Argyll, Scotland.

Stock intermoult animals were maintained in large, plastic tanks (45L) supplied with clean, aerated circulating seawater (Salinity 30.0-31.0‰ S; T^{o} = 10°C). Following collection, animals were kept in the aquarium for about one week before transfer to experimental tanks for salinity and/or temperature acclimation. This procedure was to ensure that any tidal rhythms of activity and oxygen consumption (Naylor, 1958), of ventilation volumes and scaphognathite reversals (Arudpragasam & Naylor, 1964b) were lost and that their spontaneous appearance would be avoided during experiments. The animals did, however, retain their light/dark activity rhythms in which they tended to be most active during darkness and least active during the daylight hours. Consequently, the work described here was carried out during daylight hours unless otherwise stated. The animals were maintained in a light/dark regime similar to the pertaining natural photoperiod.

For given salinity experiments the animals were allowed to acclimate for at least 10 days in smaller plastic aquaria (30L) with recirculating water at 10.0, 13.5, 19.0 or 30.0‰ S and 15°C. The animals were fed every third day on chopped Mytilus or fish flesh. However, no experimental animals were fed for 3 days before or during the experiments unless otherwise stated.

2.2. Haemolymph Sampling

Haemolymph samples were obtained by capillary puncture through the arthrodial membrane at the base of a walking leg. In all experiments 200-250µl of haemolymph was obtained from each individual and collected in a microcentrifuge vial (L.I.P. Ltd). Normally the blood was centrifuged immediately at 3000g for 5 mins before being analyzed, subsampled or stored at -20°C, depending on the nature of the experiment. In experiments where whole blood samples were required (e.g. analysis of haemolymph carbohydrates) the original blood was subsampled before centrifugation (Page No.**58**). The time taken for blood collecting was kept to between 20-30 seconds at all times in order to minimize the stress to the animals caused by handling and sampling. This time restriction of handling time was particularly important in experiments involving the estimation of haemolymph glucose and/or carbohydrate estimation (Page No.**57**), since a hyperglycemic response to handling stress has been shown to occur in decapod crustaceans (Roche & Dumazert, 1935; Kleinholtz & Little, 1949; Lynch & Webb, 1973; Telford, 1974; Walters, 1980). In short term hypoxia (Page No.**21**) or aerial exposure (Page No.**57**) experiments, the animals used were bled serially as an alternative to using different groups of animals at each sampling. Serial bleeding

was used, with corresponding control experiments, because of the degree of individual variation shown by haemolymph constituent concentrations in normoxic conditions and the variability of responses to any given stress shown by animals in any group. Serial bleeding was also used in the investigations of protein synthesis during long term hypoxia (Page No. 41) because considerable individual variability of protein concentration among normoxic individuals of many species has been shown (Uglow, 1969a; Djangmah, 1970).

2.3. Haemolymph Analysis

2.3.1. Atomic Absorption Spectrophotometry

For atomic absorption spectrophotometry measurements, haemolymph and seawater samples were diluted 1000x in distilled water for sodium analysis, or for calcium and magnesium samples 200x and 500x respectively in 0.1% Lanthanum chloride solution (Hopkin and Williams). The lanthanum chloride solution was used to overcome the supressive effects of phosphate ions and protein on calcium absorption and the slight suppressive effect on both calcium and magnesium absorption by high sodium concentrations (Willis, 1960).

Analyses were made using a Perkin Elmer Model 103 Atomic absorption spectrophotometer coupled to a Perkin Elmer Model 56 chart recorder. The calibration standards used in the analyses were 0.1-0.4mMl^{-1} for sodium; 1.0-5.0µg.ml^{-1} for calcium, and 0.1-0.6µg.ml^{-1} for magnesium. A fresh set of standards were prepared from stock solutions, containing Analar grade reagents, for each set of samples. The haemolymph ion concentrations were obtained from the standard curves prepared for each ion being analyzed.

2.3.2. Haemolymph Lactate Determination

Haemolymph lactate concentrations were determined using Boehringer Test Combination No. 124842 (Boehringer Corporation London Ltd). The test principle is based on the reaction:-

$$\text{L - Lactate} + NAD^{+} \overset{LDH}{\rightleftharpoons} \text{Pyruvate} + NADH + H^{+}$$

where the concentration of the reduced form of Nicotinamide adenine dinucleotide (NADH) is proportional to the L-Lactate concentration.

In the procedure, 100µl of each haemolymph sample was added to 200µl of ice cold 0.6N Perchloric acid, mixed and centrifuged at 3000g for 5 min to obtain a deproteinized supernatant. A 200µl aliquot of the supernatant was added to 2ml of Glycine/Hydrazine buffer (pH 9.0), 200µl of Nicotinamide adenine dinucleotide (NAD) and 20µl of the enzyme Lactate Dehydrogenase (LDH), which initiated the reaction. The samples and a reagent blank, containing 200µl of 0.6N Perchloric Acid instead of supernatant, were incubated at 25°C for exactly 1hr. The absorbance of each samples was read against the reagent blank at 340nm using a Cecil CE 303 Grating Spectrophotometer (EEL Instruments Ltd). The concentration of L-Lactate in each sample was calculated using the table of values provided, the validity of which had been checked on each occasion by using an L-Lactate calibration standard (Boehringer Mannheim GmbH).

Graham et. al. (1983) suggested that the end point of the reaction is unstable due to interference of the assay by Cu^{2+} ions liberated into the supernatant when haemocyanin is precipitated by perchloric acid. Engel & Jones (1978) suggested that lowering the pH of the glycine/hydrazine buffer (Sigma Chemical Co.) from 9.2 to 9.0 with HCl and adding Ethylenediaminetetra-acetate, EDTA (12mM), completely

eliminated the interference and produced a stable end point. However, in the Boehringer Test Combination used in this study the pH of the glycine/hydrazine buffer was 9.0 rather than the pH 9.2 of the Sigma Chemical Co. Diagnostic Kit and stable end points have been obtained without the use of EDTA.

2.3.3. Haemolymph Glucose Determination

Haemolymph glucose concentrations were determined using Sigma Kit 510 (Sigma Chemical Co.), which is a quantitative procedure using the enzymes glucose oxidase and peroxidase. The procedure is based on the following coupled enzymic reactions:-

i) $$\text{Glucose} + 2H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic Acid} + 2H_2O_2$$

ii) $$H_2O_2 + \underset{\text{(Colourless)}}{\text{O-Dianisidine}} \xrightarrow{\text{Peroxidase}} \underset{\text{(Brown)}}{\text{Oxidized O-Dianisidine}}$$

where the intensity of the brown colour at 425-475nm is proportional to the original glucose concentration.

A deproteinized supernatant was obtained by adding 100µl of each haemolymph sample to 200µl of 0.33N Perchloric acid, mixing and centrifuging at 3000g for 5 minutes. A 200µl aliquot of the supernatant was added to 2ml of Combined Enzyme Colour Reagent, containing the enzymes glucose oxidase and peroxidase and O-Dianisidine and the samples were incubated at 37°C for 30 minutes. A distilled water blank and glucose standards (2.5, 5.0, 10.0 and 20.0mg.100ml^{-1}) were incubated with each complete set of samples being analyzed. The intensity of the brown colour was measured at 450nm using a Cecil CE 303 Grating Spectrophotometer (EEL Instruments Ltd.). The glucose concentrations were derived from the standard curve

prepared for each sample set.

2.3.4. Haemolymph Protein Determination

Haemolymph protein concentrations were determined using the Sigma Kit 540 (Sigma Chemical Co.) which is a quantitative procedure for serum total protein based on the Biuret Method. The procedure is based on the reaction:-

$$\text{Copper} + \text{Serum proteins} \xrightarrow{\text{NaOH}} \text{Copper protein complexes (purple)}$$

where copper in biuret reagent, stabilized by tartrate, reacts with the peptide bonds of serum proteins to form a purple colour with an absorption maximum at 540-545nm. The colour development at standard time is proportional to the total protein concentration in the serum.

In the procedure, 25µl of haemolymph serum were added to 2.5ml of Biuret reagent, and the samples, glass distilled water blank and an 80mg.ml^{-1}. Protein (Human albumin/Human globulin) standard were incubated at 20^{o}C for 15 minutes. The absorbance of each sample was read against the reagent blank at 540nm using a Cecil CE 303 Grating Spectrophotometer (EEL Instruments Ltd.). The total protein concentrations were calculated with reference to the standard using the formula:

$$\text{Haemolymph Protein Concentration (mg.ml}^{-1}) = \frac{\text{(Absorbance of sample) (Concentration of sample)}}{\text{(Absorbance of standard)}}$$

2.4. Recording of Heart and Scaphognathite Rates

The Impedance Technique

In all experiments where heart and scaphognathite beat rate data were required, organ activities were recorded using impedance techniques. The transducers used were the "Classic H7 Impedance Pneumographs" (Scientific Instruments Centre) coupled to a 2 channel oscillograph (George Washington Ltd.). The experimental arrangement is represented diagrammatically in Figure 2.1.

A large common (earth) electrode placed in the water of the tank supplies several impedance transducers. The movements of particular organs under investigation are detected via single electrodes affixed in close proximity to the organ under study, and each associated with a single transducer. The pneumograph produces a small, oscillating current between the disparate sized electrodes and any perturbations close to the smaller (organ) one are detected, filtered and amplified.

The outputs of each transducer could be monitored on an oscilloscope (Telequipment) or produced as chart records on a chart recorder (George Washington Ltd.) so that qualitative and quantitative analyses of them could be made.

Electrode anchoring and attachment

Animals were removed from the water, their carapaces were dried thoroughly and the intended areas where electrodes were to be anchored were roughened using emery paper. A small hand-held electric drill (12v Expo) with a dental burr, was used to drill holes in the carapace at the sites indicated in Figure 2.2. When drilling the hole for the heart electrode, care was taken to avoid blood loss by not

puncturing the pericardium beneath the carapace.

The recording electrodes were made from 0.25mm diameter plastic coated miniature solid wire (R.S. Components Ltd.), which had the last 2-3mm bared and bent through 180°. The electrodes were inserted into the drilled hole and positioned against the wall of the branchial chamber for each of the scaphognathites or positioned between the pericardium and carapace for the heart electrode. A low melting point black wax was used to seal the electrodes in place at the sites and to anchor the electrodes to the carapace. The scaphognathite electrodes were laid over the back of the animal and each was secured at an additional point on the carapace before all three electrodes were secured in the region of the heart electrode (Figure 2.2). The black wax attachments were strengthened by applying expoxy resin adhesive (Araldite) around their margins, which was allowed to air dry before the animals were returned to the holding tank.

Heart and Scaphognathite Recording

The animals were held in partitioned plastic tanks (30L) supplied with circulating aerated seawater of the chosen experimental salinity at 15°C. In order to minimize extraneous optical stimuli, such as shadows, the tank dividers were of opaque material and the tank was covered with black plastic. After electrode implantation the animals were held in the tank for 24 hours before any recordings were made, thereby enabling the organ rates to return to non-stressed levels (See Cumberlidge and Uglow, 1977a).

The particular experimental conditions were achieved as described in the relevant section (Page 21 for hypoxia, page 61 for aerial

exposure). Recordings (ca. 2 minutes each) were made of each organ (heart and left and right scaphognathites). Both scaphognathites were monitored because the organs do not always beat synchronously under normoxic conditions (Uglow, 1973).

Figure 2.1. Diagram of the system for monitoring the activity of the heart and both scaphognathites in a single brachyuran. The small electrodes (H, L_s and R_s) are attached near to the heart, left and right scaphognathites respectively. The large metal electrode (E) is used as a common earth electrode for the impedence pneumographs (I.P.), the output from each of which is passed through an amplifier (A) before being recorded.

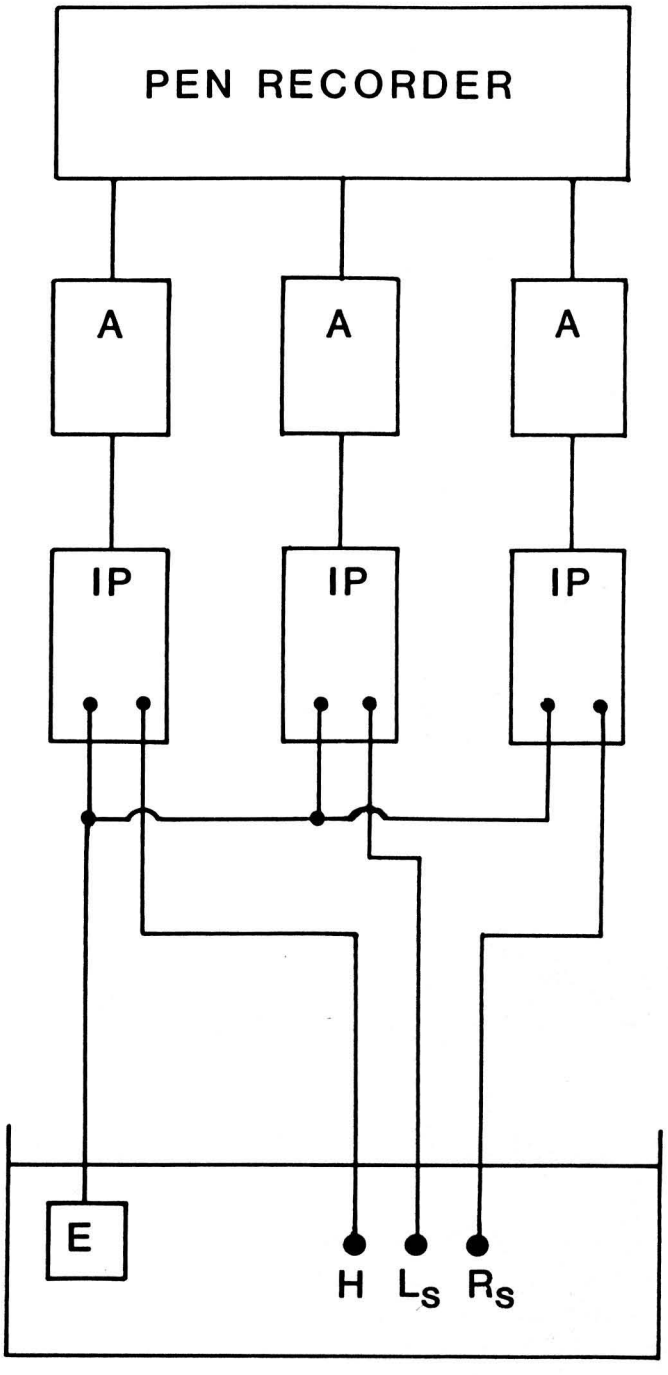
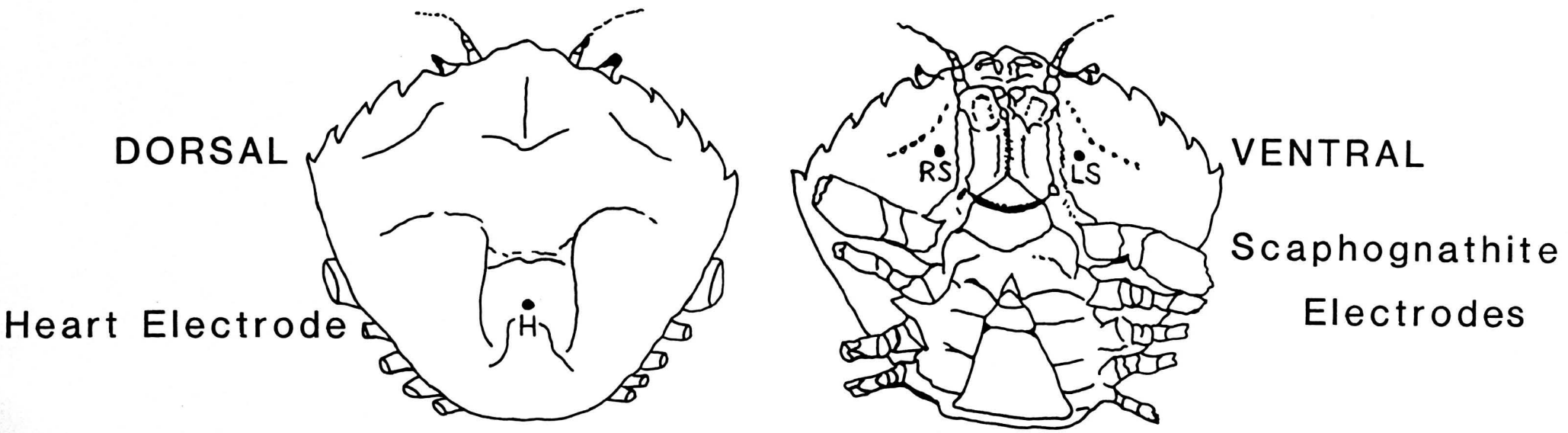


Fig. 2.1

Figure 2.2. *Carcinus maenas*: sites of electrode insertion for heart (H), left (LS) and right (RS) scaphognathites.



Sites of Electrode Insertion

Fig. 2.2

CHAPTER 3

SOME EFFECTS OF HYPOXIA ON THE ION/OSMO-REGULATION AND ACID-BASE BALANCE OF *Carcinus maenas* (L.) AT 4 EXPERIMENTAL SALINITIES.

3.1. Introduction

Carcinus maenas (L.) is a hyper/hypo-osmoregulating species which is able to maintain some control over its internal osmotic status in media covering a wide salinity range. The species is also known to regulate strongly various haemolymph ions (Webb, 1940; Robertson, 1960; Lockwood and Reigal, 1969; Zanders, 1980). The maintenance of a hyperosmotic haemolymph over a wide range of external salinity depends on various physiological processes such as an active transport of ions against an ionic gradient, a low "surface" permeability to water and ions, and an ability to control the volume and/or the ionic composition of the urine. Such processes may be energy-consuming and there is some evidence that certain species may expend an appreciable proportion of their available energy on the maintenance of ionic regulation (Hagerman, 1970; Taylor, 1976; Spaargaren, 1977; Taylor et al., 1977a).

Inactive Carcinus in fully marine conditions have been shown to possess respiratory independence of their external media oxygen tensions (P_wO_2) down to a critical value (P_{cr}) in the region P_wO_2 = 60-80 torr (Taylor, 1976). At oxygen tensions $<P_{cr}$ respiration becomes dependent on P_wO_2 and, at such low oxygen levels, may have important implications with regard to the energy budget of the animal.

However, the critical oxygen tension is a variable parameter and has been described by Herreid (1980) as dependent both on the environmental conditions and the physiological state of a given animal. Herried stated also that the P_{cr} value increases as a consequence of an increase in oxygen consumption ($\dot{M}O_2$). In crustaceans an increase in $\dot{M}O_2$ can result from changes in environmental conditions

(e.g. external salinity and temperature), physiological status (e.g. through physical activity or handling stress) or moult condition. In addition, the P_{cr} is dependent on the ability of the animal to supply oxygen to the tissues. Any impairment of this ability, as would result from a reduction in circulation and/or ventilation, or a decrease in the surface area for gas exchange, would result in the P_{cr} increasing to a higher external value (i.e. an increased PO_2 value). Consequently, the experimental conditions and the animals physiological status have to be carefully defined before a critical oxygen tension can be stated for a given species.

In summer months especially, the normal distribution of Carcinus includes the intertidal zone and estuaries; areas which may be subject to periodic low salinity/low oxygen conditions. Although some attention has been paid to the combined effects of low salinity /hypoxic media on decapods, the main objectives of such studies have principally been concerned with appraisals of respiratory and/or cardiac and ventilatory responses (e.g. Taylor et al. 1977b). Rather less attention appears to have been paid to the possible effects of such combinations of environmental variables on the osmo- and ionic regulating performances of decapods. The strongly-regulating shrimps Palaemon adspersus and Crangon crangon have been shown to incur a loss of haemolymph Cl^-, presumably to the medium, during periods of hypoxia at low salinities. The extent of such losses was related to the combined effects of medium salinity and P_wO_2 and the exposure time at such conditions (Hagerman and Uglow, 1981, 1982).

The capacity of decapods for ion regulation has implications for their haemolymph acid-base balance in addition to that of their ionic status (Henry & Cameron, 1982). Cameron (1979) reviewed this subject

and stated that, from available evidence, it would appear that the principal means of controlling haemolymph pH in water-breathing invertebrates is not *via* changes in ventilation but rather by the modulation of ion-exchange mechanisms in the gills, namely the $Na^+ - H^+$ and $Cl^- - HCO_3^-$ systems (Cameron, 1978b; De Pew and Towle, 1979). It is the difference in the rates of H^+ and HCO_3^- excretion *via* these pathways that act to change pH, i.e. the strong ion difference (S.I.D.) (Stewart, 1978) is controlled rather than the partial pressure of carbon dioxide in the blood (P_{CO2}).

The aim of these experiments was to investigate some of the effects of hypoxic stress, and subsequent re-oxygenation, at various salinities on the circulating levels of various solutes and acid-base balance of the haemolymph of *C. maenas*.

3.2. Materials and Methods

The holding procedures used for C. maenas are described in detail in the General Materials and Methods (Page No.**9/10**). Specimens used had been acclimated at the given salinity for at least 7 days and were not fed for three days before, or during the experiments.

3.2.1. Control of External Oxygen Tension

Required oxygen tensions were obtained by flushing the experimental media with high purity nitrogen gas (Air Products). The P_wO_2 values were monitored constantly throughout each experiment using an EIL Portable Oxygen Meter, 1520. The P_wO_2 was lowered from the initial normoxic level (ca 130 torr) to the experimental hypoxic level over a period of a) 30 minutes (P_wO_2 = 40 torr) or b) 45 minutes (P_wO_2 = 20 torr) as shown in Figure 3.1. The oxygen tension was maintained at the prescribed level for the duration of the experiment, small fluctuations being adjusted by flushing with air or nitrogen gas. In the experimental tank the water was circulated, thus to ensure its uniformly distributed P_wO_2. On completion of the hypoxic phase of the experiment, the medium was flushed with air for 30 minutes to restore normoxic conditions and sampling proceeded at this new P_wO_2 for a further 4hrs.

3.2.2. Haemolymph Sampling

At given intervals (time 0, 150, 270, 420, 540 minutes) prebranchial samples (200-250µl each) were withdrawn through the arthrodial membrane at the base of a walking leg, the same animals being used throughout an experiment. Where pH was to be measured, sampling was carried out only at 0, 270 and 540 minutes. The samples were centrifuged at 3000g for 5 minutes in a Quickfit Micro-centrifuge.

In practice, the time involved in handling and sampling each animal was restricted to less than 30 secs in order to minimize handling stress.

3.2.3. Haemolymph Analysis

Haemolymph samples were used variously for Na^+, Ca^{2+}, Mg^{2+} and Cl^- determinations, osmolarity and lactate analyses and pH measurements. For the chloride determinations, 20µl of haemolymph was diluted 1:4 with glass-distilled water and aliquots (20µl) were measured for chloride concentration coulimetrically using a Radiometer CMT10 Titrator.

The Na^+, Ca^{2+} and Mg^{2+} concentrations in the haemolymph were determined by atomic absorption spectrophotometry (AAS) the details of which are given in the General Materials and Methods (Page No.**11**). Diluted haemolymph and seawater samples were analysed using a Perkin Elmer Model 103 atomic absorption spectrophotometer coupled to a Perkin Elmer Model 56 chart recorder.

In the osmolarity analyses, 75µl of haemolymph were diluted 1:1 with double-distilled water and measured using a Knauer Semi Micro-Osmometer. A 100µl haemolymph sample was used for lactate determinations and concentrations were measured spectrophotometrically using a Boehringer lactate diagnostic pack (No. 124842). The principle and procedure involved in the assay are detailed in the General Materials and Methods (Page No.**12**).

The pH of the haemolymph samples was measured immediately after collection using a Beckman Combination Microelectrode 3950 connected to a Beckman 4500 digital pH meter. The electrode was standardised to pH 7.0 and thermostatted at a constant 20°C.

3.2.4. Heart and Scaphognathite Rate Measurements

The principle of the organ monitoring technique and the procedure for electrode attachment are described in detail in the General Materials and Methods (Page No.**15/16**). In order to allow the organ rates to return to non-stressed levels the animals were in tanks (30L capacity) supplied with aerated circulating seawater at prescribed salinity values for 24hr following electrode implantation. To minimize undesigned stresses, such as shadow stimuli and to prevent aggressive behaviour the tanks were provided with black plastic covering and partitioned internally with opaque dividers.

At each experimental P_wO_2/salinity combination, the rates of the heart and both scaphognathites of each animal were measured initially in normoxic conditions for at least one minute. The oxygen tension in the tank was lowered, as described in Section 3.2.1. (Page No.**21**), to the final P_wO_2 value required (Figure 3.1) and at all times the medium oxygen tension was monitored with an EIL Portable Oxygen Meter 1520. The organ rates (beats.min^{-1}) of each animal were measured at given times (60, 150, 270, 390 and 510 minutes) during the period of hypoxic exposure, again each recording being of at least one minutes duration. Both scaphognathites were monitored simultaneously and a mean value was obtained ($^{[L+R]}/2$ - see Uglow 1973).

3.2.5. Statistical Treatment of Data

The measured values are given as means ± S.E. of mean with the number of observations in parenthesis. The significance of any apparent difference between the mean values of variables was tested using Students t-test and the word "significant" means that the difference is significant at the 95% level of confidence ($P<0.05$).

Figure 3.1. Diagram showing the changes in medium oxygen tension during short-term hypoxia experiments at i) P_wO_2 = 40 torr or ii) P_wO_2 = 20 torr). The times at which haemolymph samples were taken are indicated (↓).

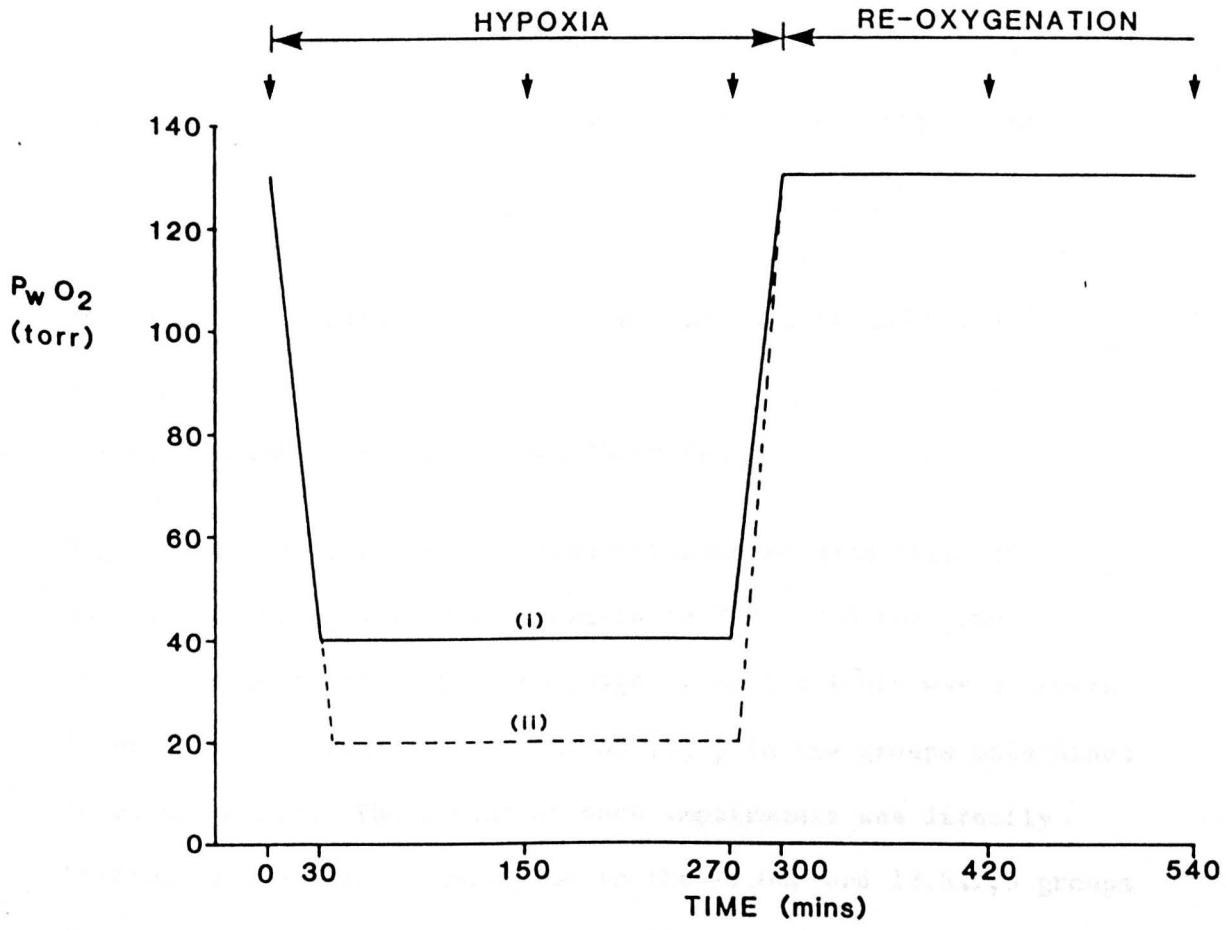

HYPOXIA
RE-OXYGENATION
$P_w O_2$ (torr)
140
120
100
80
60
40
20
0
(I)
(II)
0
30
150
270
300
420
540
TIME (mins)

Fig.3.1

3.3. Results

The haemolymph chloride ($[Cl^-]$) regulation curve obtained for groups of acclimated crabs in normoxic media (P_wO_2 = 130 torr) is shown in Figure 3.2. The $[Cl^-]$ was isoionic with that of the medium at a salinity of approximately 25.0‰ S (413mM Cl^- l^{-1}). In lower salinity media, down to *ca* 10.0‰ S *Carcinus* showed strong regulation of haemolymph chloride.

Figures 3.3 and 3.4 show the data obtained at each salinity when the external oxygen was lowered to P_wO_2 = 20 torr and 40 torr respectively. At both oxygen tensions there was a general lowering of the regulation level of $[Cl^-]$ in the groups maintained in dilute media. The extent of such impairments was directly related to the test salinity and in the 10.0‰ and 13.5‰, S groups the changes were significant at the 5% level. Conversely, in hyperosmotic media at P_wO_2 = 40 torr the regulated level of $[Cl^-]$ was higher than at normoxia, although this difference was not significant at the 5% level. At P_wO_2 = 20 torr, in 30.0‰ S media there was no change in $[Cl^-]$ levels following the 4hr period of hypoxia.

On re-oxygenation of the medium, $[Cl^-]$ levels returned to values not significantly different to the initial normoxic levels. Although in certain groups $[Cl^-]$ was not restored to original values during the subsequent 4hr normoxic period, there was no significant difference between initial and final values at any salinity ($P>0.05$) in any group. Control group data (P_wO_2 = 130 torr) are shown in Figure 3.5 for each salinity and no significant changes were observed due to the sampling procedure ($P>0.05$ in all cases).

The changes in $[Cl^-]$ levels following a period of 4hr in hypoxic medium (P_wO_2 = 20 or 40 torr) are shown in Figure 3.6. When P_wO_2 levels were lowered the haemolymph chloride was regulated at new levels which were nearer to those in the external medium (i.e. there was a trend towards isochlorinity at the lower end of the experimental salinity range).

The haemolymph calcium ($[Ca^{2+}]$) values obtained in fully oxygenated (P_wO_2 = 130 torr) and hypoxic (P_wO_2 = 20 or 40 torr) media at the given salinities are given in Table 3.1. Because of the differences in original levels of circulating $[Ca^{2+}]$ in the two groups, and the observed difference in response to hypoxic media among groups, analyses of data were performed on transformed data (i.e. transformed to relative data as % change from original value for each individual).

The $[Ca^{2+}]$ levels increased with hypoxia at all the P_wO_2/salinity combinations tested (Table 3.1). However, analyses of these data showed that only the changes in the groups at 10.0‰ S (P_wO_2 = 40 torr) and 10.0‰, 13.5‰ and 19.0‰ S (P_wO_2 = 20 torr) were significant ($P<0.05$). On return to normoxic conditions, $[Ca^{2+}]$ levels returned to values not significantly different ($P>0.05$) from original levels. Control experiments at all salinities, which duplicated the sampling times and procedures but were carried out at normoxia only, produced no significant changes to the $[Ca^{2+}]$ levels ($P>0.05$ in all cases).

In Figure 3.7 the relative changes in $[Ca^{2+}]$ (i.e. % change from original normoxic value) after a 4hr period in oxygenated (P_wO_2 = 130 torr) or hypoxic (P_wO_2 = 20 or 40 torr) media are shown

for groups at each experimental salinity. It would appear that at all experimental salinities there is an increase in $[Ca^{2+}]$ as the external oxygen tension is lowered, the magnitude of such changes being salinity dependent.

The quantitative data obtained on haemolymph sodium and magnesium concentrations at the various experiment P_wO_2/salinity combinations are shown in Tables 3.2 and 3.3 respectively. At no test salinity did a 4hr period of hypoxia (at P_wO_2 = 20 or 40 torr) induce any significant changes in the circulating levels of sodium and magnesium ($P>0.05$ in all cases). In common with haemolymph calcium values there was considerable individual variation in normoxic haemolymph magnesium concentrations within groups at a given salinity.

The haemolymph lactate levels measured at each salinity at P_wO_2 = 20 or 40 torr are shown in Figures 3.8 and 3.9 respectively. At P_wO_2 = 40 torr, all groups showed increases in blood lactate levels but only those associated with the 10.0‰ and 13.5‰ S groups were significant ($P<0.05$). At all salinities, increases in blood lactate at P_wO_2 = 20 torr were significant ($P<0.05$). During the subsequent 4hr period of normoxia, the elevated lactate levels declined and all except the 13.5 and 19.0‰ S groups at P_wO_2 = 20 torr had returned to values not significantly ($P>0.05$) different from the original, normoxic values. Control group data (P_wO_2 = 130 torr) are shown in Figure 3.10 and reveal that in the control groups, there were

small but non-significant ($P>0.05$) increases in blood lactate. These presumably reflected the responses to either a natural rhythm or the handling procedures or both. The data in Figure 3.11 suggest that the rate and extent of lactate production are salinity-dependent responses.

The changes in haemolymph pH measured at the various P_wO_2/salinity combinations during periods of hypoxia and on subsequent re-oxygenation are shown in Figure 3.12. In normoxic conditions, haemolymph pH was higher in 13.5‰ S than 30.0‰ S media, while under hypoxic conditions (P_wO_2 = 20 torr), significant ($P<0.05$) increases in blood pH were recorded at both 13.5 and 30.0‰ S. The pH increase was greatest in the most dilute medium. On return to normoxic conditions, for 4hrs at both salinities, the haemolymph pH returned to levels not significantly different from the initial levels. In the control experiments, at each salinity, there was a small decrease in haemolymph pH during serial sampling although such reductions were not significant ($P>0.05$) at either salinity.

The cardiac and ventilatory rate responses to given P_wO_2/salinity combinations are shown in Figures 3.13 and 3.14 respectively. In normoxic conditions animals acclimated to low salinity media (13.5‰ S) showed higher mean heart (f_h) and scaphognathite (f_{sc}) rates than animals from full salinity media (30.0‰ S). When the external oxygen tension was lowered f_h decreased, though not significantly ($P>0.05$), and f_{sc} increased significantly ($P<0.05$) at all the P_wO_2/salinity combinations. At both 13.5‰ and 30.0‰ S the decrease in f_h was more pronounced as the P_wO_2 declined from 40 to 20 torr. However, at both salinities the changes in f_{sc} were greatest when the P_wO_2

was lowered to 40 torr rather than 20 torr. The changes at any given P_wO_2 value were larger in the low salinity (13.5‰ S) than the full salinity (30.0‰ S) media.

In Table 3.4 the measured changes in strong ion difference (S.I.D.) expressed as $(Na^+ - Cl^-)$ are given for the groups of animals at the given P_wO_2/salinity combinations. In this study the difference between the concentrations of the two major ions, sodium and chloride, in the haemolymph $(Na^+ - Cl^-)$ is used as an index of the S.I.D. The values given are the measured changes in the S.I.D. between initial normoxic values and those after 4hr (130 torr) or 4hr hypoxia (P_wO_2 = 20 or 40 torr). After a 4hr period of hypoxia at a given salinity the S.I.D. increased with decreasing P_wO_2. Although changes in S.I.D. were also observed in control experiments, (P_wO_2 = 130 torr) the apparent hypoxia-induced S.I.D. increases remained after these had been taken into consideration. In addition there was an apparent salinity-dependent increase in S.I.D. with decreasing salinity at a given P_wO_2, the increase in S.I.D. being greatest in the 10.0‰ S media.

The quantitative data obtained on haemolymph osmolality at the various experimental P_wO_2/salinity combinations are shown in Table 3.5. At neither 13.5 or 30.0‰ S, did a 4hr period of hypoxia (P_wO_2 = 20 torr) induce any significant changes in the circulating blood osmolality levels ($P>0.05$ in all cases).

3.4. Discussion

Carcinus maenas responds to a lowering of external water oxygen tensions with an elevation of haemolymph pH, the extent of which is apparently dependent on a combination of the degree of hypoxic stress experienced and the pertaining environmental salinity. The pH change was greater in low salinity (13.5‰ S) animals than those in full salinity (30.0‰ S) when tensions were lowered to P_wO_2 = 20 torr. These hypoxia-induced increases in blood pH are of a similar magnitude to those found by Truchot (1975c); Taylor *et.al.*, (1977b); McMahon *et.al.*, (1978), Burnett (1979) and Burnett and Johansen (1981). The hypoxia-induced alkalosis is characterized also by an increase in haemocyanin oxygen affinity (i.e. decrease in P_{50}) resulting from a Bohr shift (Truchot, 1975d). In crustaceans, such an increased affinity has adaptive value, enabling a moderately hypoxic haemolymph to be highly oxygenated at the gills (McMahon *et.al.* 1978 ; Burnett, 1979).

Haemolymph pH is determined by three independent quantities; the total weak acid present, the partial pressure of carbon dioxide in the blood (P_{CO_2}) and the strong ion difference (S.I.D.) (Stewart, 1978) which is the difference between strong acid anions and strong base cations.

In water-breathing invertebrates the control of the haemolymph CO_2 content has important implications for acid-base status. The partial pressure of CO_2 in the blood may be changed by metabolic CO_2 production or altered ventilation. Hypoxic stress resulted in increased scaphognathite rates (f_{sc}) at all salinities, the changes in 13.5‰ S being greater than those in 30.0‰ S media. At a given salinity the increase in f_{sc} was greater on reduction of the P_wO_2 to 40torr rather than 20torr, these results being consistent with the findings of Uglow (1973) and Taylor (1976) for

Carcinus. Taylor (1976) also showed that an increase in scaphognathite rate during hypoxia was indicative of an increase in ventilation volume since no change in stroke volume was measured. Therefore, an increase in ventilation volume in response to a reduction in oxygen tension may result in an increased rate of CO_2 excretion, apparent as a decline in haemolymph P_{CO_2}. Burnett and Johansen (1981) found that Carcinus responded to hypoxia with a fall in haemolymph P_{CO_2}. They stated that a hypoxia-induced hyperventilation did not account fully for an observed haemolymph alkalosis and that a small metabolic component was apparent after 2hr hypoxia (P_wO_2 = 20-25 torr). However, it is apparent that there are serious implications associated with the use of ventilation as a mechanism to adjust blood acid-base status. In water breathing invertebrates, ventilation rates are controlled by the animal's oxygen requirements, which are considerably greater than the CO_2 excreting requirements (Randall and Cameron, 1973; Cameron, 1978b). In a review Cameron (1979) stated that, from available evidence, it would appear that the principal means of controlling pH in water breathing invertebrates is the modulation of the ion exchange mechanism in the gills, namely the Na^+-H^+ and Cl^--HCO_3^- systems (Cameron, 1978b, De Pew and Towle, 1979). It is the difference in the rates of H^+ and HCO_3^- excretion via these pathways that effect pH changes, i.e. the 'S.I.D.' is controlled rather than P_{CO_2}.

The 'S.I.D.' value can be effected by organic acid production, mobilization of carbonates from large body stores in the hepato-pancreas and/or exoskeleton, and ionic, excretory or uptake mechanisms located either in the antennary glands or in the gills (Truchot, 1983).

The production of lactic acid has been shown as a response to hypoxic stress. The extent of the accumulation of circulating lactate being dependent on the combination of the degree of hypoxia and the pertaining environmental salinity. The levels of lactate measured were greater in animals at P_wO_2 = 20 torr than at P_wO_2 = 40 torr for all experimental salinities, while at a given P_wO_2, the increase in circulating blood lactate was greater as the medium salinity was lowered. This finding is consistent with the work of Taylor et.al. (1977b) who showed that the P_{cr} for $\dot{M}O_2$ in Carcinus increases with decreasing salinity below 18°C.

Gabbott (1976) stated that, in response to hypoxia and depending on the degree of hypoxia experienced, invertebrates showed a shift in emphasis from aerobic to anaerobic metabolism. Such a shift from aerobic metabolism to anaerobiosis is characterized by the formation of lactic acid, as shown in Figure 3.15, and accumulation of lactate as observed in the present study. Hypoxia induced accumulation of lactate in haemolymph and muscle has been shown to occur in many crustaceans including Carcinus maenas (Taylor, et.al. 1977b; thalassinid mud shrimps (Pritchard and Eddy, 1979) and Pagurus bernhardus (Walters, 1980). Burke (1979) found that medium oxygen tensions of P_wO_2 = <20 torr induced Carcinus to abandon aerobic respiration and to increase lactate production. A shift from aerobic respiration may also result in a decline in metabolic CO_2 production, thereby affecting the haemolymph P_{CO_2}. Although a decrease in CO_2 production should result from the conversion of pyruvate to lactate rather than through the tricarboxylic acid (TCA) cycle to CO_2, the available evidence to substantiate this is

limited. Huggins (1966) found a predominance of labelled alanine and lactic acid and very low activities of tricarboxylic acid intermediates on injection of Carcinus with labelled glucose ($|^{14}C|$), sodium acetate ($|2\text{-}^{14}C|$), succinic acid ($|1{:}4\text{-}^{14}C|$) and sodium 2-oxoglutarate ($|5\text{-}^{14}C|$). His results suggest that pyruvate tends to be transaminated or reduced rather than oxidized via the tricarboxylic acid cycle. This situation could well account for the difficulty experienced by Scheer and Scheer (1951) in demonstrating labelled CO_2 production from ^{14}C-glucose in spiny lobsters.

Re-oxygenation of the medium is accompanied by a drop in the circulating lactate over a period of 4hrs and thus presumably reflects the repayment of an oxygen debt by the re-oxidation of the accumulated end-products of anaerobiosis. Lactate is re-synthesized to glucose via the gluconeogenic pathway, as shown in Figure 3.15. However, controversy exists (Thabrew et. al., 1971; van Weel, 1974; Phillips et. al., 1977) regarding the actual site of such conversions. Here, the rate of lactate removal (ca 4-6hr duration) found in Carcinus upon re-oxidation of the hypoxic medium is rapid compared to rates described for other crustacean species, e.g. Bridges and Brand (1980) found recovery times (to control values) of 6hr in A. roturdatus and N. norvegicus and 20hr in G. strigosa following 4-5hr in 30.0‰ S media at P_wO_2 = <20 torr. This may be a physiological adaptation to the likelihood of encountering hypoxic media in its natural environment, as has been suggested by Bridges and Brand (1980). Indeed, Hochachka and Somero (1973) have emphasised that the ability to convert lactate to glucose is an essential mechanism for an animal that has to tolerate high levels of lactate.

Changes in haemolymph Ca^{2+} and Cl^- ion concentrations were also found to be responses to hypoxic stress, but the anions Na^+ and Mg^{2+} did not alter during hypoxia and no significant changes in blood total osmolarity were recorded at any of the P_wO_2/salinity combinations tested.

An increase in circulating haemolymph Ca^{2+} level was found in the "low salinity" groups (10.0 and 13.5‰ S) at P_wO_2 = 40 torr and in all salinity groups at P_wO_2 = 20 torr, although only the 10.0‰ S (P_wO_2 = 40 torr) and 10.0, 13.5 and 19.0‰ S (P_wO_2 = 20 torr) groups showed changes in blood Ca^{2+} which were significant at the 5% level. The increase in circulating Ca^{2+} in certain P_wO_2/‰ S combination groups may reflect a mobilization of calcium carbonate i.e. $[HCO_3^- + CO_3^{2-}]$ via the calcified exoskeleton or stores in the hepatopancreas. Truchot (1975c) found a small increase in haemolymph buffer base concentration in C.maenas as a response to a reduction in oxygen tension from normoxia to P_wO_2 = 55 torr. However, Burnett and Johansen (1981), found a reduction in the Carcinus haemolymph bicarbonate pool during hypoxia (P_wO_2 = 20-25 torr). McMahon et. al. (1978) found that during moderate hypoxia (P_wO_2 = 50-55 torr) H.vulgaris showed an initial respiratory alkalosis, induced by a fall in haemolymph P_{CO_2}, which was gradually superceded by a metabolic alkalosis resulting from an increase in the haemolymph $[HCO_3^- + CO_3^{2-}]$. The metabolic component of the alkalosis became apparent after 6hrs and became increasingly responsible for the elevated haemolymph pH.

In dilute media (10.0 and 13.5‰ S) hypoxia (P_wO_2 = 20 or 40 torr) induced significant ($P<0.05$) reductions for haemolymph Cl^-.

Presumably such losses were to the external medium. Similar hypoxia-induced losses have been shown to occur in the shrimps Palaemon adspersus and Crangon crangon (Hagerman and Uglow, 1981, 1982) and the rate of such loss was shown to be dependent upon medium salinity, P_wO_2 and exposure time. Hagerman and Uglow (1982) have also suggested that hypoxia-induced $[HCO_3^- + CO_3^{2-}]$ levels may facilitate the outward flux of Cl^- ions in dilute media, possibly via a Cl^-/HCO_3^- exchange system in the gills. In neither the 19.0 or 30.0‰ S media were significant changes in haemolymph Cl^- observed during hypoxic (P_wO_2 = 20 or 40 torr) stress which is in accordance with the findings of Burnett and Johansen (1981) for Carcinus in 17.0 and 35.0‰ S media at P_wO_2 = 20-25 torr).

Spaargaren and Kraay (1973) found that the blood chloride of Crangon crangon was less strongly regulated than other blood electrolytes (e.g. Na^+, K^+ and Mg^{2+}). This led them to suggest that Cl^- was not of primary importance in electrolyte regulation. The present data, which show that circulating levels of Na^+ and Mg^{2+} are P_wO_2 independent lends some support to their contention.

Haemolymph Mg^{2+} concentrations were found to be maintained at about 30-40% of the values pertaining in the medium. This low value is achieved by the active excretion of Mg^{2+} into the urine from the antennal glands (Riegal and Lockwood, 1961). Robertson (1953) postulated a relationship for invertebrates between their general activity (e.g. maximum rates of movement) and haemolymph Mg^{2+} levels. Walters and Uglow (1981) demonstrated an inverse relationship between relative heart activity and haemolymph

magnesium concentration in a series of marine decapods. Here, the observed strict regulation of haemolymph magnesium and the small reduction in heart rate (i.e. bradycardia) observed at each P_wO_2/salinity combination tested may be the reflection of an important implication for Carcinus, whereby the speed of its reactions in unstable media is largely maintained to confer a competitive advantage.

In this study the observed bradycardia at both salinities was more pronounced at P_wO_2 = 20 torr compared to P_wO_2 = 40 torr. These results support those of Taylor (1976) for this species who found that the bradycardia was concommitant with a reduction in cardiac output and an increase in the ventilation/perfusion ratio.

A further response to hypoxia was an apparent P_wO_2-dependent change in the haemolymph strong ion difference (S.I.D.) at a given salinity. Henry and Cameron (1982) found an increase in S.I.D. and a haemolymph alkalosis on transferring C.sapidus from 865 to 260m.Osmol salinity media. They suggested that the S.I.D. increase was correlated with the change in acid-base status. In this study, an increase in S.I.D. was shown to occur in C. maenas as P_wO_2 values fell at a given salinity. As an elevation of blood pH also occurred during falling P_wO_2 in both 13.5 and 30.0‰ S media, it would seem that the changes in blood acid-base status during hypoxia may be related to an increased S.I.D.

During hypoxia there was found to be a distinct shift towards isochlorinity in the haemolymph chloride of those animals in the more dilute media. In the natural population this may have the effect of lowering the tolerance to low salinities should hypoxic conditions persist and thus restrict the boundaries of the local

distribution of Carcinus. The occurrence of hypoxia in certain low salinity environments is increasing (see Jørgensen, 1980). Such a phenomenon has implications for the total energy budget of a strongly regulating species. In dilute media (>10.0‰ S) the energy required for ionic regulation, and through the S.I.D., blood acid-base balance is reputedly very small (Spaargaren, 1975). Although such energy costs may not change in hypoxic media, the total energy available will probably become limited because of lactate formation and perforce, be re-allocated (Spaargaren, 1977). The extent of any such changes will be dependent upon the energy limitation incurred during hypoxia and the ability of the species to cope with such changes. It would appear from the present studies that Carcinus may suffer some restriction of its range during long-term hypoxia, but would seem able to cope with short-term periods of hypoxia of some hours duration such as may occur frequently in parts of its normal range of habitat (e.g. the exposed intertidal regions of the shore).

Figure 3.2. *Carcinus maenas*: Regulation curve of haemolymph chloride against medium chloride ($T^{o}=15^{o}C$). Values given are means ± S.E. for 5-10 animals in each case.

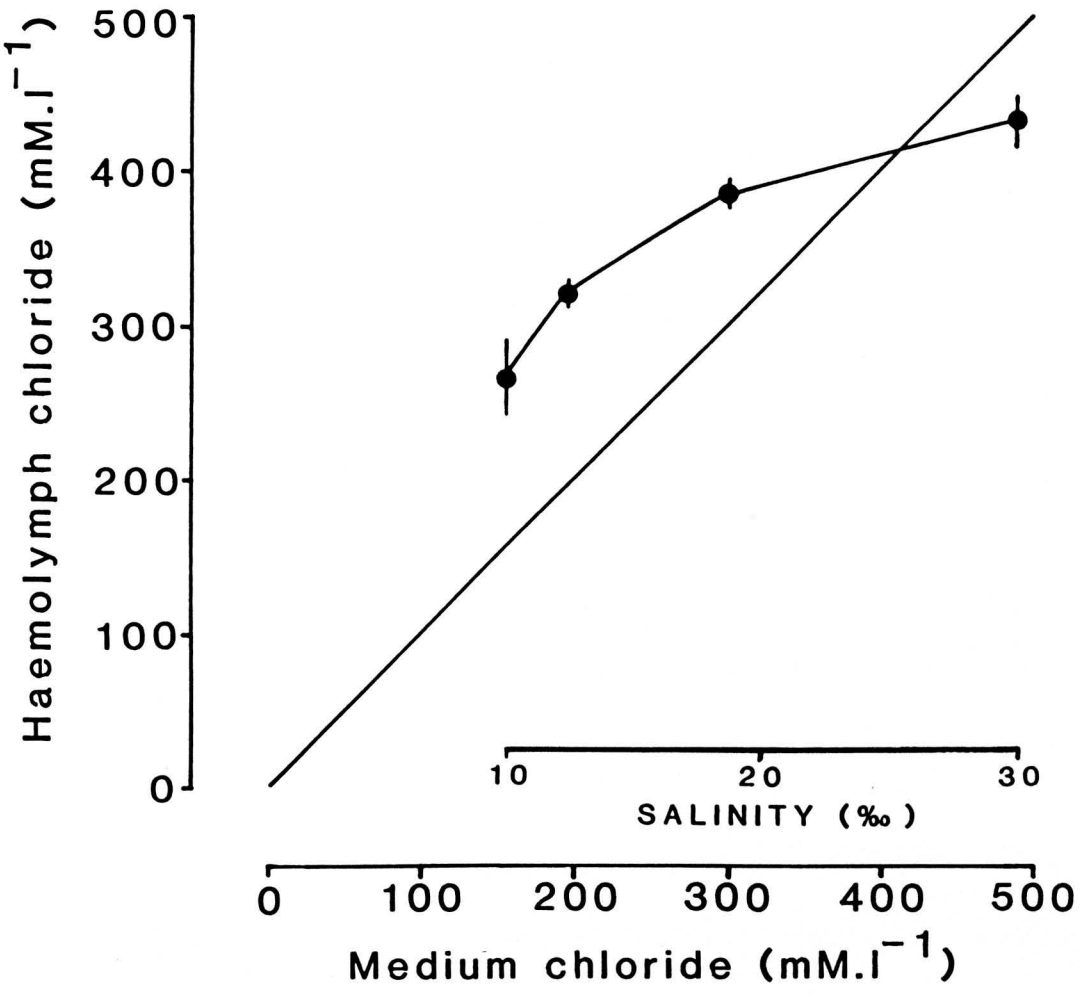

Haemolymph chloride (mM.l^{-1})
500
400
300
200
100
0
10
20
30
SALINITY (‰)
0
100
200
300
400
500
Medium chloride (mM.l^{-1})

Fig.3.2

Figure 3.3. *Carcinus maenas*: Time-concentration curves of haemolymph chloride during a 4hr period of hypoxia (P_wO_2=20 torr) and subsequent re-oxygenation at various salinities (T^{o}=15^{o}C). Values given are means ± S.E. for 5-10 animals in each case.

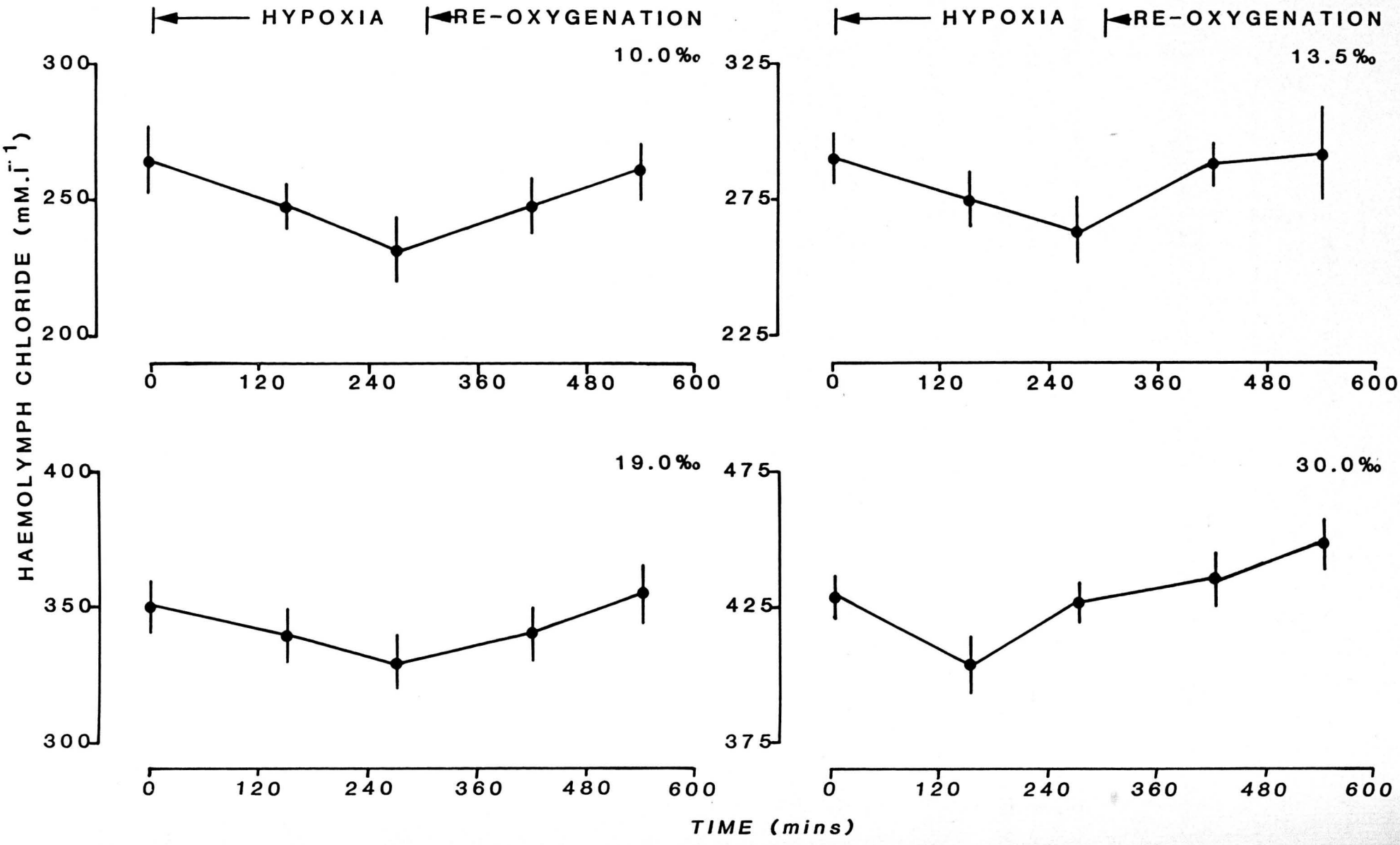

HYPOXIA
RE-OXYGENATION
10.0‰
13.5‰
19.0‰
30.0‰
HAEMOLYMPH CHLORIDE (mM.l⁻¹)
300
250
200
325
275
225
400
350
300
475
425
375
0
120
240
360
480
600
TIME (mins)

FIG.3.3

Figure 3.4. Carcinus maenas: Time-concentration curves of haemolymph chloride during a 4hr period of hypoxia (P_wO_2=40 torr) and subsequent re-oxygenation at various salinities (T^oC=15^oC). Values given are means ± S.E. for 5-10 animals in each case.

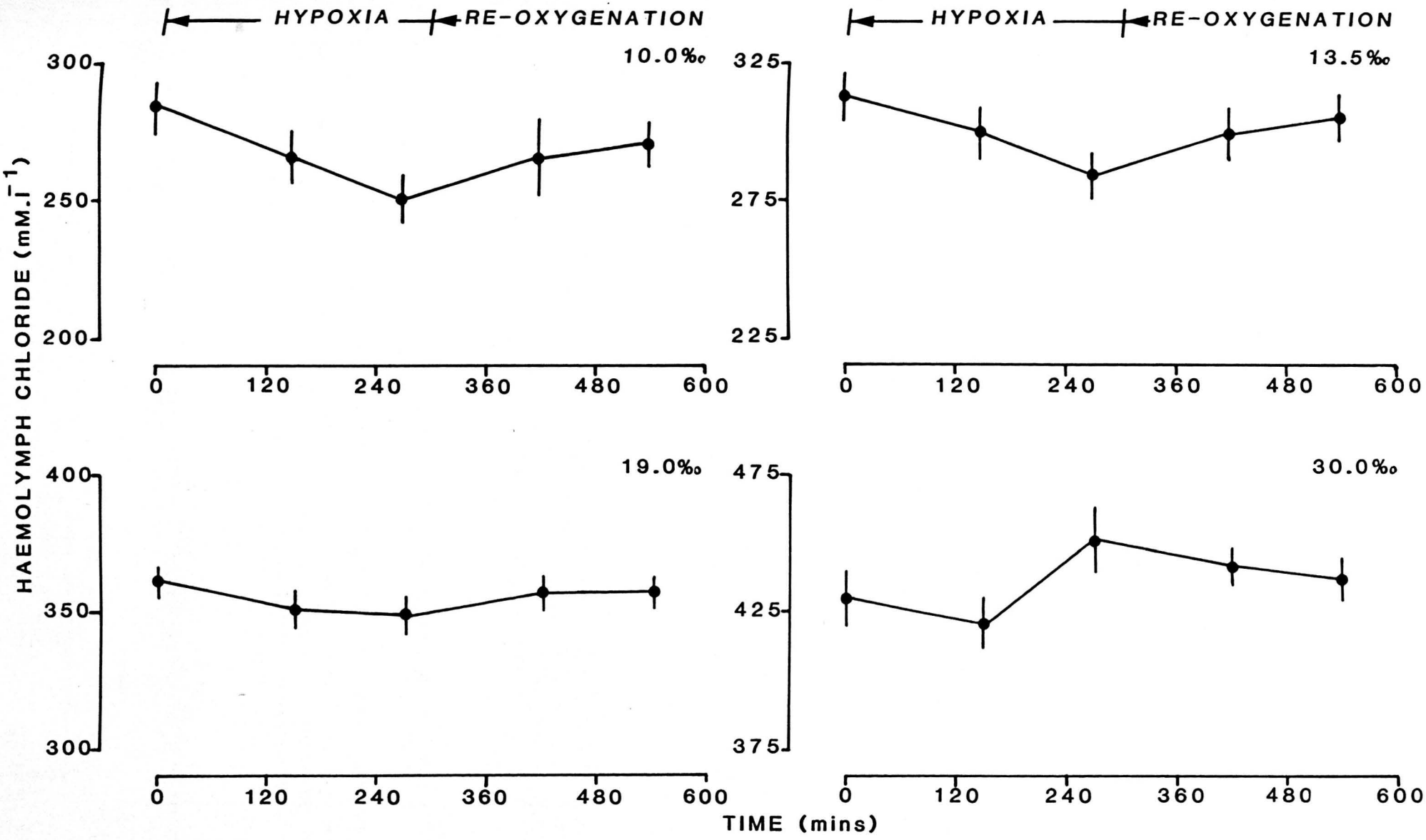

HYPOXIA
RE-OXYGENATION
10.0‰
13.5‰
19.0‰
30.0‰
HAEMOLYMPH CHLORIDE (mM.l^{-1})
300
250
200
325
275
225
400
350
300
475
425
375
0
120
240
360
480
600
TIME (mins)

FIG.3.4

Figure 3.5. Carcinus maenas: Time-concentration curves of haemolymph chloride during normoxia (P_wO_2=130 torr) at various salinities (T^oC=15°C). Values given are means ± S.E. for 5-10 animals in each case.

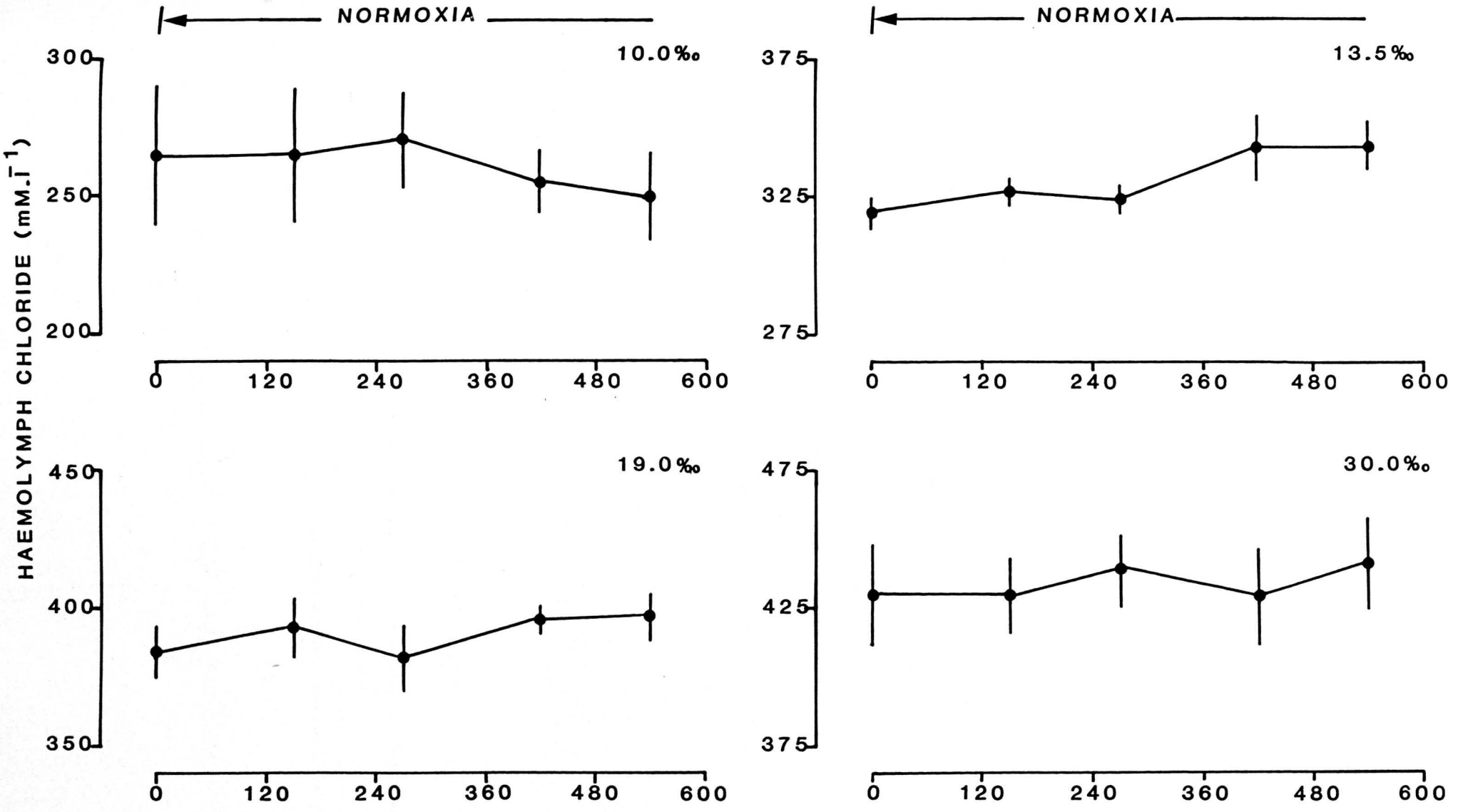

NORMOXIA
10.0‰
13.5‰
19.0‰
30.0‰
HAEMOLYMPH CHLORIDE (mM.l⁻¹)
300
250
200
375
325
275
450
400
350
475
425
375
0
120
240
360
480
600
TIME (mins)

FIG.3.5

Figure 3.6. *Carcinus maenas*: Differences ($mM.l^{-1}$) between haemolymph and medium chloride during normoxia (P_wO_2 = 130 torr) and after 4hr hypoxia (P_wO_2 = 20 or 40 torr). Values given are means for 5-10 animals in each case.

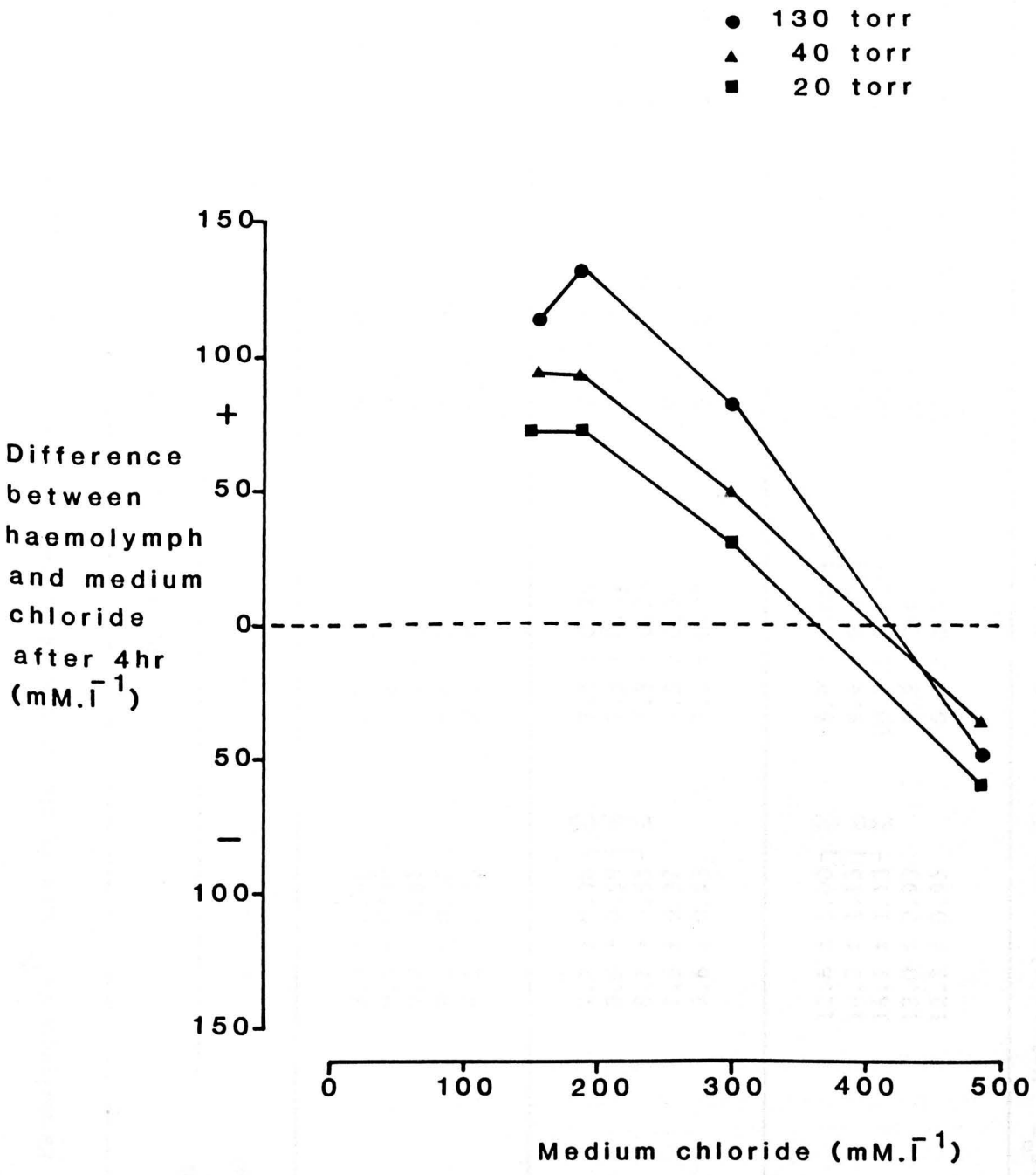

130 torr
40 torr
20 torr
150
100
+
50
0
50
−
100
150
Difference between haemolymph and medium chloride after 4hr (mM.l⁻¹)
0
100
200
300
400
500
Medium chloride (mM.l⁻¹)

Fig.3.6

Table 3.1 C.maenas: Haemolymph Ca^{2+} data at the various seawater P_wO_2/salinity combinations.

P_wO_2 (torr)	Sampling Time (Minutes)	HAEMOLYMPH Ca^{2+} ($mM.L^{-1}$)			
		10.0‰	13.5‰	19.0‰	30.0‰
130	0	9.3 ± 0.61	8.2 ± 0.51	9.4 ± 0.68	20.6 ± 0.31
	150	9.1 ± 0.58	7.8 ± 0.35	8.3 ± 0.93	20.9 ± 0.55
	270	9.2 ± 0.81	8.1 ± 0.32	9.0 ± 1.12	20.1 ± 0.40
	420	9.2 ± 0.57	8.6 ± 0.94	8.5 ± 0.94	21.8 ± 0.52
	540	9.1 ± 0.53	8.7 ± 0.90	9.4 ± 0.90	20.7 ± 0.80
40	0	7.2 ± 0.36 ┐ P<0.05	7.5 ± 0.28	9.6 ± 0.25	12.1 ± 0.74
	150	8.0 ± 0.59 ┘	7.5 ± 0.31	9.6 ± 0.31	12.1 ± 0.87
	270	8.1 ± 0.55	7.8 ± 0.23	9.7 ± 0.30	12.3 ± 0.82
	420	7.5 ± 0.37	7.5 ± 0.30	9.7 ± 0.27	12.0 ± 0.77
	540	7.6 ± 0.43	7.2 ± 0.37	9.7 ± 0.25	11.7 ± 0.77
20	0	11.6 ± 1.00 ┐ P<0.05	8.9 ± 0.46 ┐ P<0.05	17.5 ± 0.46 ┐ P<0.05	16.6 ± 1.77
	150	14.2 ± 1.13 ┘	9.4 ± 0.55 ┘	18.4 ± 0.96 ┘	15.9 ± 1.69
	270	14.7 ± 1.13	10.7 ± 0.73	19.8 ± 1.10	17.2 ± 1.78
	420	13.0 ± 0.93	8.8 ± 0.47	18.5 ± 0.49	16.3 ± 1.45
	540	12.2 ± 0.95	8.2 ± 0.26	17.3 ± 0.29	16.4 ± 1.77

Seawater temperature 15°C. Values given are means ± S.E. of 5-10 animals in each case.

Figure 3.7. Carcinus maenas: Changes in relative Ca^{2+} levels at the various salinity/P_wO_2 combinations. Values given are means ± S.E. for 5-10 animals in each case.

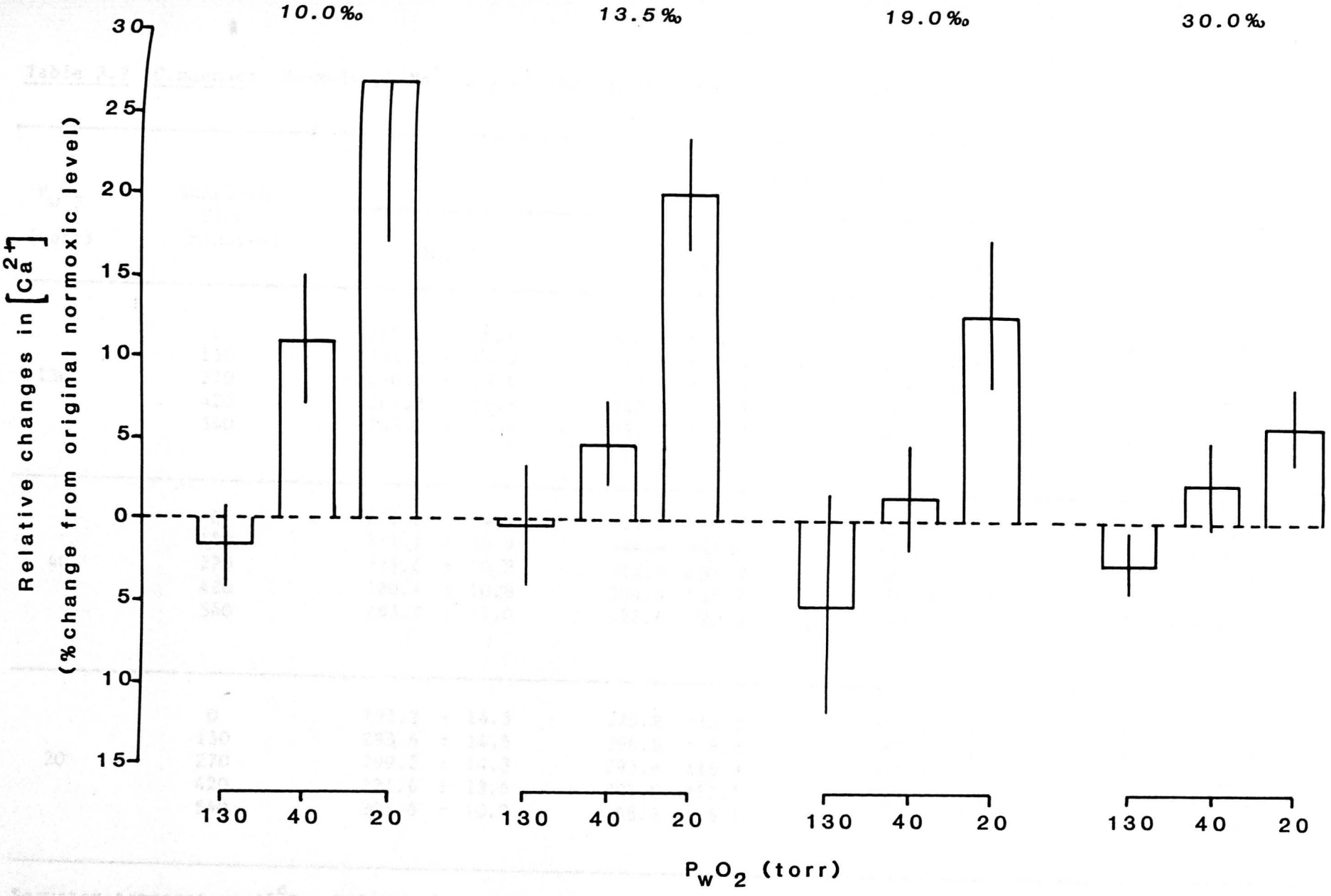

10.0‰
13.5‰
19.0‰
30.0‰
30
25
20
15
10
5
0
5
10
15
Relative changes in [Ca2+]
(%change from original normoxic level)
130
40
20
PwO2 (torr)

Fig.3.7

Table 3.2 C.maenas: Haemolymph Na^+ data at the various seawater P_wO_2/salinity combinations.

P_wO_2 (torr)	Sampling Time (Minutes)	HAEMOLYMPH Na^+ ($mM.L^{-1}$)			
		10.0‰	13.5‰	19.0‰	30.0‰
130	0	243.2 ± 18.1	330.4 ± 4.0	400.0 ± 6.2	437.6 ± 21.2
	150	254.8 ± 16.4	346.8 ± 2.9	401.6 ± 13.6	433.2 ± 17.1
	270	250.8 ± 13.1	335.4 ± 3.5	408.4 ± 4.1	432.4 ± 13.2
	420	261.2 ± 11.3	342.2 ± 7.7	410.0 ± 9.0	443.6 ± 8.0
	540	253.2 ± 14.1	352.6 ± 9.9	409.6 ± 13.0	452.4 ± 11.6
40	0	270.2 ± 14.7	316.6 ± 30.7	354.4 ± 5.8	497.2 ± 15.2
	150	271.2 ± 16.9	344.4 ± 27.1	367.8 ± 3.5	506.2 ± 20.4
	270	275.6 ± 10.8	312.8 ± 22.8	357.6 ± 9.5	514.3 ± 23.8
	420	280.4 ± 10.8	304.4 ± 18.1	366.0 ± 10.1	479.0 ± 8.2
	540	283.2 ± 11.0	327.6 ± 20.2	368.0 ± 10.8	484.8 ± 17.5
20	0	291.2 ± 14.3	278.8 ±13.3	364.4 ± 13.9	439.2 ± 8.6
	150	293.6 ± 14.5	296.8 ± 8.6	361.2 ± 15.9	435.6 ± 8.7
	270	299.2 ± 14.3	293.6 ±16.3	363.2 ± 16.9	435.6 ± 6.5
	420	321.6 ± 13.5	301.0 ±17.1	384.8 ± 20.0	424.4 ± 18.8
	540	305.6 ± 10.9	296.2 ± 6.1	382.0 ± 12.9	424.4 ± 18.8

Seawater temperature 15°C. Values given are means ± S.E. for n=5-10 animals in each case.

Table 3.3 C.maenas: Haemolymph Mg^{2+} data at the various seawater P_wO_2/salinity combinations.

P_wO_2 (torr)	Sampling time (Minutes)	HAEMOLYMPH Mg^{2+} ($mM.L^{-1}$)			
		10.0‰	13.5‰	19.0‰	30.0‰
130	0	6.8 ± 0.47	7.5 ± 0.48	13.5 ± 0.94	10.7 ± 0.54
	150	6.3 ± 0.31	8.0 ± 0.40	13.1 ± 0.56	10.1 ± 0.62
	270	6.1 ± 0.34	7.3 ± 0.27	12.8 ± 0.63	9.9 ± 0.58
	420	6.6 ± 0.43	7.5 ± 0.36	13.5 ± 0.82	10.0 ± 0.64
	540	6.7 ± 0.16	7.6 ± 0.23	12.9 ± 1.17	10.1 ± 0.40
40	0	7.0 ± 0.34	8.7 ± 0.41	11.1 ± 0.77	16.5 ± 0.59
	150	7.0 ± 0.20	8.6 ± 0.33	11.1 ± 0.71	15.7 ± 0.50
	270	6.9 ± 0.28	8.7 ± 0.37	11.5 ± 0.84	16.1 ± 0.50
	420	6.8 ± 0.31	8.5 ± 0.37	11.3 ± 0.76	15.5 ± 0.44
	540	7.3 ± 0.37	8.4 ± 0.26	11.2 ± 0.66	15.5 ± 0.46
20	0	10.0 ± 0.16	6.6 ± 0.35	12.9 ± 0.68	12.7 ± 0.50
	150	10.2 ± 0.26	6.4 ± 0.20	13.5 ± 0.88	12.4 ± 0.82
	270	11.3 ± 0.35	6.9 ± 0.27	13.9 ± 0.48	12.3 ± 0.44
	420	10.9 ± 0.38	6.6 ± 0.33	12.4 ± 0.28	11.6 ± 0.44
	540	10.6 ± 0.04	6.6 ± 0.38	13.5 ± 0.46	11.4 ± 0.16

Seawater temperature 15°C. Values given are means ± S.E. of 5-10 animals in each case.

Figure 3.8. Carcinus maenas: Time-concentration curves of haemolymph lactate during a 4hr period of hypoxia (P_wO_2=20 torr) and subsequent re-oxygenation at various salinities (T^oC=15^oC). Values given are means ± S.E. for 5-10 animals in each case.

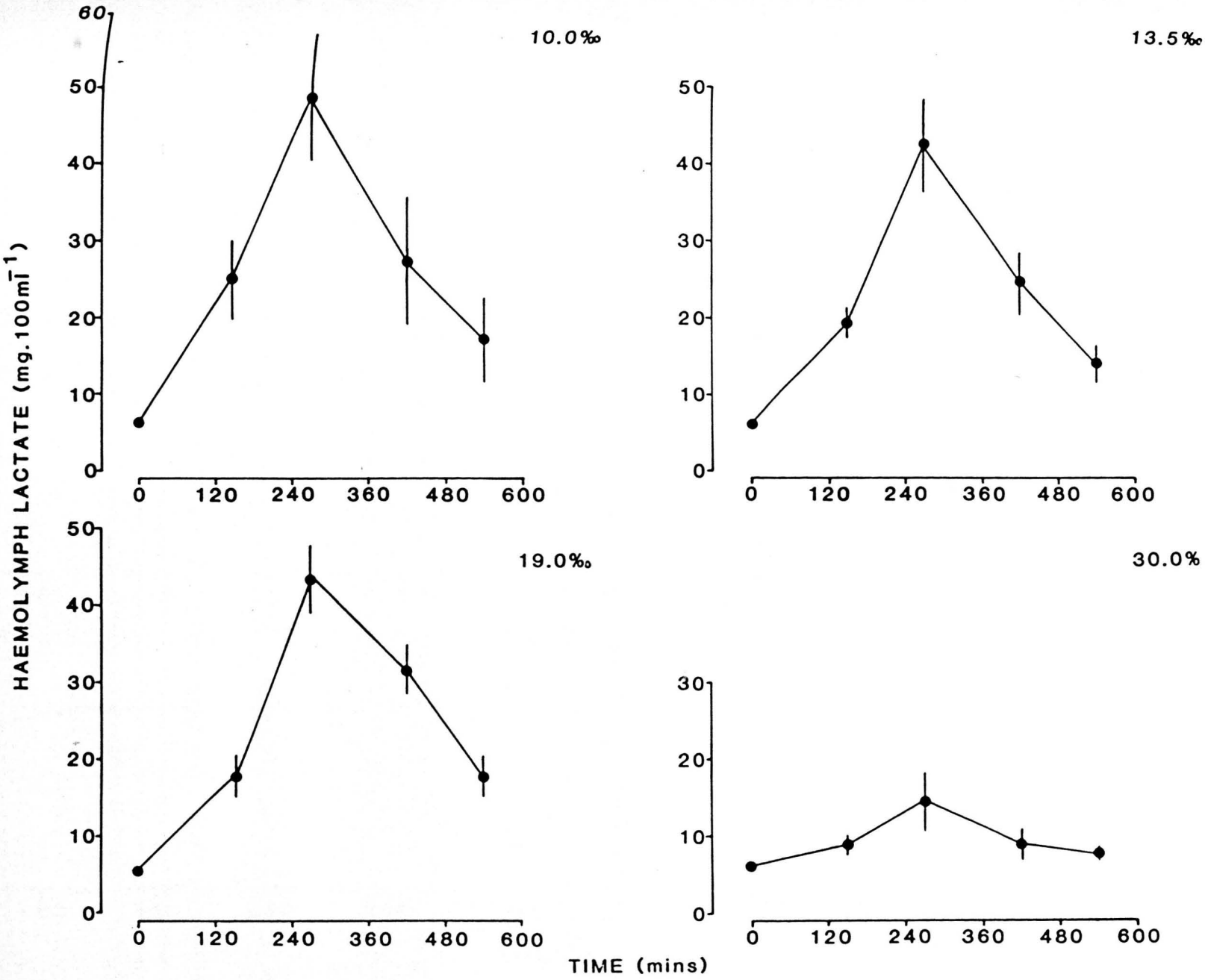

10.0‰
13.5‰
19.0‰
30.0%
HAEMOLYMPH LACTATE (mg.100ml⁻¹)
TIME (mins)
0
10
20
30
40
50
60
0
120
240
360
480
600

Fig.3.8

Figure 3.9. Carcinus maenas: Time-concentration curves of haemolymph lactate during a 4hr period of hypoxia (P_wO_2=40 torr) and subsequent re-oxygenation at various salinities (T^o=15^{o}C). Values given are means ± S.E. for 5-10 animals in each case.

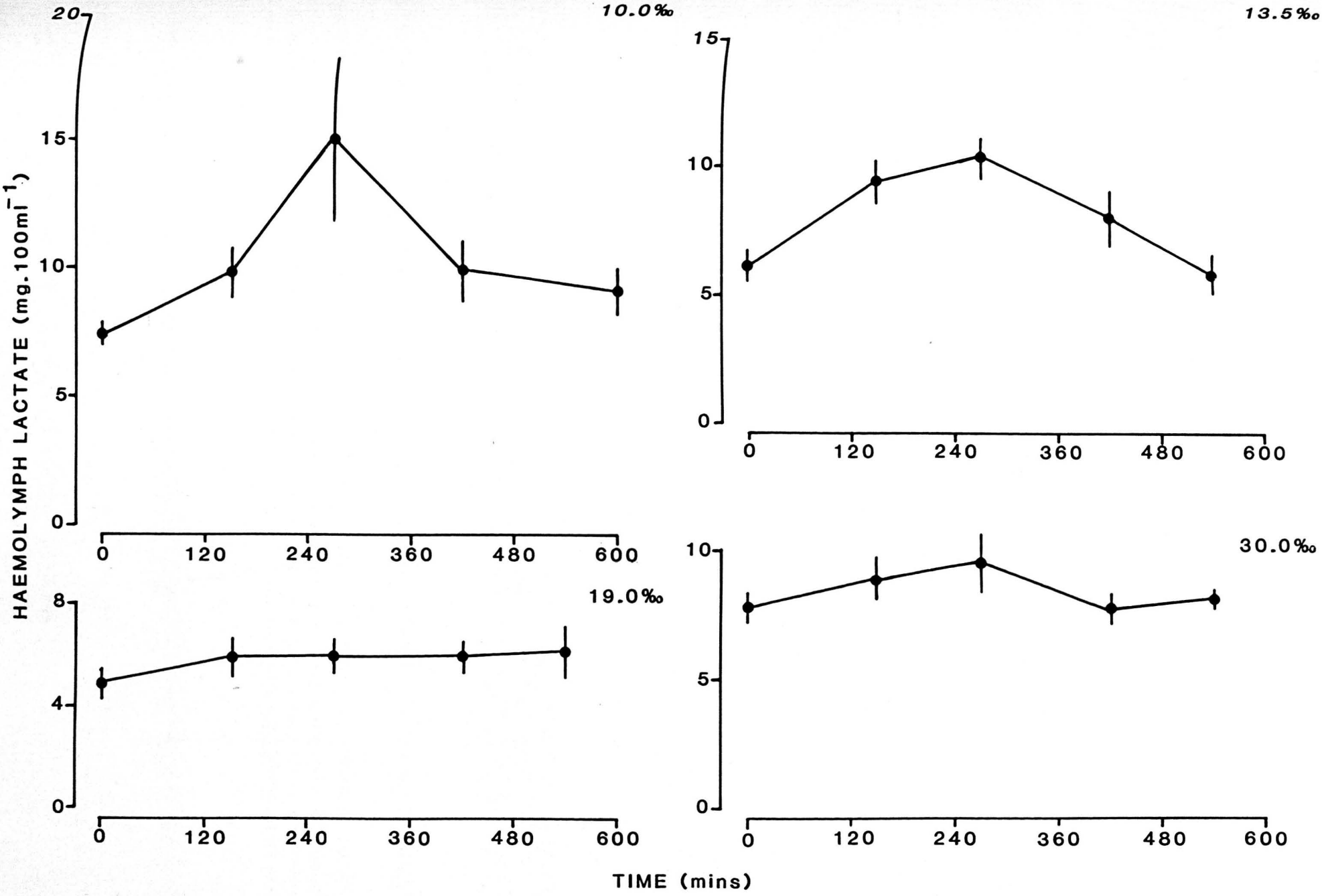

10.0‰
13.5‰
19.0‰
30.0‰
HAEMOLYMPH LACTATE (mg.100ml⁻¹)
TIME (mins)
0
120
240
360
480
600
0
5
10
15
20
0
4
8

Fig.3.9

Figure 3.10. *Carcinus maenas*: Time-concentration curves of haemolymph lactate during normoxia (P_wO_2=130 torr) at various salinities (T^oC=15°C). Values given are means ± S.E. for 5-10 animals in each case.

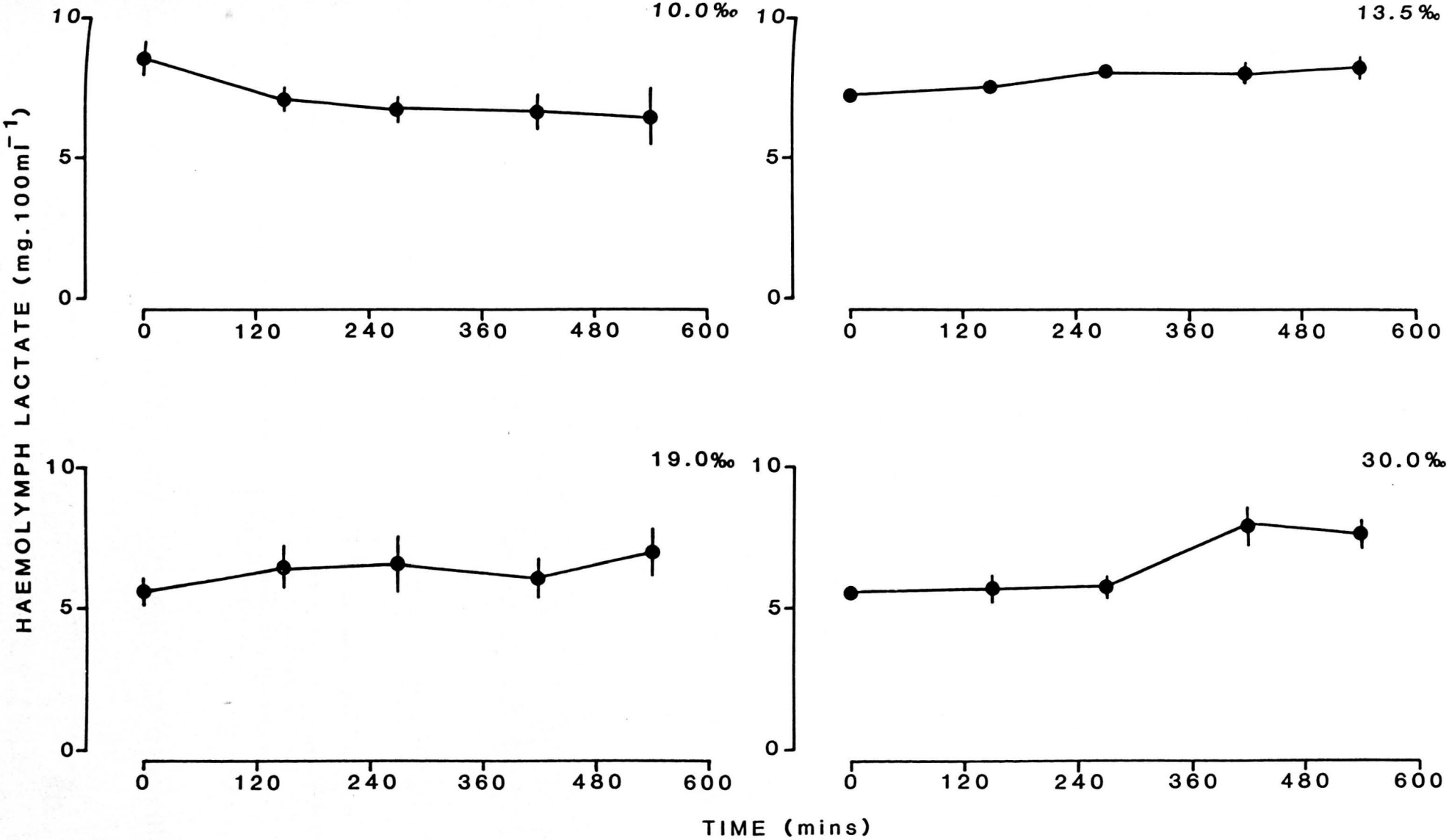

10.0‰
13.5‰
19.0‰
30.0‰
HAEMOLYMPH LACTATE (mg.100ml⁻¹)
TIME (mins)
0
5
10
0
120
240
360
480
600

Fig.3.10

Figure 3.11. Carcinus maenas: Haemolymph lactate levels after 4hr normoxia (P_wO_2 = 130 torr) or 4hr hypoxia (P_wO_2 = 20 or 40 torr) at the various salinities. Values given are means ± S.E. for 5-10 animals in each case.

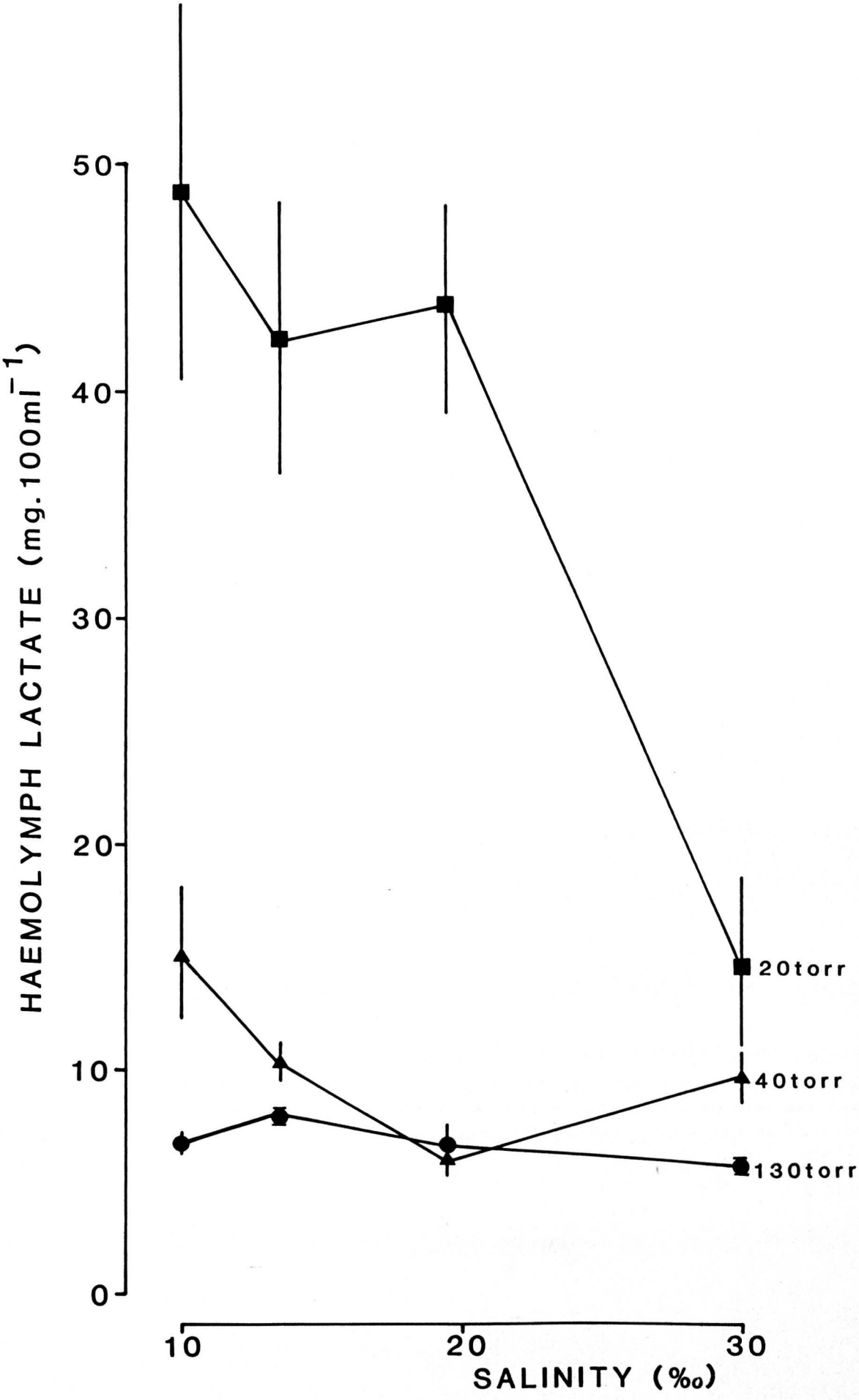

HAEMOLYMPH LACTATE (mg.100ml^{-1})
50
40
30
20
10
0
20torr
40torr
130torr
10
20
30
SALINITY (‰)

Fig.3.11

Figure 3.12. Carcinus maenas: Haemolymph pH levels during normoxia (P_wO_2 = 130 torr) or after a 4hr period of hypoxia (P_wO_2 = 20 torr) with subsequent re-oxygenation in groups at 13.5‰ and 30.0‰ S, (T°=15°C). Values given are means ± S.E. for 6-10 animals in each case.

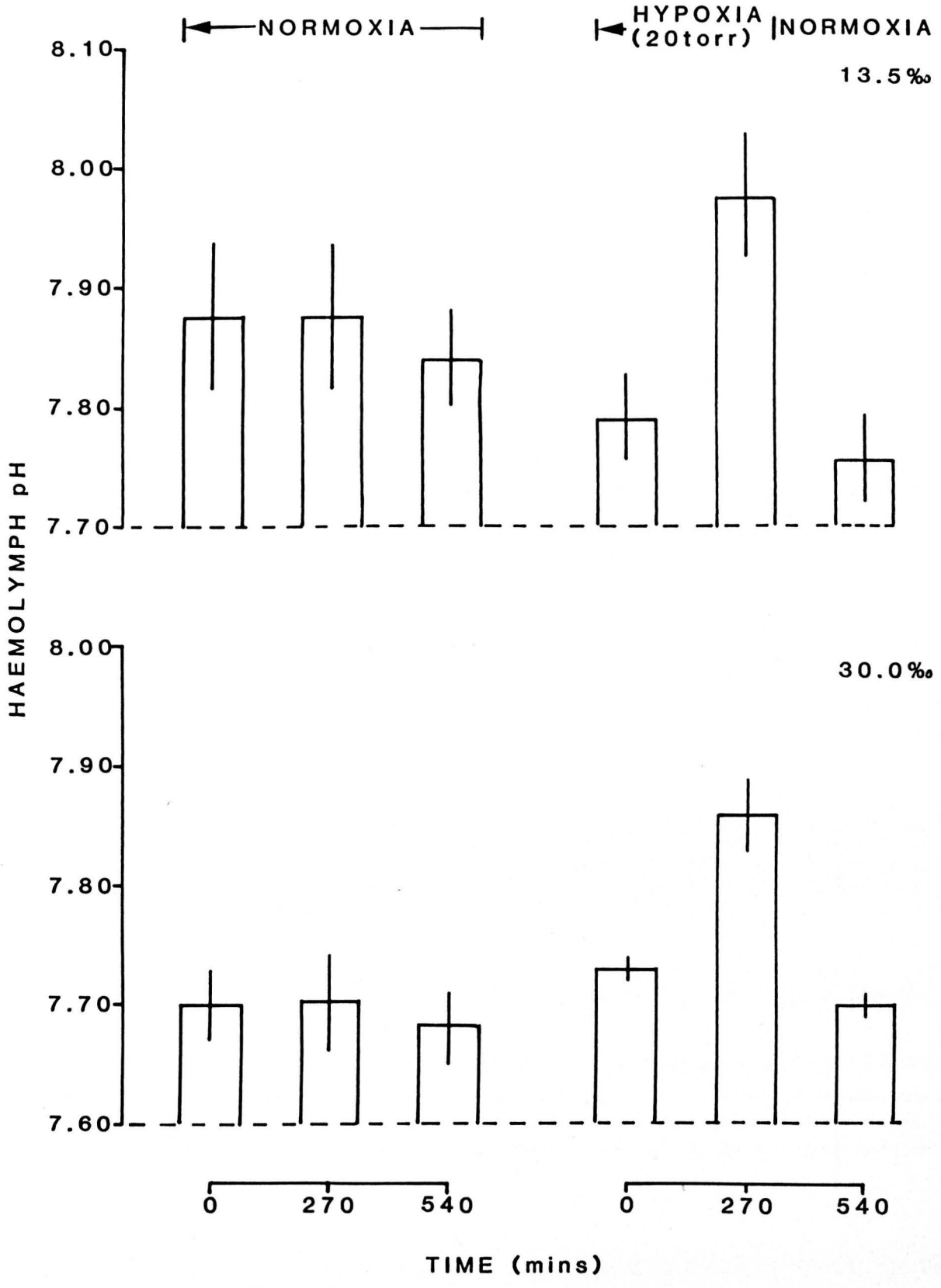

NORMOXIA
HYPOXIA (20torr)
NORMOXIA
13.5‰
30.0‰
8.10
8.00
7.90
7.80
7.70
8.00
7.90
7.80
7.70
7.60
HAEMOLYMPH pH
0
270
540
0
270
540
TIME (mins)

Fig.3.12

Figure 3.13. Carcinus maenas: The cardiac responses to hypoxia (P_wO_2 = 20 or 40 torr) of groups of animals (n=6) acclimated to 13.5‰ and 30.0‰ S, (T^o=15^{o}C).

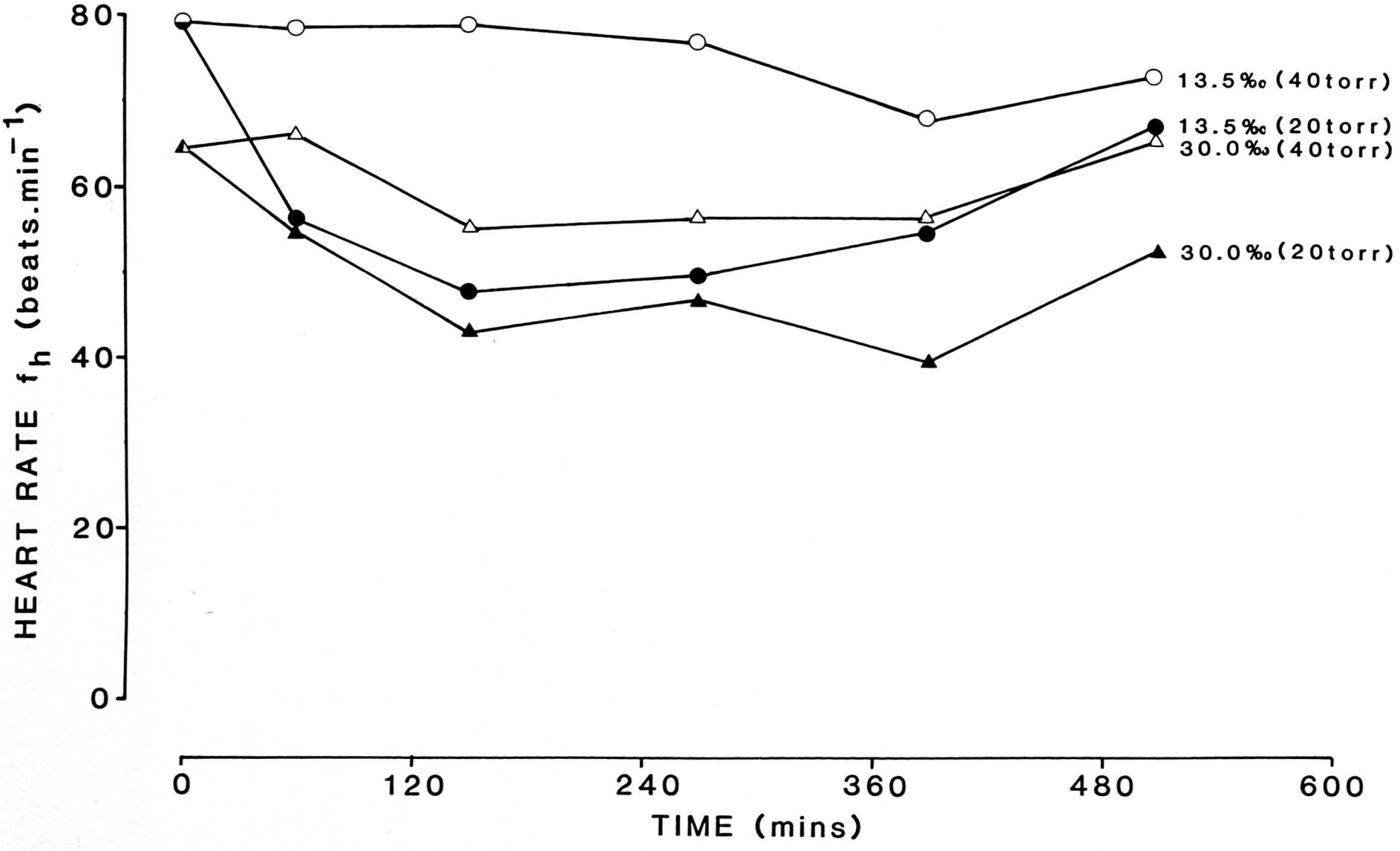

80
60
40
20
0
HEART RATE f_h (beats.min^{-1})
13.5‰ (40torr)
13.5‰ (20torr)
30.0‰ (40torr)
30.0‰ (20torr)
0
120
240
360
480
600
TIME (mins)

Fig.3.13

Figure 3.14. Carcinus maenas: The ventilatory responses to hypoxia (P_wO_2 = 20 or 40 torr) of groups of animals (n=6) acclimated to 13.5‰ and 30.0‰ S (T^oC=15^oC).

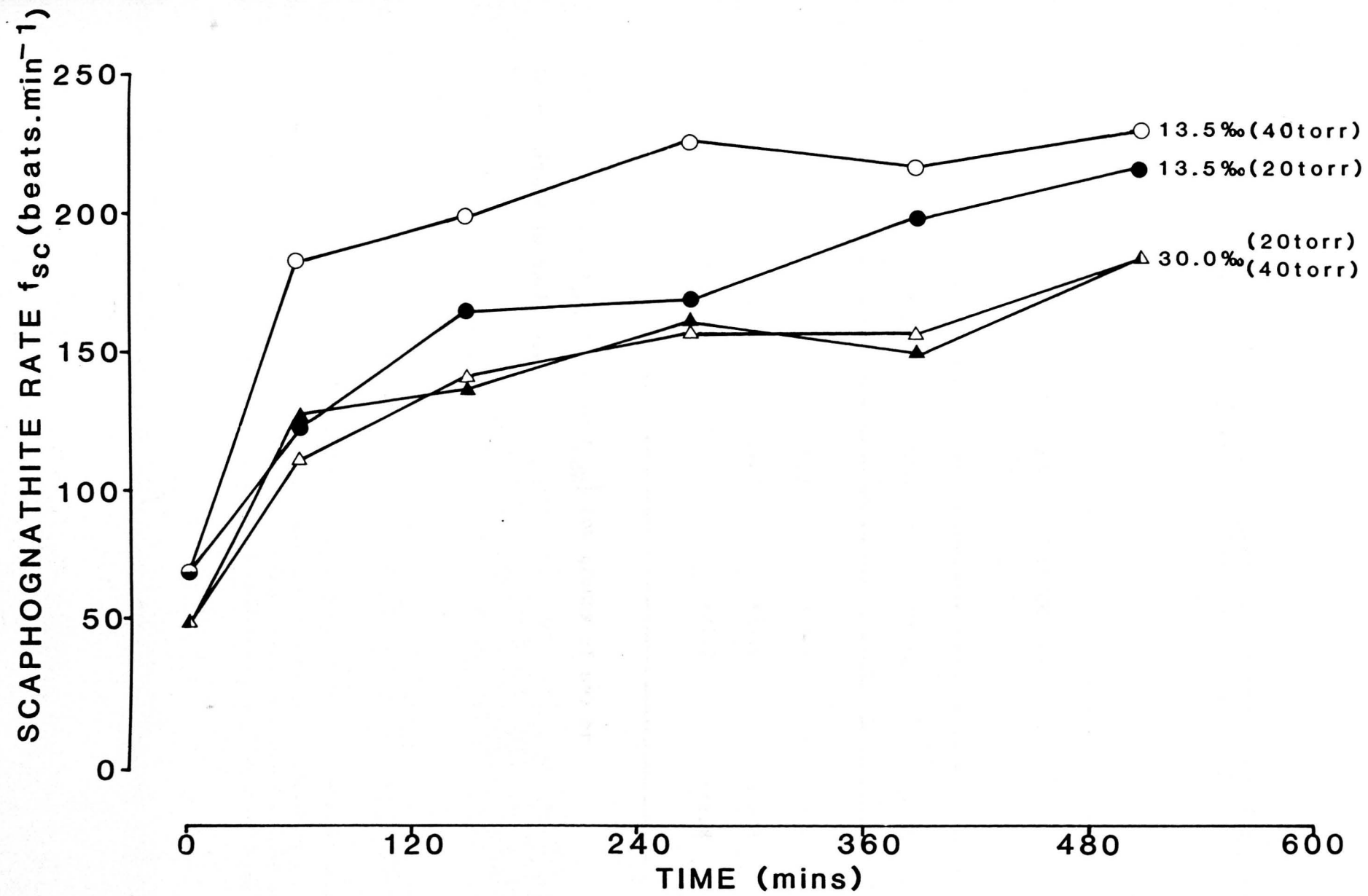

SCAPHOGNATHITE RATE f_{sc} (beats.min^{-1})
250
200
150
100
50
0
0
120
240
360
480
600
TIME (mins)
13.5‰(40torr)
13.5‰(20torr)
30.0‰(20torr)
(40torr)

Fig.3.14

Table 3.4. C.maenas: Strong ion difference data at the experimental salinity/P_wO_2 combinations ($T^oC=15^oC$).

P_wO_2 (torr)	STRONG ION DIFFERENCE (S.I.D.) ($[Na^+] - [Cl^-]$)			
	10.0‰	13.5‰	19.0‰	30.0‰
130	+ 2.6	+ 6.9	+ 9.7	-13.8
40	+39.9	+24.9	+21.4	- 3.9
20	+42.5	+41.9	+21.2	- 2.8

Values = $[(S.I.D.)_{4HR} - (S.I.D.)_{OHR}]$ for groups of n=5 or more animals in each case.

Table 3.5. C.maenas: Haemolymph osmolality data at the experimental salinity/P_wO_2 combinations (T^o=15°C).

P_wO_2 (torr)	Sampling Time Minutes	Haemolymph Osmolality (mOsmol.KgH_2O^{-1})	
		13.5‰	30.0‰
130	0	653.0 ± 21.8	923.3 ± 11.0
	270	651.0 ± 14.1	933.7 ± 7.8
	540	668.3 ± 15.7	915.0 ± 6.3
20	0	711.7 ± 31.5	901.1 ± 11.0
	270	666.7 ± 23.3	896.6 ± 5.6
	540	707.2 ± 28.3	895.2 ± 6.9

Values given are means ± S.E. for 6-11 animals in each case.

Figure 3.15. Abbreviated diagram showing the pathways of glycolysis and gluconeogenesis. The ordinary double arrows (⇌) signify reactions that are found to proceed in either direction, and dashed arrows (⇢) those reactions where different enzymes catalyze the forward and reverse directions.

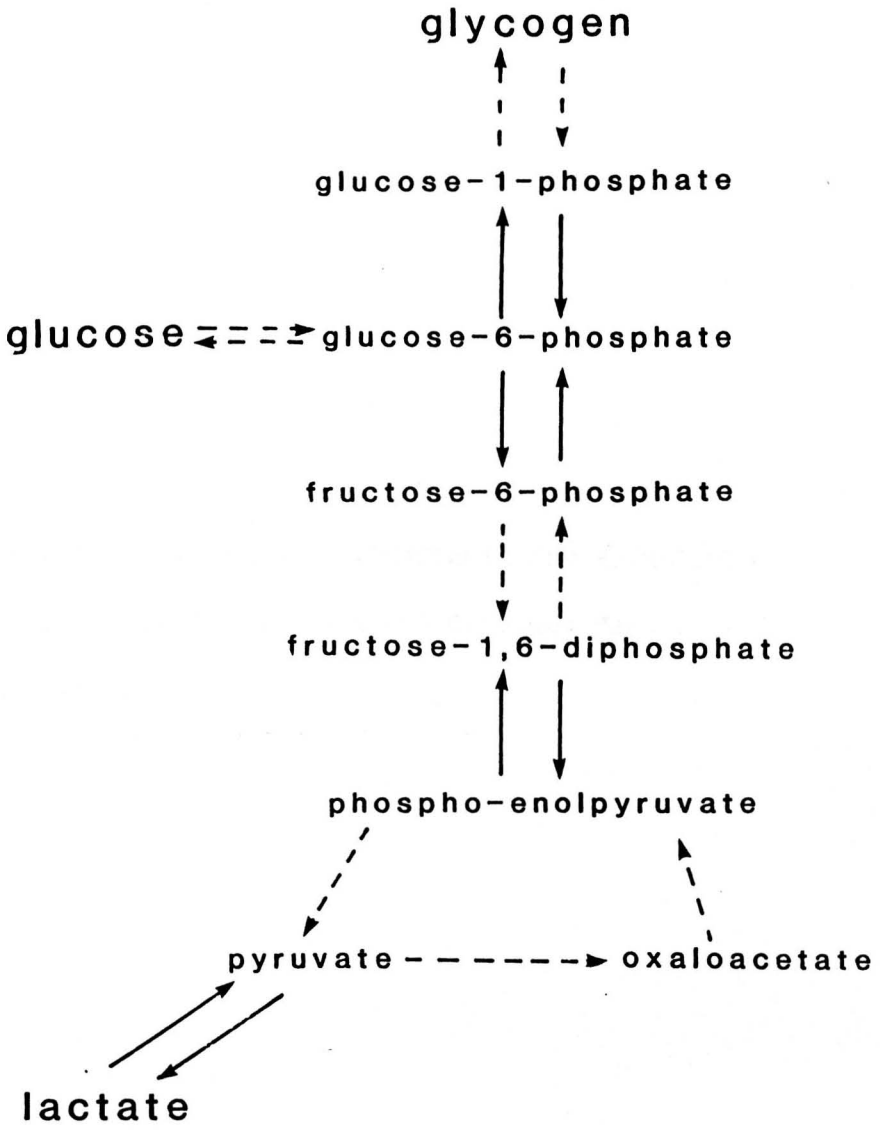

glycogen
glucose-1-phosphate
glucose
glucose-6-phosphate
fructose-6-phosphate
fructose-1,6-diphosphate
phospho-enolpyruvate
pyruvate
oxaloacetate
lactate

Fig.3.15

CHAPTER 4

SOME EFFECTS OF HYPOXIA ON HAEMOCYANIN AND HAEMOLYMPH PROTEIN LEVELS IN FED AND STARVED *Carcinus maenas* (L.) AT 2 SALINITIES.

4.1 Introduction

Haemocyanin has been shown to be the principal blood protein of decapod crustaceans (Tyler and Metz, 1945; Frentz, 1954, 1958; Uglow, 1969a,b). It is widely accepted that, by virtue of its ability to combine reversibly with oxygen, haemocyanin plays a major respiratory role in decapod crustaceans. However, Zuckerkandl (1960) found that haemocyanin in *Maia squinado* reached extremely low levels at early intermoult stages and Bottoms (1977) found a virtual absence of haemocyanin in a Scottish population of *Cancer pagurus*. These findings suggest that the respiratory necessity for haemocyanin is, possibly, not as great as thought by earlier workers. Furthermore, it has been postulated that haemocyanin may have a role as a store of organic reserves in addition to that of respiratory protein (Wieser, 1965; Uglow, 1969a,b; Schoffeniels, 1976; Boone and Schoffeniels, 1979).

The site of haemocyanin synthesis in crustaceans has not been conclusively established and evidence has been advanced for synthesis in the heptopancreas (Zuckerkandl, 1960; Senkbiel and Wriston, 1981a) and the gastric mill (Ghiretti-Magaldi *et al.*, 1977).

In decapod crustaceans wide variations of intraspecific haemolymph total protein concentrations have been reported in *Callinectes sapidus* (Horn and Kerr, 1963), *Carcinus maenas* (Uglow, 1969a), *Crangon crangon* (Djangmah, 1970) and *Homarus gammarus* (Hagerman, 1983). Drilhon-Courtois (1934) used pooled samples and found that female specimens of *C.maenas* had higher protein concentrations than males, but Uglow (1969a) found no significant sexual difference in total

protein levels in Carcinus.

Blood protein levels of crustaceans have been shown to vary with external salinity (Gilles, 1977; Pequeux et al., 1979; Boone and Schoffeniels, 1979) and with such physiological conditions as moult stage (Zuckerkandl, 1960; Glynn, 1968; Ghiretti-Magaldi et al., 1977; Hagerman, 1983) and nutritive status (Horn and Kerr, 1963; Uglow, 1969b; Djangmah, 1970; Hagerman, 1983). Medium dilution has been shown to be accompanied by haemocyanin synthesis in Carcinus (Boone and Schoffeniels, 1979) and a number of decapod species (Pequeux et al., 1979) but the large intraspecific variability of haemocyanin levels complicates the drawing of conclusions from these findings.

Moult-dependent changes of haemolymph protein concentrations have been shown for Maia squinado (Zuckerkandl, 1960). Glynn (1968) concluded that the low post-moult levels of blood protein in Homarus vulgaris were the combined result of water uptake and catabolic utilization for exoskeleton synthesis. Ghiretti-Magaldi et al., (1977) consider post moult water uptake to be only a small part of protein level changes at this time. Hagerman (1983) found that in juvenile H.gammarus (vulgaris) protein levels were lowest immediately after the moult (Drachstage B_2) and showed a gradual increase through stages C and D.

Decreased serum protein levels associated with reduced food intake have been found in many crustacean species e.g. Callinectes sapidus (Horn and Kerr, 1963), Marinogammarus marinus (Wieser, 1965), Carcinus maenas (Uglow, 1969b), Crangon crangon (Djangmah, 1970) and Homarus gammarus (Hagerman, 1983). In addition to food availability

the quality of the available food has been shown to effect blood haemocyanin concentrations in juvenile H.gammarus (Hagerman, 1983).

Although there is much information on the effects of environmental and physiological variables on crustacean haemolymph protein concentrations, few studies have appraised the effects of hypoxia on haemolymph protein levels. The aim of this study was to investigate the effect of hypoxic stress on blood haemocyanin and total protein concentrations in groups of C.maenas maintained at low salinity (13.5‰) or fully marine (30.0‰) conditions. The extent to which feeding level affected protein levels was also investigated at the salinity/P_wO_2 combinations.

4.2. Materials and Methods

The holding procedures for specimens of C.maenas are described in detail in the General Materials and Methods (Page No.9/10). All animals were maintained for at least 10 days in aerated recirculating aquaria at 13.5 and 30.0‰ S and 15°C. Unless otherwise stated, only intermoult males were used in the experiments. The animals were fed every third day on 1.5-3.0g of C.crangon or fish flesh. For experiments at each salinity, animals in the 'fed' group were given food on the day of the experiment whereas the 'starved' group were not fed for 3 days immediately before or during the experiment.

4.2.1. Control of External Oxygen Tension

The procedure used to obtain the required oxygen tension in the experimental media has been described in detail in the Chapter 3 Materials and Methods (Page No. 21). The P_wO_2 was lowered from the initial normoxic level (ca 130 torr) to the experimental hypoxic level of 20 torr over a period of 45 min by flushing the medium with high-purity nitrogen gas (Air Products). The experimental P_wO_2 was maintained for the duration of the experiment, small fluctuations being adjusted by flushing appropriately with compressed air or nitrogen gas. In the experimental tank, the water was continuously circulated to ensure a uniformly distributed P_wO_2 throughout the experimental tank. The P_wO_2 values were monitored constantly throughout each experiment using an EIL Portable Oxygen Meter 1520.

4.2.2. Haemolymph Sampling

At given intervals (time 0 and 24hr) pre-branchial haemolymph samples (125-150µl) were withdrawn through the arthrodial membrane at the base of a walking leg using a fine capillary tube. Each sample was centrifuged at 3000g for 5 minutes in a Quickfit Microcentrifuge. The same animals were used throughout any particular experiment in order to avoid the complications of the considerable intraspecific variability of protein concentration which has been shown among normoxic individuals of this species (Uglow, 1969a). The use of restricted serial bleeding has been shown to result in slight but non-significant reductions in protein concentration (Uglow, 1969b).

4.2.3. Haemolymph Analysis

The principle and procedure used in the assay for haemolymph total protein are described in detail in the General Materials and Methods (Page No.**14**). A 25µl aliquot of haemolymph was used for total protein determinations using a Sigma total protein kit (No. 540) which is based on the Biuret method. Protein concentrations were determined spectrophotometrically at 540nm using a Cecil CE303 Grating spectrophotometer (EEL Instruments Ltd).

For haemocyanin analysis, a 100µl blood sample was diluted 1:9 with distilled water. The haemocyanin concentration was measured in a 1cm quartz microcuvette at 335nm on a Unicam SP800A U.V. spectrophotometer. The absorption spectra of haemocyanin is such that there are peaks at 280-290, 335 and 580nm. The peak at

280-290nm is characteristic of all proteins whereas that at 335nm is indicative of oxyhaemocyanin. As haemocyanin is rapidly oxygenated *in vitro* the absorption peak can be used as an effective measure of pigment concentration using an extinction coefficient E^{mM}_{1cm} of 17.26. The E^{mM} value was calculated from an $E^{1\%}_{1cm}$ value of 2.83 given by Nickerson and van Holde (1971) and Antonini and Brunori (1974) on the basis of a minimal functional haemocyanin unit of 74000. Haemocyanin concentrations were expressed as mg Hcy.ml^{-1} blood.

4.2.4. Statistical Treatment of Data

The measured values are given as means ± S.E. of means and the number of observations are stated. The significance of any apparent difference between the mean values of variables was tested using Students' t-test and the word 'significant' means that the difference is significant at the 95% level of confidence ($P<0.05$). Any additional statistical analyses carried out are described in the relevant section of this study.

4.3. Results

Table 4.1 shows the haemolymph total protein and haemocyanin concentration data for 13.5‰ and 30.0‰ S acclimated groups of C.maenas in normoxic conditions. The mean total protein value of the full salinity group was lower than that for the low salinity animals, although this difference was not significant at the 5% level. However, the mean haemocyanin concentration of the 13.5‰ S group was lower than the value for the 30.0‰ S group and this difference was significant ($P<0.05$). The intraspecific variability of haemocyanin and total protein values complicates comparisons of the two groups when absolute values are used. Consequently, the absolute haemocyanin values given in Table 4.1 were transformed to relative haemocyanin values (i.e. % of blood total protein values) and expressed against total protein concentrations for both salinity groups in Figure 4.1. From these data it is evident that the relative haemocyanin level shows a negative, linear relationship with the total protein value. In the 13.5 and 30.0‰ S groups there were total blood protein concentrations below which the animals possessed only haemocyanin as a blood protein, these being 15.2 $mg.ml^{-1}$ for the 13.5‰ S group and 32.9 $mg.ml^{-1}$ for the 30.0‰ S group. The regression lines of these data have similar slopes but differ in position. Covariance analysis of these regression data show that the differences between the two slopes was not significant ($P>0.05$) but differences of position were significant ($P<0.05$). Thus the ratio of haemocyanin to total protein varied in the same manner in the two groups of animals but the low salinity group had relatively lower haemocyanin values than the full salinity group at any given blood total protein value.

Table 4.2 shows the data obtained on hypoxia-induced (P_wO_2 = 20 torr) changes to the blood haemocyanin and total protein levels of fed and starved groups of Carcinus acclimated either to 13.5‰ or 30.0‰ S media. The data are given as relative values, with the absolute values obtained initially (i.e. at time 0hr) being taken as 100%. From these data hypoxia-induced increases of blood haemocyanin and total protein were apparent in 'fed' groups at both salinities. Under hypoxic stress the starved groups at both salinities showed increases in blood haemocyanin and total protein levels, although these were smaller than those for comparable 'fed' groups. In both 'fed' and 'starved' groups the hypoxia-induced increases in haemocyanin and total protein levels were more marked in animals acclimated to 30.0‰ S than those animals in the 13.5‰ S group. However, multi-way analysis of variance of these data revealed no significant changes due to any individual factor (e.g. salinity, nutritional status or oxygen level) or interaction between factors, as is shown by the 'anova' data in Tables 4.3 and 4.4 for haemocyanin and total protein respectively.

4.4. Discussion

In groups of C.maenas acclimated to either low salinity (13.5‰) or to fully marine (30.0‰) conditions, considerable intraspecific variability of blood total protein levels were found. This finding is consistent with those of Uglow (1969a,b) for this species, Horn and Kerr (1963) for Callinectes sapidus, Djangmah (1970) for Crangon crangon and Hagerman (1983) for Homarus gammarus.

In this study, haemocyanin was found to be the major protein component in the haemolymph of both groups of animals, a finding consistent with those of Tyler and Metz (1945), Frentz (1954, 1958) and Uglow (1969a,b). The other protein components of the blood were not investigated in this study although Uglow (1969a) analyzed Carcinus maenas blood electrophorectically and found in addition to the 'fast' and 'slow' haemocyanins, an apohaemocyanin and other minor constituent proteins, such as glycoproteins and fibrinogen. Horn and Kerr (1969) showed that the blood of Callinectes sapidus contained electrophorectically 'fast' and 'slow' haemocyanin, amylase and 'complex' and 'clotting' proteins.

The mean blood total protein concentration in Carcinus was greater in animals acclimated to 13.5‰S than those in 30.0‰S media, although the difference was not significant due to the considerable intraspecific variation measured. Uglow (1969b) found similar mean total protein values in Carcinus at full salinity. Gilles (1977) also found an elevation of blood serum protein levels in Carcinus acclimated to 50% seawater (cf. values in full seawater) and specimens of Eriocheir sinensis and Astacus fluviatilis acclimated to low osmolality media (cf. values in high osmolality media).

The mean haemocyanin level of the 13.5‰ S group was significantly higher ($P<0.05$) than that of the 30.0‰ S group over the whole range of total blood protein concentrations recorded, with the 13.5‰ S group showing lower relative haemocyanin levels (i.e. expressed as % of blood total protein) than the 30.0‰ S group. Thus both groups showed a negative linear relationship between the relative amount of haemocyanin present in the blood and the total protein level. The regression lines of these relationships showed no significant variation in slope ($P>0.05$) but did exhibit a significant difference ($P<0.05$) in position.

Boone and Schoffeniels (1979) found a 250% increase in the blood haemocyanin levels of Carcinus following the transfer of the animals from 32‰ to 16‰ S media. Pequeux et al. (1979) also found such changes in several species, although individual variability was large enough to preclude any statistical significance being attached to the values. In this study, the lower haemocyanin values measured in the low salinity group animals are taken here to be the probable consequence of an increase in other blood protein concentrations, particularly apohaemocyanin, which would not be measured in the haemocyanin assay since the copper moeity is not present in this protein. The values given here relate to animals acclimated for 2 weeks at the low salinity and it is possible that any initial elevations of haemocyanin which may have occurred have subsequently been reduced as an acclimation response to the low salinity medium.

In both groups, haemocyanin comprised essentially all of the circulating protein at total protein concentrations of <30-35 $mg.ml^{-1}$. This may

reflect the range of values around the maximum required to fulfill a respiratory function, as has been speculated upon by Uglow (1969b). Any haemocyanin present in amounts greater than a minimal essential level for respiratory function may possibly subserve a role as an organic reserve as posulated by Wieser (1965). Uglow (1969a,b) on the basis of seasonal quantitative variability, and on the changes accompanying food deprivation, reasoned that the electrophoretically 'fast' haemocyanins may fulfill the basic respiratory requirement whilst electrophoretically 'slow' haemocyanins and the apohaemocyanin may act as the organic reserve. Schoffeniels (1976) has extended this hypothesis and postulated that the haemocyanin, or apohaemocyanin, may act as a store for free amino acids which are liberated from the cells during hypo-osmotic stress and which can be released from the haemocyanin or apohaemocyanin during hyper-osmotic stress when they are required as intracellular osmolytes. The salinity-induced haemocyanin synthesis in dilute media has been taken as further evidence for this hypothesis.

In this study, small increases in haemolymph haemocyanin and total protein concentrations in response to hypoxic stress (P_wO_2 = 20 torr) were measured in animals at both salinities. Although these changes were not statistically significant they can probably be regarded as indicative of more marked increases with increasing length of hypoxic stress. This finding is in accordance with the hypoxia-induced haemocyanin synthesis measured in Homarus americanus (Senkbiel and Wriston, 1981a) and Nephrops norvegicus under moderate hypoxia (P_wO_2 = 60 torr) and 10-13 days (Hagerman and Uglow, in press). However, Wilkes and McMahon (1982) found no change of blood Cu^{2+} or total protein levels in Orconectes rusticus subjected to long term

(3.5 weeks) moderate hypoxia (P_wO_2 = 50-55 torr). Such an increase in circulating haemocyanin as a response to hypoxia could have some adaptive value through an increase in the oxygen carrying capacity of the blood. However, Mangum and Johansen (1975) have suggested that any such increased haemocyanin concentration might produce an elevation of colloid osmotic pressure sufficient to cause a fluid imbalance between intra- and extra-cellular compartments. An increased colloid osmotic pressure may also affect water balance across the gills, particularly in freshwater species, and so increase haemolymph viscosity and possibly add to the energy required for perfusion of the gills and tissues. Crustaceans generally have a high cardiac output (cf. aquatic vertebrates) which is required to balance the low oxygen carrying capacity of the haemolymph (Mangum, 1983 for review).

The hypoxia-induced increases in haemolymph haemocyanin were apparently greater in the 30.0‰ S groups than in the 13.5‰ S acclimated groups. From data obtained on blood lactate accumulation in response to extreme hypoxic stress (P_wO_2 = 20 torr) it was suggested in Chapter 3 that the allocation of the total available energy in animals at 13.5‰ S, (P_wO_2 = 20 torr) may be lower than in animals at 30.0‰ S (P_wO_2 = 20 torr). Consequently when under hypoxic stress in 13.5‰ S medium animals may not have sufficient energy available for haemocyanin synthesis as compared to animals in 30.0‰ S medium where the energy may be available.

In full salinity media the increases in haemolymph haemocyanin under hypoxic stress were more marked than those for total protein in both 'fed' and 'starved' groups. However, in dilute media haemocyanin

increases were less than those for total protein. Presumably, this is because non-haemocyanin proteins also are synthesized in response to hypoxia in low salinity media.

The hypoxia-induced increases in blood haemocyanin and total protein measured at either salinity were more marked in fed than starved animals; a finding consistent with that of Hagerman and Uglow (in press) for Nephrops norvegicus.

Under all conditions the mean haemolymph haemocyanin and total protein levels were lower in starved animals than fed ones. The conclusion that a reduction in food intake is accompanied by decreased serum protein levels is consistent with the findings of Uglow (1969b) for this species, Horn and Kerr (1963) for Callinectes sapidus, Djangmah (1970) for Crangon crangon and Hagerman (1983) for Homarus gammarus. Furthermore Hagerman (1983) has shown that in addition to food availability the quality of the available food has an effect on the blood haemocyanin concentration.

In this study haemocyanin levels decreased relatively less than did blood total protein values in respone to starvation at all salinity/ P_wO_2 combinations. Uglow (1969b) also found haemocyanins decreased to a lesser extent than non-haemocyanin proteins during starvation in C.maenas. The maintenance of the haemocyanin concentrations at the expense of non-haemocyanin proteins might be expected to have adaptive value, particularly under conditions of hypoxic stress.

Studies have shown that in response to prolonged stress (e.g. starvation, extensive serial bleeding) there is a measure of protein catabolism. Horn and Kerr (1963) concluded that an increased

haemocyanin : total protein ratio in serially-bled Callinectes sapidus was due to the maintenance of haemocyanin levels by the conversion of apohaemocyanin to haemocyanin by the addition of copper from a stored source such as the hepatopancreas. However, Uglow (1969b) showed that apo-haemocyanin levels in Carcinus decreased to a similar extent in both fed and starved groups, whereas starved animals may have been expected to show greater apohaemocyanin losses than fed ones on the basis of the conclusion of Horn and Kerr (1963).

The biosynthetic pathway for haemocyanin is not established. In support of the evidence of Zuckerkandl (1960) which implicated the hepatopancreas as the site of haemocyanin synthesis, Senkbiel and Wriston (1981a) have demonstrated in Homarus americanus the incorporation of labelled aspartate into an immunologically recognized haemocyanin in the hepatopancreas, but not in several other tissues. However, Ghiretti-Magaldi et al. (1977), in work with C.maenas, postulated the presence of a 'lymphocytogenic' organ associated with the 'gizzard' (gastric mill) in which cyanoblast cells contained immunologically and morphologically recognisable haemocyanin molecules. The turnover-time for the haemocyanin pool has been stated to be relatively long with a half time of 26 days (Senkbiel and Wriston, 1981b). However, Hagerman and Uglow (in press) have concluded that the biosynthesis and catabolism of haemocyanin in Nephrops norvegicus may be more rapid than a half-time value of 26 days.

In conclusion it is contended that Carcinus responds to a hypoxic stress, when in 13.5‰ S or 30.0‰ S media, with an increased haemocyanin synthesis and an elevated mean blood total protein level

in fed animals and with maintained levels of haemocyanin and total protein in starved groups. Although small, such changes which were to an extent salinity-dependent, may have adaptive value by increasing or maintaining the blood oxygen carrying capacity during periods of hypoxic stress.

Figure 4.1. *Carcinus maenas*: The relationship between haemolymph total protein and the relative blood haemocyanin (i.e. as % of total protein) in groups of animals (n=11) acclimated to 13.5‰ and 30.0‰ S (T^{o}=15^{o}C). The calculated regression lines and regression formulae are included.

13.5‰ ○ Y = −1.00X+115.1
30.0% ● Y = −1.14X+137.6
Relative Haemocyanin (% of total protein)
100
90
80
70
60
50
40
10
20
30
40
50
60
70
80
Haemolymph Total Protein (mg.ml⁻¹)

Fig.4.1

Table 4.1: Summary of blood protein and haemocyanin data of *Carcinus maenas* at 2 experimental salinities.

	SALINITY (‰)	
	13.5	30.0
Blood total protein ($mg.ml^{-1}$)	55.9± 11.5	48.1± 7.9
Range of total protein values	41.9 - 71.0	31.5 - 59.4
Blood haemocyanin ($mg.ml^{-1}$)	32.0± 0.8	39.4± 2.8
Range of haemocyanin values	30.2 - 33.0	31.5 - 42.9
Relative blood haemocyanin (% of total protein)	59.4 ± 11.6	82.7± 9.5

Values given are mean± S.E. for 11 animals

Table 4.2 C.maenas: summary of changes in the relative haemocyanin and total protein levels in fed and starved groups at given salinity/P_wO_2 combinations ($T^oC=15^oC$)

SALINITY (‰)	P_wO_2 (torr)	SAMPLING TIME (hr)	Relative Haemocyanin			Relative Total Protein		
			FED	STARVED	% diff. due to starvation	FED	STARVED	% diff. due to starvation
13.5	NORMOXIA (130 torr)	0	100.0	100.0	-	100.0	100.0	-
		24	105.0 ± 4.7	99.1 ± 1.6	(-6.0)	104.2 ± 1.6	97.4 ± 2.2	(-6.8)
	HYPOXIA (20 torr)	0	100.0	100.0	-	100.0	100.0	-
		24	111.0 ± 5.3	101.1 ± 0.6	(-9.9)	114.4 ± 4.6	103.5 ± 5.0	(-10.9)
	% difference due to hypoxia after 24hr		(+5.9)	(+2.0)	-	(+10.2)	(+6.1)	-
30.0	NORMOXIA (130 torr)	0	100.0	100.0	-	100.0	100.0	-
		24	100.7 ± 4.5	103.0 ± 3.3	(+2.3)	103.4 ± 3.7	99.2 ± 1.6	(-4.2)
	HYPOXIA (20 torr)	0	100.0	100.0	-	100.0	100.0	-
		24	113.9 ± 1.9	112.8 ± 2.6	(1.1)	110.4 ± 2.4	103.5 ± 4.4	(-6.9)
	% difference due to hypoxia after 24hr		(+13.2)	(+9.8)	-	(+7.0)	(+4.3)	-

Values given are mean ± S.E. for 6 animals in each case.

Table 4.3: *Carcinus maenas:* *Summary of the analysis of variance of the quantitative data pertaining to haemocyanin levels in fed and starved animals maintained at 2 salinities and 2 oxygen levels.*

Source of variation	df	SS	MS	F
Salinity	1	1255	1255	26.2 ns
Feeding	1	1362.4	1362.4	28.4 ns
Oxygen level	1	3553.4	3553.4	74.0 ns
INTERACTIONS				
Salinity x Feeding	1	920.2	920.2	19.2 ns
Salinity x Oxygen level	1	691.9	691.9	14.4 ns
Feeding x Oxygen level	1	91.2	91.2	1.9 ns
Salinity x Feeding x Oxygen level	1	48	48	-
TOTAL	7			

Table 4.4 *Carcinus maenas*: *Summary of the analysis of variance of the quantitative data pertaining to total protein levels in fed and starved animals maintained at 2 salinities and 2 oxygen levels.*

Source of variation	df	SS	MS	F
Salinity	1	356.5	356.5	3.6 ns
Feeding	1	2317.9	2317.9	23.3 ns
Oxygen level	1	5090.4	5090.4	51.2 ns
INTERACTIONS				
Salinity x Feeding	1	729.6	729.6	7.3 ns
Salinity x Oxygen level	1	5.1	5.1	< 1.0 ns
Feeding x Oxygen level	1	737.3	737.3	7.4 ns
Salinity x Feeding x Oxygen level	1	99.4	99.4	-
TOTAL	7			

CHAPTER 5

SOME EFFECTS OF AERIAL EXPOSURE ON THREE SPECIES OF CRABS.

5.1. Introduction

Any aquatic organism which has to withstand periods of aerial exposure is subject at such times to the stresses of desiccation and thermal fluctuations. Water loss from the organism is a passive process the rate of which is influenced by relative humidity, exposure temperature and body size (Ahsanullah and Newell, 1977). The animal has also to be able to maintain ventilation at a level sufficient to survive the period of aerial exposure.

Organisms which are principally high-shore or intertidal in their distribution have been shown to have greater heat tolerances (Newell, 1970 for review) and a higher resistance to desiccation (Newell, 1976 for review) than low shore or sub-tidal animals. In many cases upper shore species have structural and physiological adaptations which both minimize the effect of, and increase the resistance to, dehydration (for review, Newell, 1976).

However, water loss may not always be restricted and many intertidal species respond to heat stress by heat loss through transpiration. In Uca sp. water loss by evaporation results in a marked depression of the body temperature both under natural conditions and in the laboratory (Edney, 1960; Wilkens and Fingerman, 1965). Ahsanullah and Newell (1977) showed that the depression of body temperature below the ambient temperature resulted in enhanced survival of Carcinus at high air temperatures. In the same study they showed that under conditions of heat stress Carcinus moves from aquatic to aerial conditions thereby facilitating heat loss by evaporative cooling.

The crustaceans are among the few animal groups which have exploited both aquatic and aerial environments. The ability to respire in both air and water is of particular importance and many species rely on the same respiratory structure, the gill, in both media (Wolvenkamp and Waterman, 1960). Only in highly specialised air breathers (e.g. Birgus latro) is the lining of the gill chamber vascularised and gill numbers decreased so that effectively the respiratory apparatus becomes a lung (Diaz and Rodriguez, 1977; Taylor and Greenaway, 1979).

Respiration in water presents different problems to an animal compared with those associated with aerial respiration. The energy required to ventilate the respiratory surface with water is considerably higher than that for air because of the higher density and viscosity and the lower oxygen carrying capacity of water compared with air. Consequently, water-breathing animals have higher energetic requirements for ventilation (Dejours, 1975). As a result of the high solubility of carbon dioxide in water the excretion of this gas is accomplished more readily than in the aerial environment. Therefore, the haemolymph P_{CO_2} is lower in water breathers and in water and air different strategies of acid-base balance operate (Dejours, 1975; Cameron, 1979).

Air and water breathers differ in their control of respiration. Water breathers are sensitive to changes in ambient oxygen tension whereas air-breathing animals respond primarily to changes in CO_2 tensions. Although these generalizations have evolved largely as the result of investigations on vertebrates (Rahn and Howell, 1976) their general applicability would appear to be justified by the results of studies on crustacean respiration (Cameron, 1981b;

Taylor, 1982 for review).

An animal can respond to aerial exposure by either i) maintaining, with no marked reductions, active respiration in air or ii) reducing the ventilation rate with a consequent shift from aerobic to anaerobic metabolism and an accumulation of metabolites such as lactate or succinate.

The extent to which either strategy is followed is dependent on the normal habitat of the animal. Brachyurans have been found to inhabit a range of environments from sub-tidal (e.g. Callinectes sapidus), intertidal (e.g. Carcinus maenas), semiterrestrial (e.g. (Cardiosoma guanhumi) to terrestrial (e.g. Birgus latro).

Previous investigations of aerial exposure on decapods have primarily been concerned with respiratory and circulatory responses of species from different habitats. Thus measurements have been made of rates of oxygen consumption of Carcinus maenas, in water and in air using juvenile (Wallace, 1972; Newell et al., 1972) or mature animals (Taylor and Butler, 1978), and terrestrial species by Cameron and Mecklenburg (1973) and Cameron (1975). O'Mahoney and Full (1984) measured aquatic and aerial respiration in three crab species, which vary in their degree of terrestrial adaptation. The heart rates of Carcinus maenas (Ahsanullah and Newell, 1971; Newell et al., 1972; Taylor and Butler, 1978) and Austropotambius paiipes (Taylor and Wheatley, 1980) have been measured in air and in water. Gray (1957) made a comparative study of the relative gill areas of crabs and showed a negative relationship between gill area and adaptation to the terrestrial habitat.

Acid-base balance adjustments made in response to aerial exposure have been studied in C.maenas (Truchot, 1975b; Taylor and Butler, 1978) and terrestrial crabs (Cameron, 1981b). Young (1973) investigated the variation in the functional properties of haemocyanin with respect to terrestrial habitat in a range of decapod crustaceans. Bliss and Mantel (1968) described the regulation of body temperature and body fluid salt concentration and water balance in crabs exposed to the air.

In this study the effects of short (4hr) and longer term (24hr) aerial exposure were investigated in three crab species which experience varying degrees of exposure to the air in their normal range of habitat. The shore crab Carcinus maenas is commonly an intertidal animal whereas Cancer pagurus is a low littoral to sub-littoral species. The third species, the velvet swimming crab, Liocarcinus puber is a sub-tidal, infrequent intertidal, animal which is highly specialized for aquatic life. The aim of the following experiments was to determine the effects of aerial exposure on blood chemistry, the relative capabilities of the given species to maintain oxygen uptake and ultimately survival.

5.2. Materials and Methods

The holding procedures used for specimens of C.maenas, C.pagurus and L.puber in Hull are described in the General Materials and Methods (Page No.**9/10**). All species were held for at least 10 days in aerated recirculating aquaria at 30.0‰ S and 15°C. At the Marine Biology Laboratory, Helsingor, Denmark, specimens of C.maenas were held in aerated recirculating aquaria at 28.0‰ S and 15°C. Unless otherwise stated only males were used in the experiments because of the extreme variability of haemolymph glucose levels in females. The animals were fed every third day but not immediately before, or during, the experiments.

5.2.1. Control of the External Oxygen Tension

The medium was maintained initially at a normoxic level (ca 130 torr), the P_wO_2 value being monitored constantly using an EIL Portable Oxygen Meter 1520. The animals were exposed to the air by draining the water from the experimental tank over a period of 15 minutes. The animals were held in the air at 15°C for the duration of the experiment, which ranged from short (4hr) to longer term (24hr). The relative humidity in the tanks was constantly monitored using wet and dry bulb thermometers.

In the short-term aerial exposure experiments the animals were reimmersed in water after 4hr, the water being reintroducted into the tank over a 15 minute period. The medium was then maintained at a normoxic P_wO_2 (ca 130 torr) for 4hr following aerial exposure.

5.2.2. Haemolymph Sampling

At given time intervals, prebranchial haemolymph samples (200-250µl)

were withdrawn through the arthrodial membrane at the base of a walking leg. In short-term experiments (4hr) and in blood carbohydrate investigations the same animals were sampled throughout, whereas in longer-term experiments a different group of animals was used at each sampling time. In all experiments, except blood carbohydrate measurements, the samples were centrifuged at 3000g for 5 minutes to remove cell debris. For blood carbohydrate measurements whole blood was required and samples were utilized immediately following collection.

The handling-time involved in sampling was restricted to a maximum of 20 seconds for each animal in order to minimize the stress resulting from handling and bleeding. This time restriction was particularly important in experiments involving glucose estimations since it has been shown for many species that handling and sampling results in hyperglycaemia, the magnitude of which is directly related to handling time (Florkin and Duchâteau, 1939; Telford, 1968, 1974).

5.2.3. Haemolymph Analysis

Haemolymph samples were used for Na^+ and Ca^{2+} determinations, osmolality, lactate and glucose analyses and estimations of whole-blood carbohydrates. The Na^+ and Ca^{2+} concentrations in the haemolymph were determined by AAS, the details of which are given in the General Materials and Methods (Page No.11). Diluted haemolymph samples were analyzed using a Perkin Elmer Model 103 atomic absorption spectrophotometer coupled to a Perkin Elmer Model 56 chart recorder.

In the osmolarity analyses, 75µl of haemolymph were diluted 1:1 with double-distilled water and analyzed using a Knauer Semi Micro Osmometer. The principles and procedures involved in the lactate and glucose assays are detailed in the General Materials and Methods (Page Nos.**12** and **13**). A 100µl haemolymph sample was used for lactate determinations and concentrations were measured spectrophotometrically using a Boehringer lactate diagnostic pack (No. 124842). The haemolymph glucose concentrations were determined spectrophotometrically using a 100µl blood sample and a Sigma glucose test kit (No. 510), which is based on the glucose-oxidase method.

5.2.4. Carbohydrate Analysis

The carbohydrates present in the haemolymph sample were separated into two fractions by treatment with concentrated ethanol. In the separation oligosaccharides, of less than four or five monomeric units, remained in solution whereas the polysaccharides were precipitated as a pellet.

The haemolymph samples (200-250µl) were obtained by capillary puncture through the arthrodial membrane at the base of a walking leg. Haemolymph samples (100µl) were added to 500µl of 80% ethanol and centrifuged at 3000g for 5 minutes. The supernatant was retained for analysis (oligosaccharide fraction) whereas the ethanol precipitate was washed two times with 95% ethanol and then resuspended in 500µl of distilled water (polysaccharide fraction).

Quantiative analyses were carried out on the oligosaccharide fraction for glucose and total reducing substances (T.R.S.) and on the polysaccharide fraction for glycogen. Glucose was assayed on

the basis of the glucose oxidase method using a 200µl aliquot of the oligosaccharide fraction. The sample concentrations were derived from a standard glucose curve prepared using 2.5, 5.0, 10.0 and 20.0 mg.100ml^{-1} glucose standards.

The TRS concentrations were assayed using a modification of Nelson's (1944) method. Aliquots (200µl of the oligosaccharide fraction were evaporated to dryness and the residue of each was resuspended in 500µl of distilled water. The resulting solutions were treated with 500µl of copper reagent (containing 25 vols.reagent A : 1 vol. reagent B, Table 5.1), and heated at 100^{o}C for 20 minutes in stoppered tubes. After cooling, 1.5ml of Arsenomolybdate reagent (Table 5.2) were added to each sample and incubated at room temperature for 1hr to allow for complete CO_2 evolution from the solutions. The absorbance of each solution was measured against a distilled water blank at 620nm using a Cecil CE303 grating spectrophotometer (EEL Instruments Ltd). The concentrations of T.R.S. were derived from a standard curve (Fig. 5.1) prepared from glucose standards (2.5, 10.0, 20.0 and 100mg.100ml^{-1}) which had been treated in the same manner as the samples.

Glycogen levels were estimated from acid hydrolysates of the polysaccharide fraction. Samples (500µl) were treated with 50µl of INHCL and incubated at 100^{o}C for 3hr. After cooling, the glucose units liberated were assayed by the glucose oxidase method. Standard glycogen solutions (50.0 and 100.0 mg.100ml^{-1}) were subjected to acid hydrolysis to determine the percentage of glucose units liberated.

Table 5.1: Composition of Copper reagents used for T.R.S. analysis.

Copper Reagent A:

Sodium carbonate, Na_2CO_3 (anhydrous)	25g
Sodium potassium tartrate, $NaKC_4H_4O_6.4H_2O$	25g
Sodium hydrogen carbonate, $NaHCO_3$	20g
Sodium sulphate, Na_2SO_4 (anhydrous)	200g

Dissolve in 800ml of water and dilute to 1 litre.

Copper Reagent B:

Copper sulphate $CuSO_4,5H_2O$	15g

Dissolve in 100ml of water and add one or two drops of concentrated sulphuric acid (H_2SO_4).

All reagentsused were Analar grade.

Table 5.2: Composition of Arsenomolybolate colour reagent used for T.R.S. analysis.

25g Ammonium molybdate, $(NH_4)_6MO_7O_{24}.4H_2O$ in 450ml distilled water

↓

21ml of concentrated sulphuric acid (H_2SO_4)

↓

3g Sodium arsenate, $Na_2HAsO_4.7H_2O$ (hydrated) in 25ml water

↓

Incubate at 37^oC for 24-48 hours

All reagents used were Analar grade.

Figure 5.1. Standard curve of total reducing substance (T.R.S.) concentration against sample absorbance at 620nm.

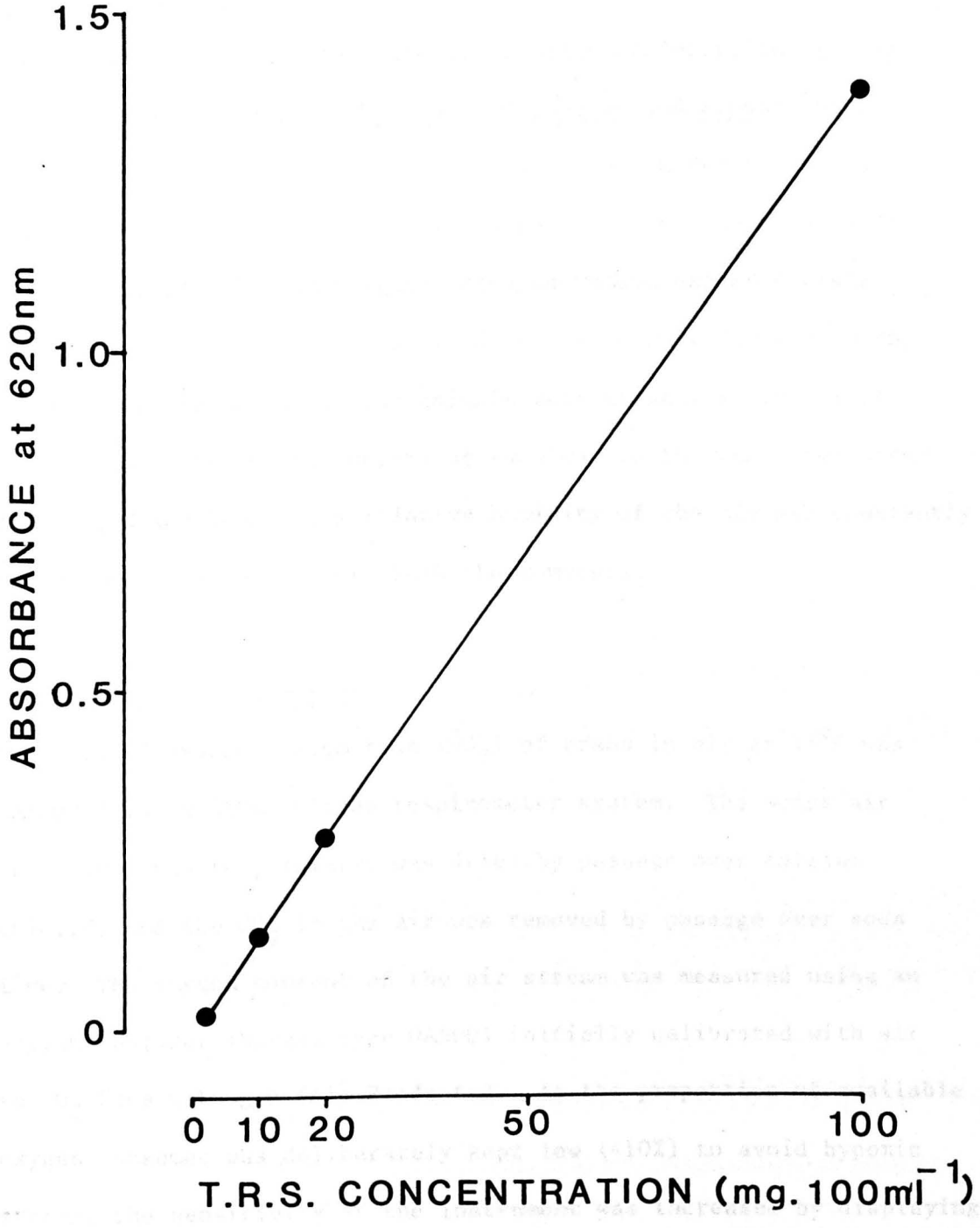

1.5
1.0
0.5
0
ABSORBANCE at 620nm
0
10
20
50
100
T.R.S. CONCENTRATION (mg.100ml⁻¹)

Fig.5.1

5.2.5. Weight Change

The weight change of eight intermoult males of intertidal (Hull) and sub-tidal (Helsingor) C.maenas, C.pagurus and L.puber were measured at 15°C, the animals being maintained under the normal holding conditions. Each animal was removed from the holding tank and the carapace was thoroughly dried to remove excess surface water before the animal was weighed to the nearest 0.01g on a top pan balance (Sartorius). The animals were maintained in air at 15-16°C for 24hr and the weight of each was further measured after 2, 4, 6, 8 and 24hr. The relative humidity of the air was constantly monitored using wet and dry bulb thermometers.

5.2.6. Oxygen Consumption

The rate of oxygen consumption ($\dot{M}O_2$) of crabs in air at 15°C was measured in an 850ml closed respirometer system. The moist air drawn from the respirometer was dried by passage over calcium chloride and the CO_2 in the air was removed by passage over soda lime. The oxygen content of the air stream was measured using an oxygen analyzer (Morgan type OA500) initially calibrated with air and O_2 free nitrogen (Air Products). As the proportion of available oxygen consumed was deliberately kept low (<10%) to avoid hypoxic stress, the sensitivity of the instrument was increased by displaying the output from the analyzer on a potentiometric pen recorder (Kipp and Zonen BD 40). The full scale deflection was set to 20.95% oxygen in air and the scale was checked using a standard gas mixture (12% O_2, 5% CO_2, 85% N_2, Corning Medical).

The respirometer was enclosed in an insulating polystyrene jacket

with a cooling element to maintain the temperature at the required level of 15-16°C. The rates of oxygen consumption were measured over a 4hr period and all values obtained for $\dot{M}O_2$ were corrected to S.T.P.D.

5.2.7. Heart and Scaphognathite Measurements

The principle of the organ monitoring technique and the procedure for electrode attachment are described in detail in the General Materials and Methods (Page No. 15). The specimens of C.maenas and L.puber were held in aerated recirculating aquaria (30 litres) at 30.0‰ S and 15°C, which were covered with black plastic and partitioned with opaque dividers to minimize undesigned external optical stresses. The animals were held in these conditions for 24hr following electrode implantation in order to allow the organ rates to return to non-stressed levels (see Cumberlidge and Uglow, 1977a).

In the experiments, the organ beat rates (left and right scaphognathites and heart) of each animal were measured initially for at least one minutes duration. The animals were exposed to the air by draining the tank over a 15 minute period and remained in the air at 15-16°C for 4hr. The relative humidity of the air was constantly monitored using wet and dry bulb thermometers. Following 4hr of aerial exposure the animals were reimmersed in water (T^{o} = 15°C) and the organ rates of each animal were measured at 10, 30 and 60 minutes following reimmersion. Each recording was made for at least one minutes duration with left and right scaphognathites being recorded simultaneously, a mean value of the two being obtained for each animal

[(L+R)/2]. This precaution was taken because of the wide disparity which has been demonstrated to occur for the rates of the two scaphognathites in C.maenas (Uglow, 1973).

5.2.8. Scanning Electron Microscopy (S.E.M.)

For examination of the gills by scanning electron microscopy (S.E.M.) animals were killed by destruction of thoracic ganglion and the two sets of gills were dissected out carefully from the branchial chamber. The gills were fixed for 24hr in a mixture of 2% Glutaraldehyde/ 2% Formaldehyde in filtered seawater. Fixed gills were dehydrated in acetone and specimens were then critical-point dried in a 'Polaron' E3000 critical point drying apparatus. The specimens were mounted on aluminium stubs and coated with a layer of gold in a Nanotech SEM Prep 2 Sputter Coater. The prepared sections were then examined by S.E.M. using a Cambridge Stereoscan 600 scanning electron microscope.

5.2.9. Statistical Treatment of Data

Measured values are given in this study as means ± S.E. of mean with the number of observations in parenthesis. The significance of any apparent difference between the mean values of variables was tested using Students t-test and the word 'significant' means that the difference is significant at the 95% level of confidence ($P<0.05$).

5.3. Results

The haemolymph calcium $[Ca^{2+}]$, values measured for each species during aerial exposure are shown in Table 5.3. Following a 4hr period of aerial exposure there were increases in $[Ca^{2+}]$ in C.pagurus and L.puber, although only that in L.puber was statistically significant ($P<0.05$). On re-immersion into normoxic conditions the $[Ca^{2+}]$ values of all species returned to values similar to the initial normoxic levels. Control experiments in normoxic conditions which duplicated the sampling procedure only resulted in no significant alterations to $[Ca^{2+}]$ levels in any of the species (Table 5.3).

In Table 5.4 the haemolymph sodium concentrations measured for each species in response to normoxia or aerial exposure are given. No significant changes ($P>0.05$) were recorded after either 4hr aerial exposure or 4hr normoxia (i.e. control experiments) for any of the species.

The haemolymph lactate levels measured for each species in response to aerial exposure are shown in Figure 5.2. In all the species there were increases in haemolymph lactate after a 4hr period in the air. However, only the increases measured in C.pagurus and L.puber were significant at the 5% level, the elevation being most marked in L.puber. Following a 4hr period of re-immersion the blood lactate levels decreased in all species and in C.maenas and C.pagurus returned to levels not significantly ($P<0.05$) different from initial normoxic values. In L.puber, however, blood lactate levels remained significantly ($P<0.05$) higher than initial values even after 4hr of re-immersion but after 6hr normoxia had decreased to levels not significantly ($P>0.05$)

different from original normoxic levels. Control experiments which duplicated the sampling procedure only, showed no significant ($P>0.05$) changes in haemolymph lactate in any of the species.

The haemolymph glucose and lactate levels recorded in each species during a 24hr period of aerial exposure are shown in Figures 5.3 and 5.4 respectively. The haemolymph glucose levels increased significantly ($P<0.05$) in all species after 4hr aerial exposure. Between 4hr and 24hr there was a decrease of the elevated haemolymph glucose level in both C.maenas and L.puber to levels not significantly ($P>0.05$) different from the initial, normoxic levels. However, in C.pagurus the haemolymph glucose concentration continued to increase throughout the 24hr period of aerial exposure. Control experiments, which duplicated the sampling procedure only resulted in no significant increases in haemolymph glucose in any of the species ($P>0.05$ in all cases).

There were significant ($P<0.05$) increases in haemolymph lactate in all species following 24hr aerial exposure, the increase in L.puber being more marked than that for C.pagurus and lowest in C.maenas. In both C.pagurus and L.puber the increases in blood lactate were significant ($P<0.05$) after 4hr aerial exposure. For each species, control experiments, which duplicated the sampling procedure only showed no significant increases in haemolymph lactate levels ($P>0.05$ in all cases).

The data obtained for the mortality of each species following a 24hr period of aerial exposure are shown in Table 5.5. In air, the rates of mortality remained at zero for C.maenas, increased slightly in C.pagurus and reached high mortalities in L.puber,

where the LT_{50} in air (i.e. lethal time for 50% mortality) for this species was calculated at 17.0 hours.

In Figure 5.5 the concentrations of the carbohydrate fractions of C.pagurus haemolymph following 24hr aerial exposure and 24hr normoxia are given. After exposure to air there were increases in haemolymph glucose and non-glucose reducing substances and a decrease in blood glycogen, although only the change in blood glucose was significant ($P<0.05$). There was a small increase in the unidentified carbohydrate fraction (i.e. total carbohydrate - measured fractions) following aerial exposure. The animals in normoxic conditions showed no significant changes in any of the blood carbohydrate fractions after 24hr ($P>0.05$ in all cases).

The L.puber haemolymph carbohydrate concentrations measured during aerial exposure are given in Figure 5.6. The animals were only exposed to the air for 8hr, as compared with 24hr for C.pagurus because of the low LT_{50} of this species. Haemolymph glucose and glycogen levels increased whereas non-glucose reducing substances declined during aerial exposure. Only the change in haemolymph glucose concentration was statistically significant ($P<0.05$). No significant ($P>0.05$) changes in haemolymph carbohydrate concentrations were recorded in animals in normoxic conditions for 8hr.

No investigation of C.maenas blood carbohydrate concentrations during aerial exposure was conducted since no significant ($P>0.05$) increase in the haemolymph glucose concentration was measured after 24hr exposure to the air (Figure 5.3).

In order to determine the yield of glucose units from glycogen as a result of the acid hydrolysis technique used in this study standard glycogen samples were subjected to acid hydrolysis, the data obtained being given in Table 5.6. A 1mg glycogen standard was found to yield 0.373mg of glucose following the acid hydrolysis treatment used for blood glycogen samples.

The changes in body wet weight and haemolymph osmolality, expressed as % change from the initial value, for intertidal (Hull) and sub-tidal (Helsingor) C.maenas, C.pagurus and L.puber are shown in Table 5.7. The rate of water loss was similar in all groups at 15°C with the changes being significant in all species after 4hr in the air. In response to aerial exposure there was an increase in haemolymph osmolality, the extent of increase being such that C.maenas < C.pagurus < L.puber. In C.maenas the % increase in haemolymph osmolality was slightly, though not significantly ($P>0.05$), greater in intertidal crabs than in sub-tidal specimens. In all species the % increase in haemolymph osmolality was greater than the % decrease in body wet weight but lower than the possible % reduction in haemolymph volume. The possible % reduction in haemolymph volume was calculated on the basis that the reduction in mass is due to evaporative water loss from the body fluids and that the extracellular space is approximately 20% of body weight (Binns, 1969).

In Table 5.8 the measured rates of oxygen consumption ($\dot{M}O_2$) in air, numbers of gill lamellae per mm. gill length and mean interlamellar distances are given for the species used. In air, the intertidal species C.maenas had a markedly higher $\dot{M}O_2$ than

C. pagurus or L.puber, which had the lowest measured $\dot{M}O_2$ in air. The values given for numbers of gill lamellae per mm. gill length and interlamellar distances were from measurements made from scanning electron micrographs. In Figures 5.7 and 5.8 scanning electron micrographs of the respective gill lamellae of C.maenas and L.puber are shown. In C.maenas the gill lamellae were less numerous and set further apart than those of L.puber. Furthermore cross bridges between gill lamellae were evident in C.maenas, but were not observed in the gill lamellae of L.puber.

The cardiac and ventilatory responses measured in C.maenas and L.puber during re-immersion following 4hr aerial exposure at 15°C are given in Figures 5.9 and 5.10 respectively. C.maenas showed no significant changes (cf. to pre-aerial exposure levels) in heart (f_H) or scaphognathite (f_{sc}) rate on re-immersion. However, L.puber showed pronounced increases (cf. original values) in f_H and f_{sc} upon re-immersion following exposure to the air. The measured tachycardia remained significantly higher ($P<0.05$) than the pre-aerial exposure level for at least 1hr in normoxia whereas the elevated f_{sc} decreased to a value not significantly different ($P>0.05$) from the initial normoxic rate after only 30 minutes in normoxia.

5.4. Discussion

The ability of decapod crustaceans to respire in both aquatic and aerial environments is to a certain extent unexpected, since in many species the same respiratory structure, the gill, has to function in both media (Wolverkamp and Waterman, 1960). Only in highly specialized air breathers (e.g. Birgus latro) is the lining of the gill chamber modified so that the branchial cavity acts as a lung (Diaz and Rodriguez, 1977; Taylor and Greenaway, 1979).

In C.maenas, the pattern of gill ventilation in water has been described by a number of workers (Borradaile, 1922; Arudpragasam and Naylor, 1964a; Hughes et al., 1969; Taylor et al., 1973). The ventilation of the branchial chamber is characterized by a predominantly forward flowing current with associated periodic reversals. Arudpragasam and Naylor (1966) found that the ventilation patterns in C.pagurus and L.puber are basically similar to that found in Carcinus, with interspecific differences in the frequency and duration of the reversals. Taylor and Butler (1978) found that Carcinus in air ventilates its gills in either an anterior or posterior direction with alternate burst of forward and reversed ventilation.

Here, on exposure to the air at 15°C the intertidal species, C.maenas had the highest measured $\dot{M}O_2$ rate (20.1µl O_2.g.hr^{-1}), the intertidal/sub-tidal C.pagurus (10.8µl O_2.g.hr^{-1}) and the sub-tidal species L.puber had the lowest value (9.8µl O_2.g.hr^{-1}). Wallace (1972) and Newell et al. (1972) showed that the $\dot{M}O_2$ of Carcinus in air is approximately 75% of the measured rate of that when submerged in aerated seawater. Their investigations were similar in that

relatively small Carcinus (15g wet weight) were used and $\dot{M}O_2$ was measured in Gilson respirometers. Such manometric respirometers require shaking to equilibrate the gas and liquid phases and this may lead to excited levels of activity in the crabs (Wallace, 1972). However, Taylor and Butler (1978) concluded that the aerial $\dot{M}O_2$ (3hr at 15°C) of larger Carcinus (56g wet weight) is similar to the value obtained in seawater after 3hr recovery from handling. In the marine crab Callinectes sapidus, the $\dot{M}O_2$ in air was found to be 35% of the rate measured in seawater (O'Mahoney and Full, 1984). Thomas (1954) measured a depressed $\dot{M}O_2$ in air of only 14% of the level in aerated seawater in the lobster Homarus vulgaris.

In association with its capacity as an air breather Carcinus showed no marked increase in heart (f_H) or scaphognathite (f_{sc}) rate after re-immersion following aerial exposure in these studies. Ahsanullah and Newell (1971) and Taylor and Butler (1978) both found no evidence of a bradycardia in Carcinus on exposure to the air, while Newell et al. (1972) found no change in f_H of Carcinus in water following aerial exposure. Taylor and Butler (1978) measured an increase in cardiac output in Carcinus on exposure to the air which, they concluded, was the result of an increase in cardiac stroke volume, since f_H was unchanged in the air. In contrast significant increases in f_H and f_{sc} were measured in L.puber in seawater following aerial exposure in these studies, and both f_H and f_{sc} remained elevated (cf. pre-exposure values) for up to 1hr following re-immersion. de Fur and McMahon (1978) found that immediately on exposure of Cancer productus to air, f_{sc} increased while f_H remained unchanged. On re-immersion, increases over pre-

immersion values of f_H and f_{sc} were recorded and these high levels persisted for 2-4hr.

In this study, the increases in circulating blood lactate, measured in response to short term aerial exposure (4hr), were pronounced in Liocarcinus, rather less marked in Cancer but Carcinus showed only a small non-significant ($P>0.05$) increase in blood lactate concentration. The finding that haemolymph lactate levels in Carcinus did not change during aerial exposure is consistent with the findings for this species by Taylor and Butler (1978). The response is not unexpected in view of the animals' intertidal habitat and maintenance of $\dot{M}O_2$ in air at values comparable to aquatic $\dot{M}O_2$. In Cancer and Liocarcinus the increases in circulating blood lactate are concommitant with the low $\dot{M}O_2$ values measured for these species, and are indicative of a switch from aerobic to anaerobic metabolism.

Re-immersion of animals in seawater is accompanied by a reduction in their circulating lactate levels and this, presumably, reflects the repayment of an oxygen debt by the re-oxidation of the accumulated end products of anaerobiosis. Lactate is resynthesized to glucose via the gluconeogenic pathway, as shown in Figure 3.15. However, controversy exists regarding the actual site of such conversions (Thabrew et al., 1971; van Weel, 1974; Phillips et al., 1977).

Haemolymph lactate levels in Cancer had returned to pre-aerial exposure values within 4hrs of re-immersion whereas Liocarcinus restored initial normoxic values only after 6hr in aerated seawater.

The rate of gluconeogenesis and thus the proportion of lactate re-synthesized is, therefore, likely to vary between different crustacean species and this may account for the marked inter-specific differences in the rate of lactate decline measured in this study. Pritchard and Eddy (1979) found that haemolymph lactate accumulation during anoxia was more marked in the mud shrimp Upogebia pugettensis than the ghost shrimp Callianassa californiensis. They suggested that the difference could be explained in terms of adaptation to the different conditions under which the two species lived.

Data from Pearse (1929) and Gray (1957) suggest that, as the habitat of crabs changes from aquatic through intertidal to terrestrial, there is a reduction in gill area per unit of total body weight. Butler (1976) quotes relative gill areas of $777mm^2g^{-1}$ for Carcinus and $1367mm^2g^{-1}$ for the sublittoral species Callinectes.

The gill lamellae of Carcinus have been shown in this study and by Taylor and Butler (1978) to be relatively well-spaced (ca. 50μm apart) and covered with a layer of chitin. This layer may confer a degree of rigidity to the lamellae as may the cross bridges which have been observed between the lamellae in Carcinus (Figure 5.7). In Liocarcinus (and presumably Cancer) the distance between the gills is considerably smaller being approximately 28μm (Figure 5.8). Furthermore Booth et al. (1982) found that the chitin layer of the gill lamellae is relatively thin in the swimming crab C.sapidus. Therefore, it is possible that the lamellar gills of Carcinus are more resistant to collapse in air

than the gill lamellae in the more predominatly sub-littoral species such as Cancer and Liocarcinus which have been found to be less rigid (Cameron, 1981a).

The chitin layer on the gills presents a barrier to the diffusion of gases (Redmond, 1955), although in Carcinus this probably confers a mechanical advantage which outweighs any potential physiological disadvantage. The shortest diffusion distance between water and haemolymph over the gill lamellae of Carcinus has been shown by electron microscopy (Taylor and Butler, 1978) to be across the processes of the pillar cells. The distance is approximately 6μm, which is similar to the equivalent barrier on the secondary lamellae of fish gills (e.g. Hughes and Wright, 1970). In Carcinus this distance consists of approximately 5μm of chitin, which has a reported diffusion constant for oxygen of only 10% of that for tissue (Krogh, 1941). Thus the effective barrier to the diffusion of oxygen across the gills of Carcinus is five to ten times that of the typical secondary lamellae of the fish gills (or possibly the gills of sub-tidal crab species). However, in spite of this disadvantage, Carcinus can generate relatively high post branchial haemolymph oxygen tensions (P_aO_2) in aerated seawater (Taylor and Butler, 1973).

During aerial exposure, P_aO_2 and C_aO_2 values in Carcinus were found to be significantly reduced by 75% and 43% respectively, (Taylor and Butler, 1978). This was taken as an indication by the authors that the capacity of the gills to exchange oxygen with the air is limited despite the ability of the species to maintain $\dot{M}O_2$ in air. However, Carcinus appears to maintain $\dot{M}O_2$ in air by drawing

on a venous reserve of oxygen (Taylor and Butler, 1978). de Fur and McMahon (1978) found that haemolymph P_aO_2 and C_aO_2 of Cancer productus declined by 80% and 72% respectively, indicating a substantial reduction in oxygen uptake.

The responses of Carcinus to short-term exposure to air (i.e. equivalent to a tidal exposure period) are consistent with those of an animal which is an effective facultative air breather and which can maintain its oxygen uptake. However, the responses of both Cancer and Liocarcinus are indicative of a marked reduction in their oxygen uptake, probably due to a decrease in the surface area for gas exchange following the collapse and clumping of their gill lamellae.

Haemolymph pH has been found to decrease during short-term aerial exposure in C.maenas (Truchot, 1975c; Taylor and Butler, 1978) and C.productus (de Fur and McMahon, 1978). This decrease has been attributed to an accumulation of carbon dioxide as shown by the increase in P_aCO_2 which all the authors reported. However, Taylor and Butler (1978) stated that accumulation of CO_2 did not appear to be a major respiratory problem to Carcinus when exposed to air during tidal exposure and this they suggest may indicate that they retain some water in their branchial chambers.

In C.pagurus and L.puber increases in haemolymph $[Ca^{2+}]$ occurred in response to short-term aerial exposure, although only the change in Liocarcinus was statistically significant ($P<0.05$). These changes are probably not the result of a decrease in haemolymph volume since no concommitant changes in haemolymph Na^+ were recorded. The absence of changes in haemolymph Ca^{2+} as found here for Carcinus is consistent with the findings of Truchot (1979) who found no changes of blood

Ca^{2+} during compensation of the respiratory acidosis following exposure of Carcinus to the air. However, de Fur et al. (1980) reported an increase of Ca^{2+} in the haemolymph of Cancer productus during aerial exposure. The increases in haemolymph Ca^{2+} are probably indicative of an increase in haemolymph $[HCO_3^- + CO_3^{2-}]$, i.e. a mobilization of calcium carbonate from the exoskeleton or stores in the hepatopancreas, to compensate for the respiratory acidosis.

A decrease in blood pH in Carcinus during aerial exposure has been shown (Taylor and Butler, 1978) to cause a Bohr shift which decreases the affinity of haemocyanin for oxygen (i.e. increases the P_{50}). Despite this decreased oxygen affinity, they concluded that an advantage may be conferred because of the greater release of combined oxygen from the venous blodd.

Reductions in body mass were found to be a response to long term aerial exposure, with the changes being significant ($P<0.05$) after 4hr in all species. Although the extent of these reductions in mass (% basis) were similar in all the species, the changes were most marked in sub-tidal C.maenas and L.puber. The increases in haemolymph osmolality found after 24hr exposure were most marked in L.puber and were smallest in C.maenas. An unexpected point was the fact that the increase in haemolymph osmolality was greater in intertidal rather than sub-tidal groups of C.maenas. It is possible that part of the reduction in mass is due to evaporation of surface water, particularly in the first 4hr when the rate of evaporative loss in all species was high. In each species the calculated reduction in haemolymph volume was far greater than the measured

increases in haemolymph osmolality after 24hr. It may be that concentration of the haemolymph leads to the movement of water from the tissue, thereby maintaining an equilibrium which negates the potential rise in haemolymph osmolality.

The results of this study are consistent with those of Ahsanullah and Newell (1977) who found that water loss was greater in the sub-tidal crab Portunus marmoreus than in Carcinus maenas. The sites of water loss, which is a passive process, were shown by the authors to be primarily the gills and walls of the branchial chambers in both species. They concluded that the greater losses which occurred in Portunus were associated with the relatively larger gill in that species (cf. C.maenas).

As stated previously, Taylor and Butler (1978) showed that Carcinus can actively ventilate its branchial chambers from either direction when exposed in air. They suggested the possibility that this ventilation pattern may, in addition to serving a respiratory function, serve to cool the crab in warm air by encouraging evaporative heat loss from the respiratory surfaces. Ahsanullah and Newell (1977) have shown heat loss by evaporation would have ecological significance and survival value in Carcinus but not Portunus.

Following long term (24hr) aerial exposure Carcinus showed no marked increase in circulating haemolymph lactate, a finding concommitant with the measurements for $\dot{M}O_2$, f_H and f_{sc}. It was also not unexpected in view of the ability of Carcinus to remain in air for prolonged periods (Perkins, 1967).

In Cancer and Liocarcinus haemolymph lactate levels continued to increase over 24hr aerial exposure. The high levels of lactate accumulated in C.pagurus during aerial exposure suggest that the respiration of this species is considerably less effective in this medium. However, only a low mortality rate (15.4%) for this species was recorded in air after 24hr. This presumably indicates that the species can tolerate anaerobiosis for at least 24 hours. In contrast in Liocarcinus the low $\dot{M}O_2$ in air resulted in high levels of accumulated lactate and a high mortality rate (76.9%). The lethal time for 50% mortality (LT_{50}) was calculated at 17.0 hours which corresponds to a haemolymph lactate level of 81.0 mg.100ml^{-1}. In Cancer it is possible that the species can maintain gluconeogenic capacity in air to maintain accumulated lactate concentrations at tolerable levels.

The source of the accumulated lactate in the haemolymph is as yet unknown. The classical Emben-Meyerhof glycolytic system, utilizing glucose as the respiratory substrate has been shown to operate in crustaceans (Hochachka et al., 1962; Huggins, 1966). Further evidence for the operation of this pathway in crustaceans was obtained by the thorough analysis of Boulton and Huggins (1970) who demonstrated the presence of all the key glycolytic enzymes in a number of crustaceans, with activities comparable to those in rat liver in most cases.

In both Cancer and Liocarcinus the blood glucose concentration increased rapidly on short term (4hr) exposure. The level continued to increase in air up to 24hr in Cancer, whereas in Liocarcinus the blood glucose level decreased from a maximum value at 4hr to values not significantly different from initial values after 24hr in air.

Although the increase in the blood glucose level of Carcinus was significant ($P<0.05$) after 4hr aerial exposure, the change may have been due to intraspecific variability of haemolymph glucose concentrations. Such intraspecific variation has been shown to occur in many crustaceans including Homarus americanus (Telford, 1965).

Increases in circulating blood sugar concentrations during aerial exposure were found in C.pagurus by Roche and Dumazert (1935) and Libinia emarginata by Kleinholz and Little (1949). Stott (1932) found that hypoxic seawater caused marked hyperglycemia in C.maenas, C.pagurus and L.puber. Such hyperglycaemic responses have been shown to be mediated via hyperglycaemic hormone (HGH) released from the eyestalk neuroendocrine system (Abramowitz et al., 1944; Kleinholz and Little, 1949; Kleinholz et al., 1950).

In decapod crustaceans, the haemolymph has been shown to consist of both plasma and haemocytes (Johnston et al., 1971; Johnston and Davies, 1972). In the plasma, glucose and other oligosaccharides (e.g. trehalose, maltose, fructose and fucose) are present whereas the haemocytes have been shown to contain the polysaccharides glycogen and chitin. Johnston and Davies (1972) and Williams and Lutz (1975) have shown that rather than simply functioning as a transport medium for low molecular weight compounds, the haemolymph, and not the hepatopancreas as was previously believed, is the important tissue for carbohydrate metabolism.

The increases in haemolymph glucose concentration recorded during aerial exposure in Cancer and Liocarcinus may be due to the conversion of circulating oligosaccharides to glucose as has been suggested by Telford (1968b). Oligosaccharides are normally present in the blood and are thought to be intermediates in the synthesis of glycogen.

In this study non-glucose oligosacchardies (i.e. with the exception of trehalose) were found to represent 5.3% and 21.5% of blood carbohydrate respectively in Cancer and Liocarcinus. These findings are consistent with values of 5.3% and 7.5% found in Carcinus and Libinia respectively by Johnston and Davies (1972).

Telford (1968b) showed that although an overall increase in both glucose and total reducing sugars occurred in H.americanus under stress conditions, the relative level of the glucose increase was markedly greater than that of T.R.S. The author attributed this to the fact that glucose was being produced from other reducing substances in the haemolymph. It is not clear whether this conversion is mediated via HGH. In crustaceans, haemolymph glucose levels have been shown to rise rapidly within 10-20 secs of the application of a handling stress (Telford, 1974; Walters, 1980). The conversion of disaccharides to glucose could account for this rapid initial response and the breakdown of glycogen could occur simultaneously to maintain a high level of glucose in the haemolymph following the initial response. Martin (1965) has emphasised the important role of glycogen in crustacean metabolism.

Glycogen is stored in the blood haemocytes but its metabolic fate is not clear and it may be either a) a store of hexose units for chitin synthesis or b) the main source of haemolymph glucose. For haemocyte glycogen to be the source of blood glucose, the glucose-6-phosphatase enzyme would have to be present within the cells, and histochemical and biochemical tests appear to have confirmed the presence of this enzyme (Johnston et al., 1973).

In C.pagurus there was a decrease in blood glycogen after 24hr aerial exposure. Although the decrease did not reach statistical significance it was found that acid hydrolysis, used to assay glycogen, gave values corresponding to only 37% of the actual glycogen concentration in a standard solution. However, the release of glucose in vivo from glycogen may be greater than that shown by acid hydrolysis because of the action of the phosphorylase system (Figure 5.11) which may produce a greater yield of glucose from glycogen breakdown.

Therefore, the decrease in blood glycogen measured in C.pagurus could be partially responsible for the increase in blood glucose measured after 24hr aerial exposure, depending on the extent to which haemocyte glycogen is converted directly to glucose. Pritchard and Eddy (1979) found no nett utilization of glycogen stores of the midgut gland or chela muscle in thalassinid shrimps. Keller and Andrew (1973) though showed that the sites of glycogen mobilization in Uca and Orconectes, under stress conditions, are the abdominal muscles, the epidermal cells and the gonads.

In view of the present results obtained for $\dot{M}O_2$ the blood lactate concentration, it would appear that, when exposed to air, C.pagurus and L.puber have to derive metabolic energy anaerobically whereas Carcinus can maintain aerobic metabolism. In this respect Carcinus is similar to a number of other littoral invertebrate species which utilize aerial respiration when exposed to the air and thereby avoid a shift to anaerobic metabolism and the consequent accumulation of lactate. Newell (1976) pointed out that the yield of ATP resulting from the aerobic route for phosphorylation is greater than that via

anaerobic metabolism. This is shown in Table 5.9 where the energy deficits calculated, in terms of μmoles ATP.g^{-1}, after 4 and 24hr aerial exposure are given for the three species under study. The degree of dependence that each species has on anaerobiosis for their energy requirements was calculated using their rates of oxygen consumption in water and air and blood lactate production as indices of aerobic and anaerobic metabolism respectively. Relative ATP yields were estimated from rates of oxygen consumption and lactate production using the values given by Bennett and Licht (1972). The values and assumptions on which they are based are:-

i) 1mg of lactate formed = 16.7 μmoles ATP (assuming glycogen is the only substrate catabolized).

ii) 1ml O_2 (STP) consumed = 290 μmoles ATP (assuming a P/O ratio of 3).

The values given in Table 5.9 are approximations but, if accepted, seem to show that the 15.4% and 76.9% mortality rates of C.pagurus and L.puber following 24hr aerial exposure are entirely predictable on the basis of their reductions in oxygen uptake in air and relative energy deficits. By the same argument, the small energy deficit calculated for Carcinus can be correlated with the demonstrated ability of this species to maintain aerobic respiration and a high level of survival compared to the other two species, during prolonged aerial exposure.

Table 5.3 Summary of blood Ca^{2+} data relating to periods of submerged normoxia (T^{o}=15^{o}C) and aerial exposure (seawater T^{o}=15^{o}C, Air T^{o}=15^{o}C, Relative humidity = 75-80%) for three species of crabs.

Sampling Time (Minutes)	HAEMOLYMPH Ca^{2+}					
	C.maenas		C.pagurus		L.puber	
	NORMOXIA (CONTROL)	AERIAL EXPOSURE	NORMOXIA (CONTROL)	AERIAL EXPOSURE	NORMOXIA (CONTROL)	AERIAL EXPOSURE
0	13.6±0.6	12.8±1.0	16.0±0.3	15.5±0.5	15.0±0.4	16.0±0.4
		n.s.		n.s.		P<0.05
270	14.0±0.5	12.4±0.8	15.4±0.1	16.1±0.7	15.0±0.5	17.3±0.5
540	14.3±0.3	12.4±0.6	15.9±0.2	15.3±0.5	15.5±0.2	16.2±0.3

Values given are mean ± S.E. for 5 animals in each case.

Table 5.4 Summary of blood Na^+ data relating to periods of submerged normoxia ($T^oC=15^oC$) and aerial exposure (Seawater $T^oC=15^oC$, Air $T^oC=15^oC$, Relative humidity = 75-80%) for three species of crabs.

Sampling Time (Minutes)	HAEMOLYMPH Na^+					
	C.maenas		C.pagurus		L.puber	
	NORMOXIA (CONTROL)	AERIAL EXPOSURE	NORMOXIA (CONTROL)	AERIAL EXPOSURE	NORMOXIA (CONTROL)	AERIAL EXPOSURE
0	450.0±16.6	444.0±7.4	404.0±8.9	404.2±6.3	385.3±10.5	405.7±4.2
270	455.3± 4.8	451.0±9.5	407.7±5.0	386.6±8.0	372.0± 7.9	398.3±6.9
540	441.0± 2.7	457.8±6.5	404.0±6.7	389.6±9.5	384.0± 7.1	395.7±6.6

Values given are mean ± S.E. for 5 animals in each case.

Figure 5.2. Haemolymph lactate concentrations of three crab species during normoxia (T^{o}=15^{o}C) or after a 4hr period of aerial exposure (T^{o}=15^{o}C, R.H.=75-80%) followed by subsequent re-immersion (T^{o}=15^{o}C). Values given are means ± for 5 animals in each case.

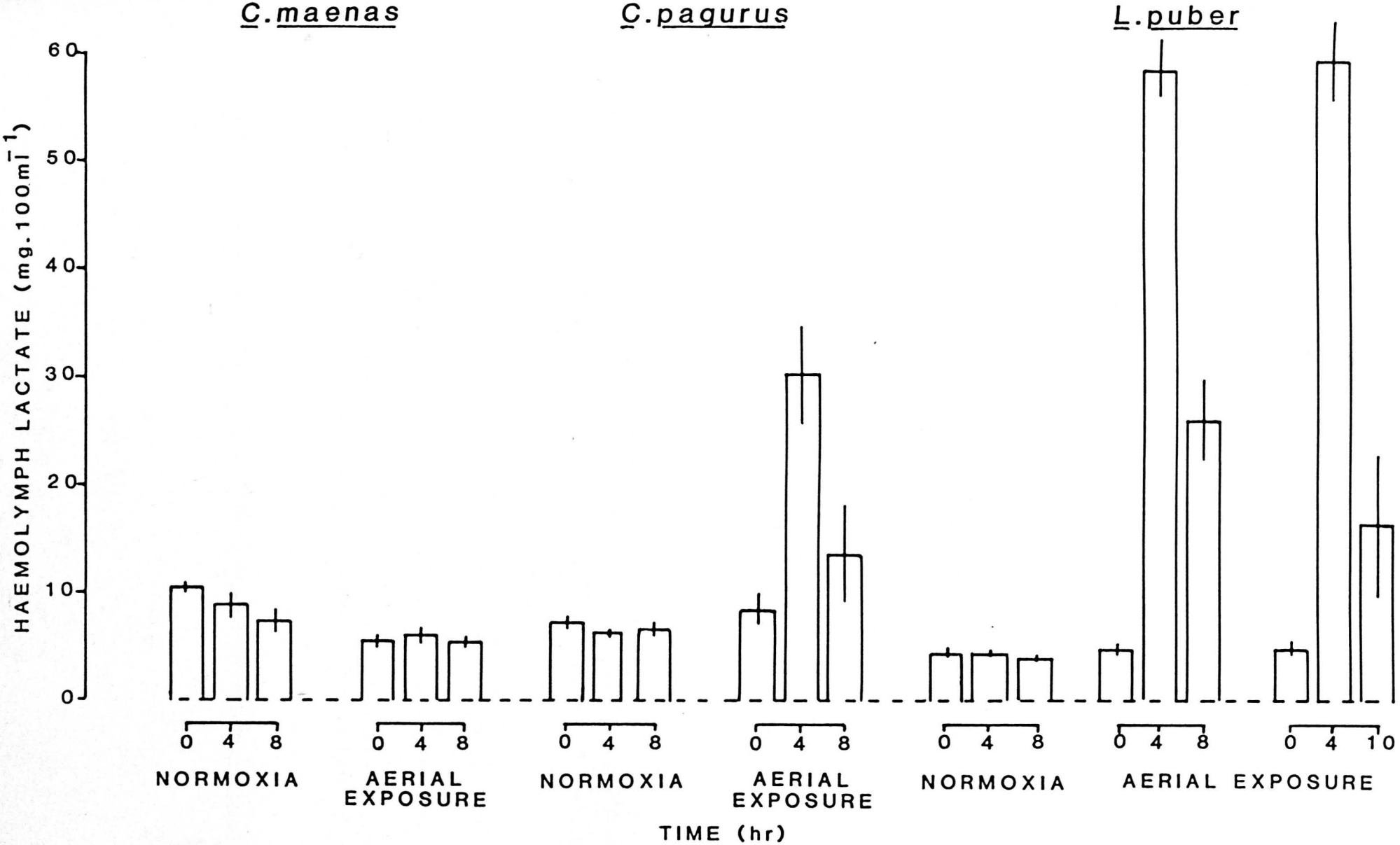

C.maenas
C.pagurus
L.puber
HAEMOLYMPH LACTATE (mg.100ml⁻¹)
60
50
40
30
20
10
0
0 4 8
0 4 8
0 4 8
0 4 8
0 4 8
0 4 8
0 4 10
NORMOXIA
AERIAL EXPOSURE
NORMOXIA
AERIAL EXPOSURE
NORMOXIA
AERIAL EXPOSURE
TIME (hr)

Fig.5.2

Figure 5.3. Haemolymph glucose concentrations of *Carcinus maenas*, *Cancer pagurus* and *Liocarcinus puber* during normoxia ○ ($T^{o}=15^{o}C$) and aerial exposure ● ($T^{o}=15^{o}C$, R.H.=75-80%). Values given are means ± S.E. for 5 animals in each case.

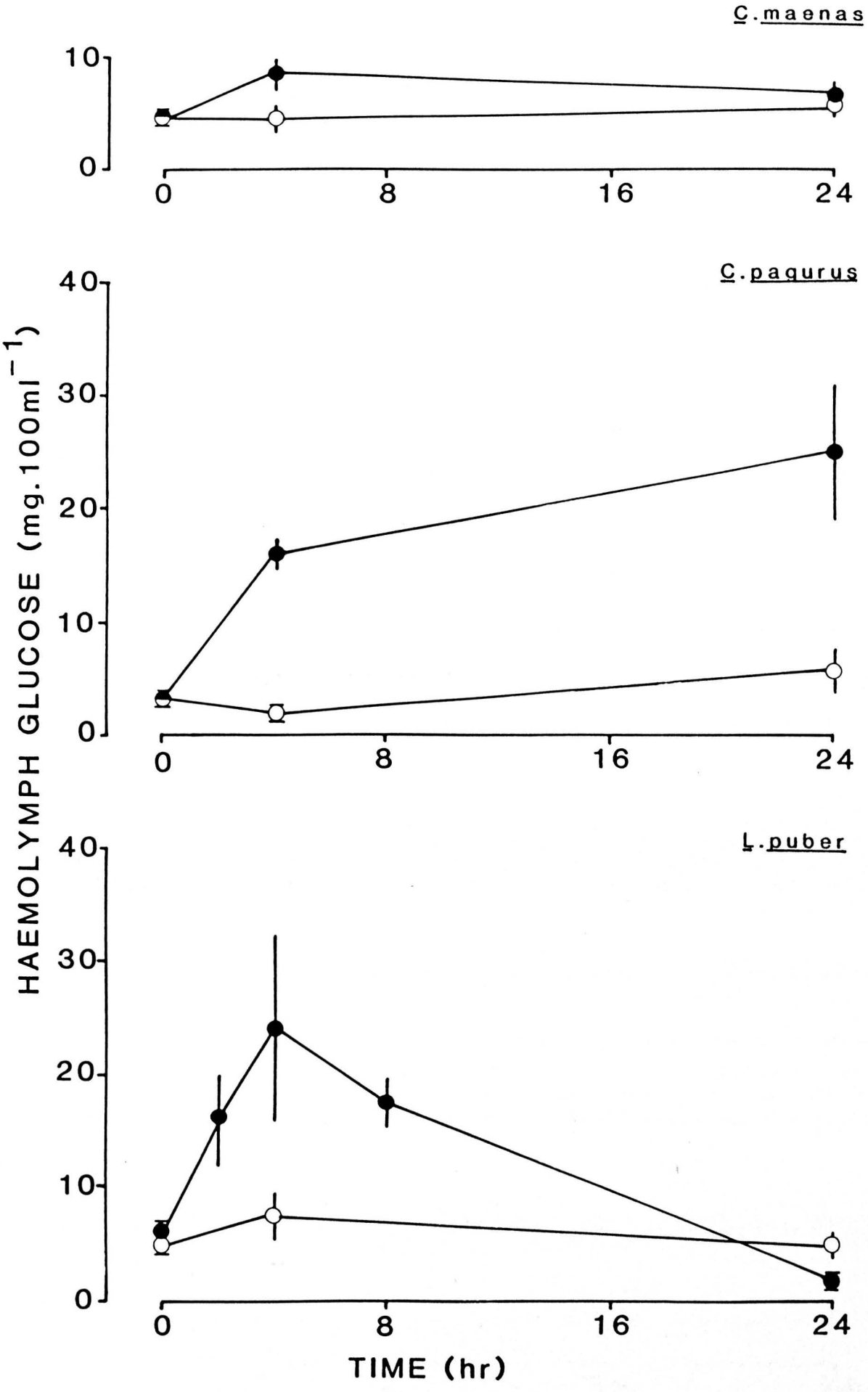

C.maenas
10
0
0
8
16
24
C.pagurus
40
30
20
10
0
0
8
16
24
L.puber
40
30
20
10
0
0
8
16
24
HAEMOLYMPH GLUCOSE (mg.100ml^{-1})
TIME (hr)

Fig.5.3

Figure 5.4. Haemolymph lactate concentrations of *Carcinus maenas*, *Cancer pagurus* and *Liocarcinus puber* during normoxia ○ (T^{o}=15^{o}C) and aerial exposure ● (T^{o}=15^{o}C, R.H.=75-80%). Values given are means ± S.E. for 5 animals in each case.

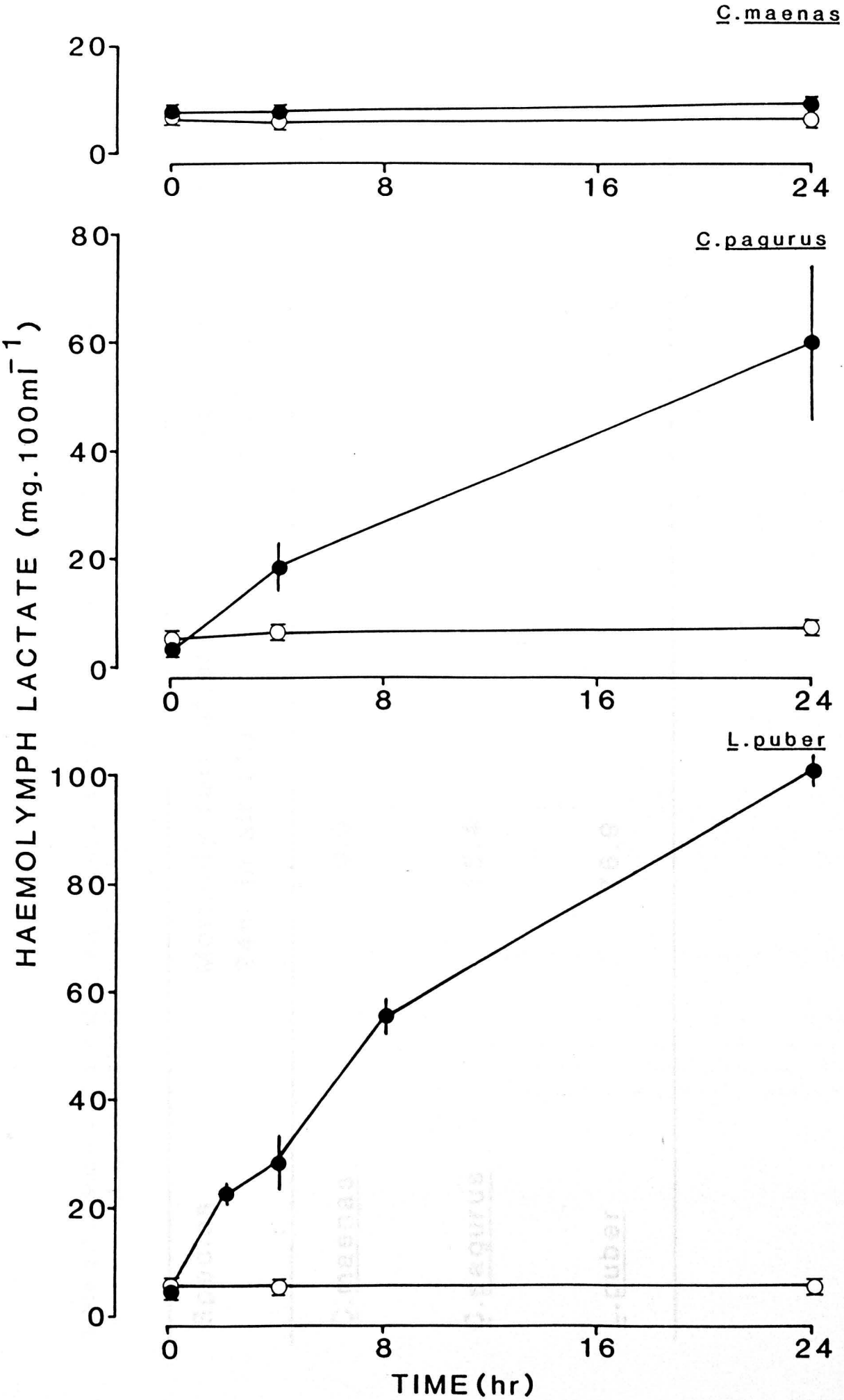

C.maenas
20
0
0
8
16
24
C.pagurus
80
60
40
20
0
0
8
16
24
L.puber
100
80
60
40
20
0
0
8
16
24
HAEMOLYMPH LACTATE (mg.100ml^{-1})
TIME (hr)

Fig.5.4

Table 5.5. Summary of mortality data relating to 24hr aerial exposure (T^{o}=15-16^{o}C, R.H.=75-80%) for 3 species of crabs (n=13 in each case).

Species	Mortality rate after 24hr in air (%)	Lethal time for 50% mortality :LT_{50} (hr)
C.maenas	0.0	-
C.pagurus	15.4	-
L.puber	76.9	17.0

Figure 5.5. Cancer pagurus: Concentrations of haemolymph carbohydrate constituents in animals sampled after 24hr normoxia (T^{o}=15^{o}C) and 24hr aerial exposure (T^{o}=15^{o}C, R.H.=75-80%). Values given are means ± S.E. for 5-6 animals in each case.

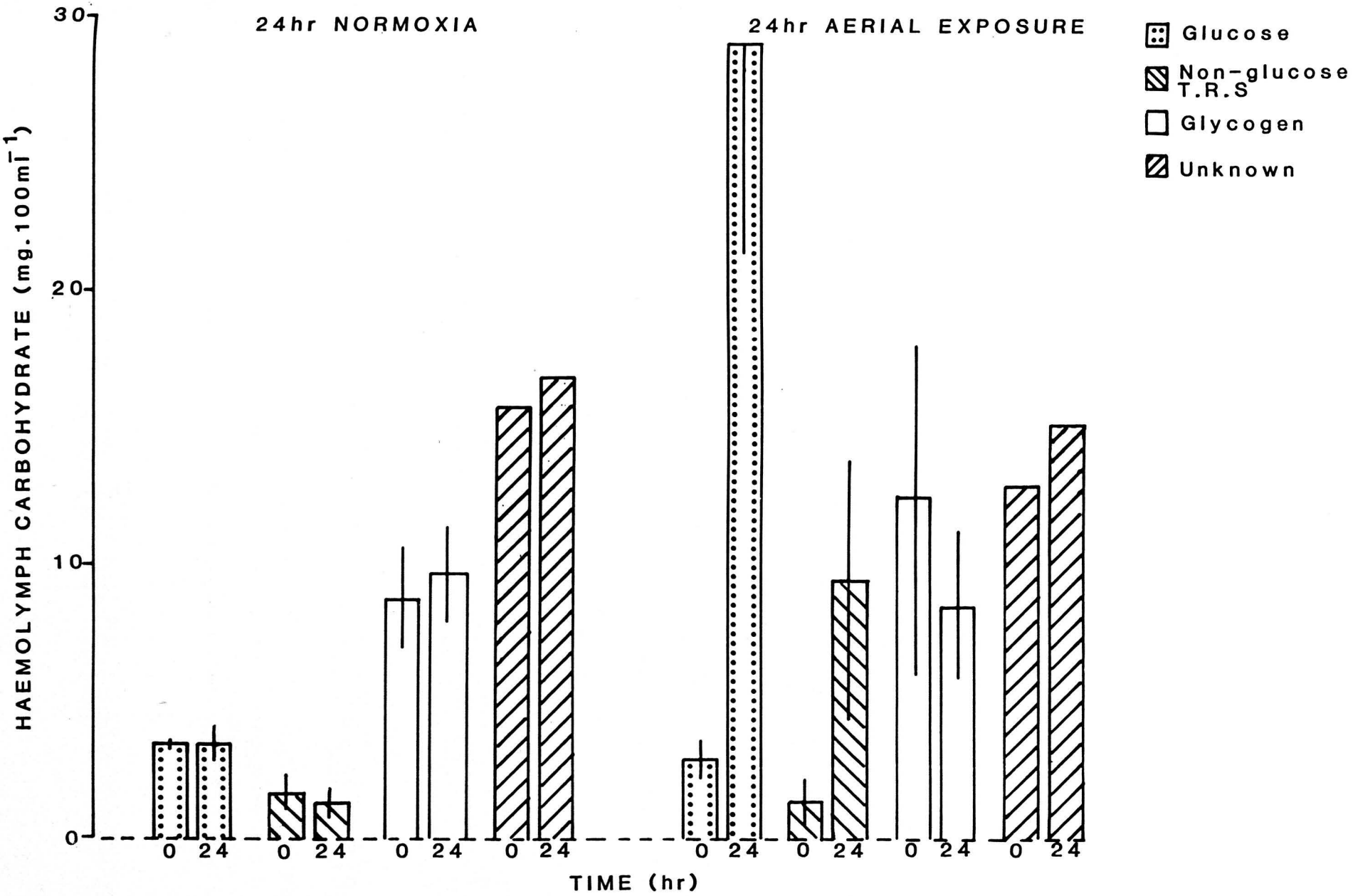

24hr NORMOXIA
24hr AERIAL EXPOSURE
Glucose
Non-glucose T.R.S
Glycogen
Unknown
HAEMOLYMPH CARBOHYDRATE (mg.100ml⁻¹)
30
20
10
0
0 24 0 24 0 24 0 24 0 24 0 24 0 24 0 24
TIME (hr)

Fig.5.5

Figure 5.6. *Liocarcinus puber*: Concentrations of haemolymph carbohydrate constituents in animals sampled after 8hr normoxia (T^{o}=15^{o}C) and 8hr aerial exposure (T^{o}=15^{o}C, R.H.=75-80%). Values given are means ± S.E. for 5-6 animals in each case.

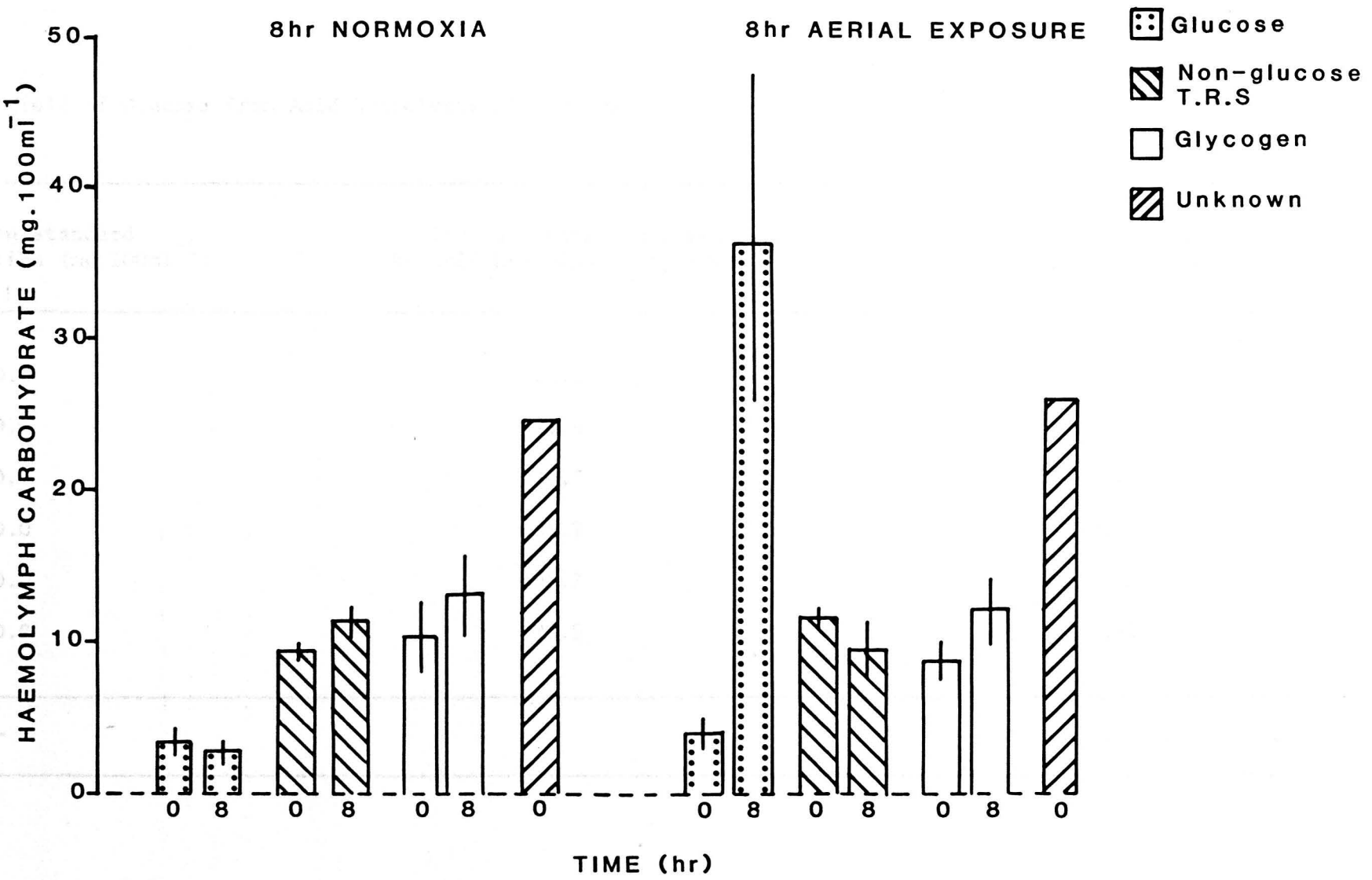

8hr NORMOXIA
8hr AERIAL EXPOSURE
Glucose
Non-glucose T.R.S
Glycogen
Unknown
HAEMOLYMPH CARBOHYDRATE (mg.100ml^{-1})
50
40
30
20
10
0
0 8 0 8 0 8 0 0 8 0 8 0 8 0
TIME (hr)

Fig.5.6

Table 5.6 Yield of Glucose from Acid Hydrolysis of Glycogen

Glycogen standard concentration ($mg.100ml^{-1}$)	Glucose units liberated by acid hydrolysis ($mg.100ml^{-1}$)	Glucose units liberated per mg. glycogen by acid hydrolysis (mg)
50.0	24.7	0.493
50.0	21.4	0.438
50.0	13.7	0.273
100.0	35.2	0.352
100.0	36.2	0.362
100.0	31.9	0.319
-	-	0.373 ± 0.033 (S.E.)

Table 5.7 Reduction in mass and increase in haemolymph osmolality after 4 and 24hr aerial exposure in humid conditions for three species of crabs.

Species	Time(hr)	Weight(g)	Reduction in mass (g)	Reduction in mass (%)	Possible reduction in blood volume (%)	Increase in blood osmolality (%)
C.maenas	4	61.67	0.84	1.36 ± 0.17	6.81	0.60 ± 1.10
(intertidal)	24	±7.81	3.09	5.00 ± 0.35	25.06	10.50 ± 1.19
C.maenas	4	41.46	0.51	1.23 ± 0.17	6.15	2.50 ± 2.79
(sub-tidal)	24	±3.13	2.67	6.44 ± 0.20	32.21	7.40 ± 1.15
C.pagurus	4	43.40	0.40	0.92 ± 0.19	4.61	2.80 ± 0.45
	24	±7.06	2.16	4.98 ± 0.29	24.88	13.50 ± 1.87
L.puber	4	94.50	0.71	0.75 ± 0.09	3.76	4.20 ± 1.34
	24	±2.84	5.22	5.52 ± 0.43	27.62	14.50 ± 2.60

Seawater Temperature 15°C; Air Temperature 15-16°C; Relative Humidity 75-80%

Values given as means ± S.E. of 8 animals in each case.

Table 5.8. Summary of data relating to oxygen consumption and gill lamellae characteristics for 3 species of crab (n=5 observations in each case).

Species	Oxygen consumption in air ($\mu l\ O_2.g.hr^{-1}$)	No. of lamellae per mm. gill length	Interlamellar gap (μm)
C.maenas	20.1 ± 1.14 (5)	9	45
C.pagurus	10.9 ± 0.87 (5)	-	-
L.puber	9.8 ± 0.96 (5)	17	28

Figure 5.7 Carcinus maenas: Scanning electron micrograph of gill lamellae showing interlamellar cross bridges.

Figure 5.8. Liocarcinus puber: Scanning electron micrograph of gill lamellae.

Acknowledgements to J.Mundy for SEM

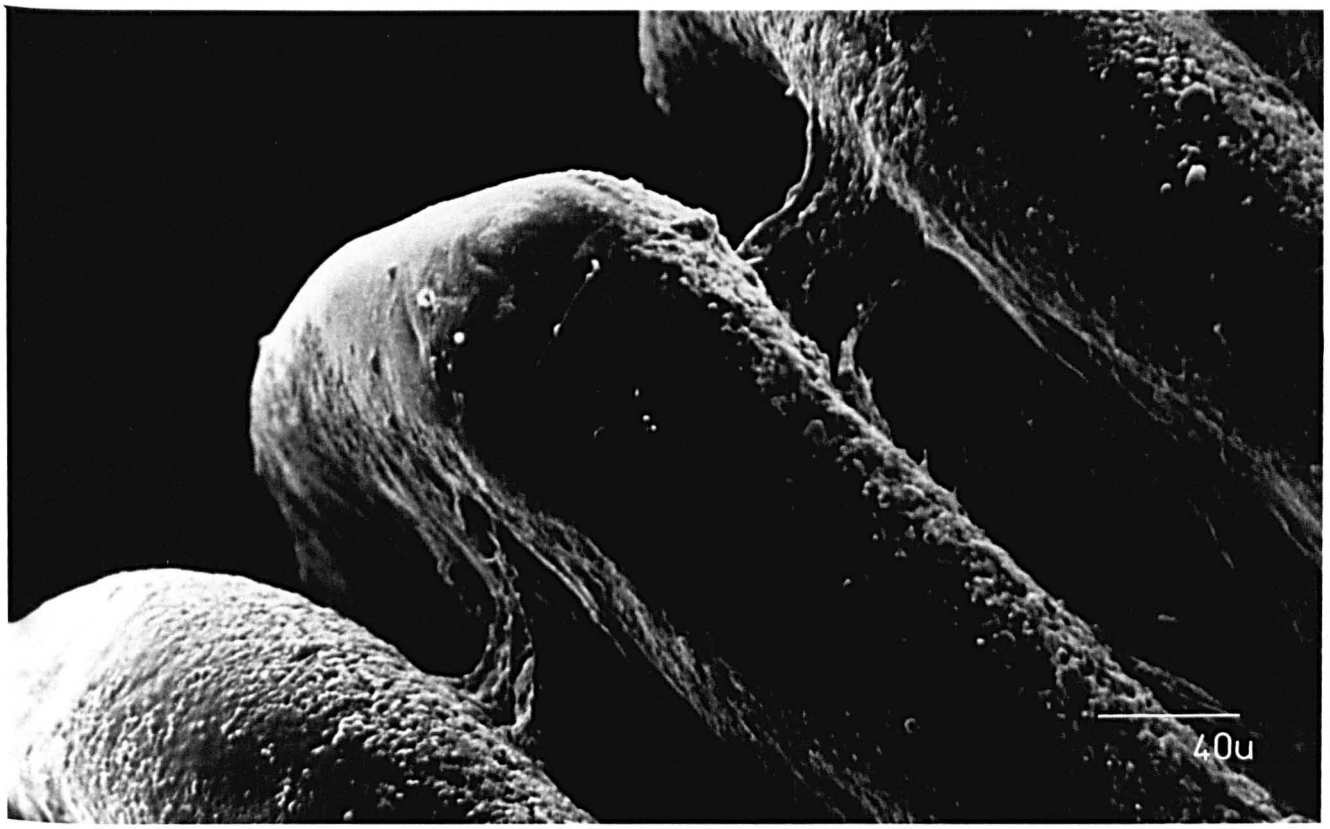


Fig. 5.7

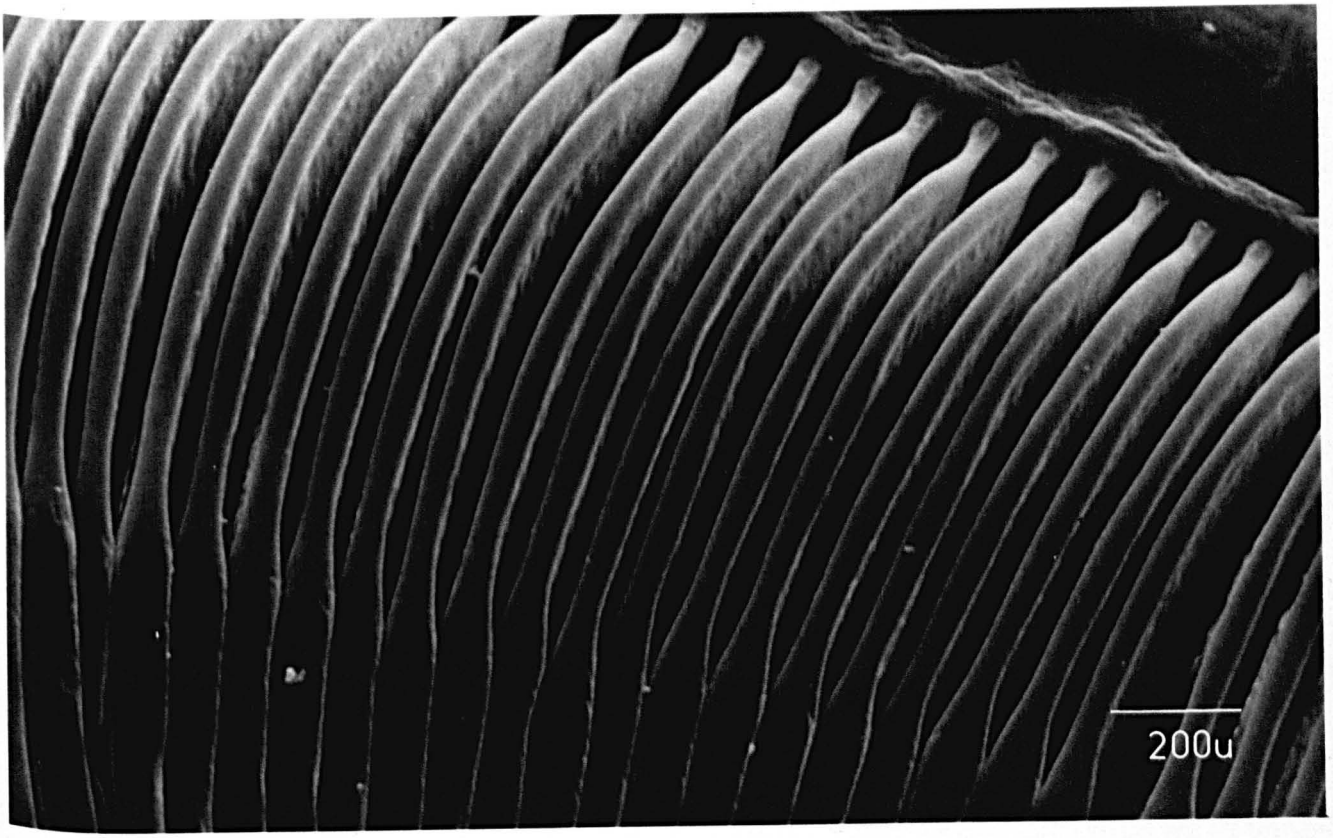


Fig. 5.8

Figure 5.9. Cardiac responses of Carcinus maenas and Liocarcinus puber to re-immersion (T^{o}=15^{o}C, S=30.0‰) following a 4hr period of aerial exposure (T^{o}=15^{o}C, R.H.=75-80%). Values given are means ± S.E. for 5 animals in each case.

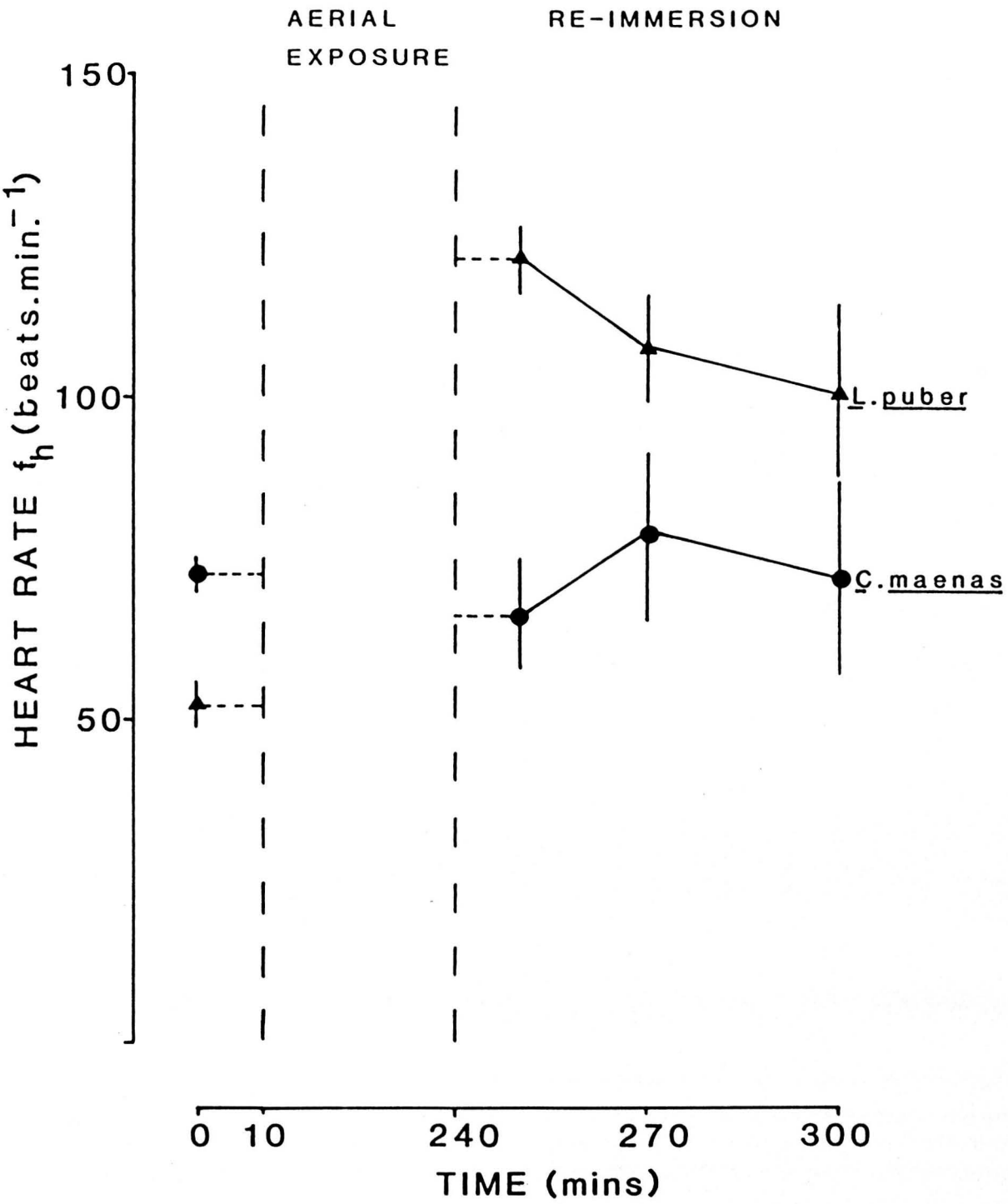

AERIAL EXPOSURE
RE-IMMERSION
150
100
50
HEART RATE f_h (beats.min.$^{-1}$)
L.puber
C.maenas
0
10
240
270
300
TIME (mins)

Fig.5.9

Figure 5.10. Ventilatory responses of Carcinus maenas and Liocarcinus puber to re-immersion (T^{o}=15^{o}C, S=30.0‰) following a 4hr period of aerial exposure (T^{o}=15^{o}C, R.H.=75-80%). Values given are means ± S.E. for 5 animals in each case.

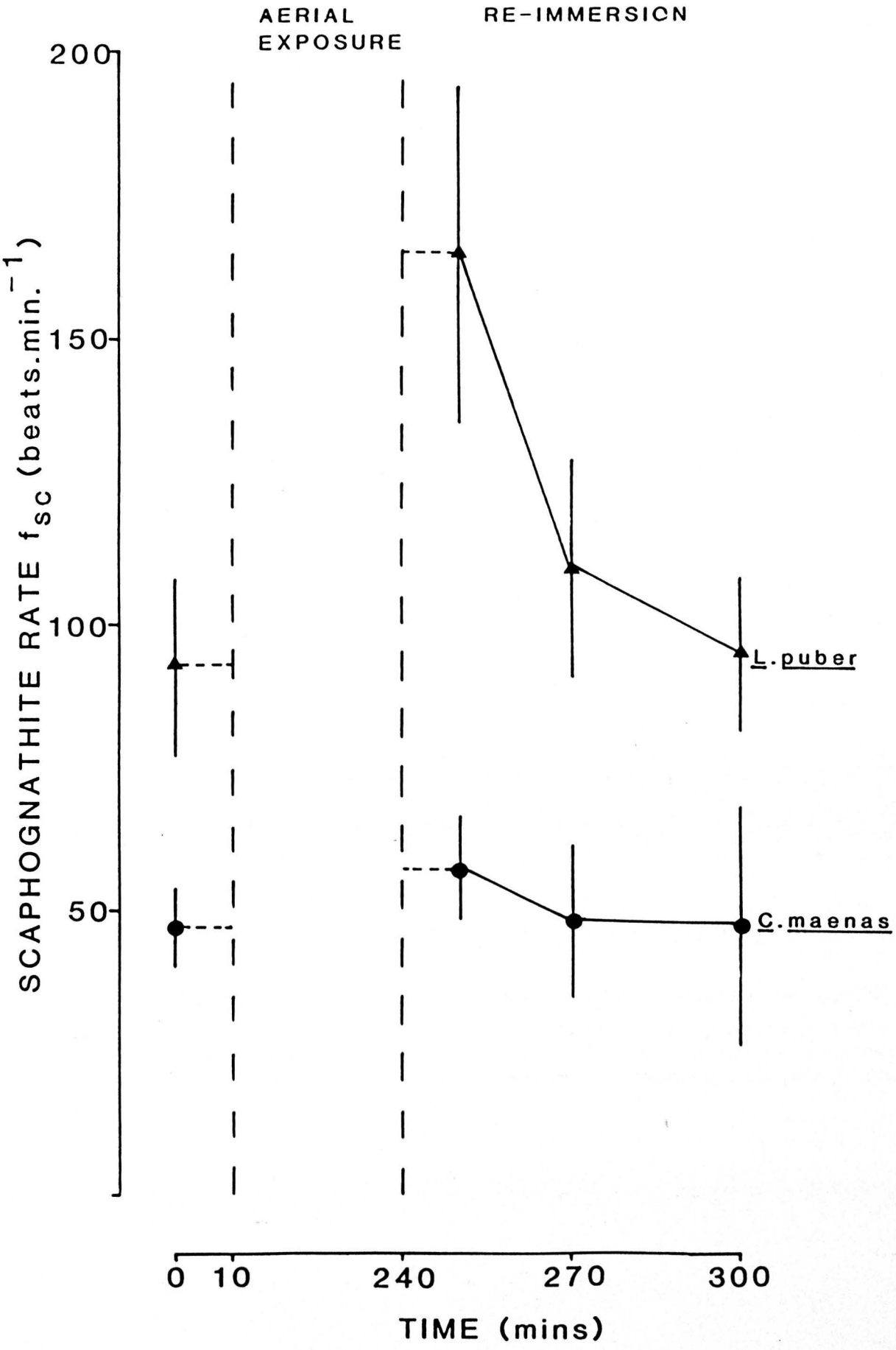

AERIAL EXPOSURE
RE-IMMERSION
200
150
100
50
SCAPHOGNATHITE RATE f_{sc} (beats.min.$^{-1}$)
L. puber
C. maenas
0
10
240
270
300
TIME (mins)

Fig.5.10

Figure 5.11. Diagram showing the breakdown of glycogen via the action of the phosphorylase system.

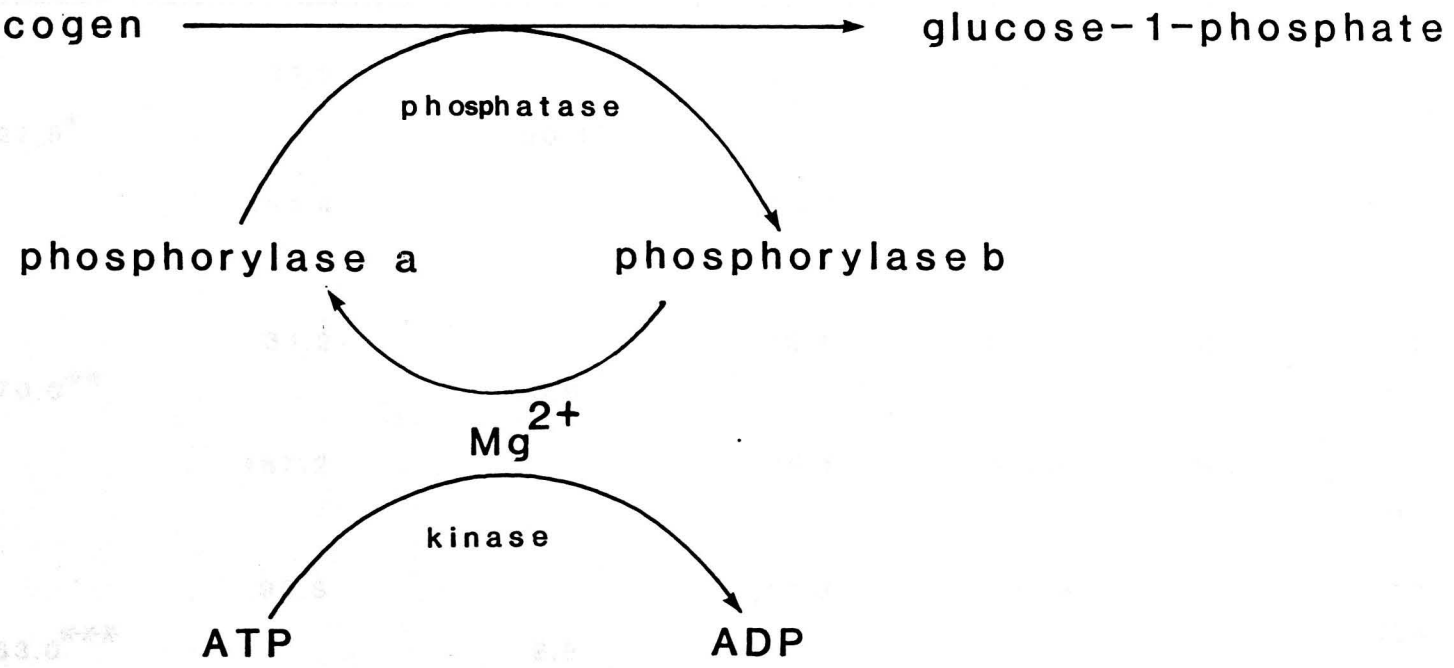

glycogen
glucose-1-phosphate
phosphatase
phosphorylase a
phosphorylase b
Mg2+
kinase
ATP
ADP

Fig.5.11

Table 5.9. *Summary of the data used to determine energy deficits during aerial exposure (T^{o}=15-16^{o}C, R.H.=75-80%) for 3 species of crabs (n=5 in all cases).*

Species	Time in air (hr)	Aquatic $\dot{M}O_2$ ($\mu lO_2.g.hr^{-1}$)	Energy from aquatic $\dot{M}O_2$ (μmolesATP.g^{-1})	Aerial $\dot{M}O_2$ ($\mu lO_2.g.hr^{-1}$)	Energy from aerial $\dot{M}O_2$ (μmolesATP.g^{-1})	Blood lactate (mg.g^{-1})	Energy from lactate (μmolesATP.g^{-1})	Energy deficit (μmoles ATP.g^{-1})
C.maenas	4	27.5*	31.9	20.1	23.2	0.004	0.1	8.6 (27.1)
	24		191.4		139.5	0.021	0.4	51.6 (27.0)
C.pagurus	4	70.0**	81.2	10.9	12.7	0.151	2.5	66.0 (80.6)
	24		487.2		76.1	0.554	9.3	401.9 (82.3)
L.puber	4	83.0***	96.3	9.8	11.3	0.244	4.1	80.9 (84.0)
	24		577.7		67.9	0.938	15.7	494.1 (85.5)

* Value from Taylor and Butler (1978)
** Average of values obtained by Ansell (1973), Aldrich (1975a) and Bottoms (1977)
*** Value from O'Mahoney and Full (1984) for C.sapidus of similar weight

CHAPTER 6

GENERAL DISCUSSION

General Discussion

In aquatic environments, particularly those such as intertidal pools or estuaries, environmental oxygen levels can be highly variable and, at times, may become hypoxic. Periods of hypoxia in such environments may be accompanied by changes in temperature and salinity which may have the consequence of increasing an animal's oxygen demand at a time of decreased oxygen availability.

The range of responses to hypoxia exhibited by invertebrates has been reviewed recently by Herreid (1980). In certain species, oxygen consumption ($\dot{M}O_2$) is dependent on the external oxygen tension (P_wO_2) over the complete P_wO_2 range, whereas in others $\dot{M}O_2$ is independent of P_wO_2 down to a critical oxygen tension (P_{cr}). However, the P_{cr}, an interspecific variable, can vary intraspecifically with environmental variables such as temperature and salinity (e.g. Wiens and Armitage, 1961; Taylor et al., 1977b; Taylor, 1981) and physiological status, such as moult stage (Thompson and Pritchard, 1969). Consequently, the P_{cr} can be considered to be the P_wO_2 at which the respiratory exchange system can no longer provide sufficient oxygen to sustain a given activity level (Hughes, 1964) or, alternatively, to maintain the normoxic level of aerobic metabolism.

In this study, a shift from aerobic to anaerobic metabolism in C.maenas, as measured by lactate formation and accumulation in the blood, was shown to be salinity-dependent. On lowering the external oxygen tension (P_wO_2) to 40 torr a significant increase in haemolymph lactate was measured only in low salinity media (i.e. 10.0 and 13.5‰S). However, at P_wO_2 = 20 torr, blood lactate increases were significant at all salinities used, although the elevations in animals maintained

in the dilute media were markedly higher than those in full seawater.

The findings in this study are consistent with those of Taylor et al. (1977b) who found the response to hypoxic stress for Carcinus was that the P_{cr} for oxygen consumption at 10°C was higher in 50% seawater (ca. 16‰ S) than in 100% seawater (ca. 32‰ S), the values being 60 torr compared to 40 torr. However, at 18°C no salinity-dependent P_{cr} was evident for $\dot{M}O_2$ (P_wO_2 = 60 torr in both 50% and 100% seawater). Thus the P_{cr} values in proportion with the relative $\dot{M}O_2$ of the species, which can be correlated with a general elevation in $\dot{M}O_2$ in lower salinity media and greater conformity in full salinity media (Bayne, 1973; Sanders, 1973).

The cardiac and ventilatory responses, measured in this study as heart and scaphognathite beat rates were consistent with those found for this species by Taylor (1976) and Taylor et al. (1977b). A lowering of heart rate (f_H) during hypoxic stress was found in animals both in low (13.5‰) and full (30.0‰) salinity media the extent of the bradycardia being greater at P_wO_2 = 20 torr rather than 40 torr. In C.maenas acclimated to fully marine conditions, Taylor (1976) found both an increased bradycardia and a reduced cardiac output as the P_wO_2 decreased below the P_{cr}. Here, during hypoxic stress the scaphognathite rate (f_{sc}) increased at all salinity/P_wO_2 combinations tested the hypoxia-induced increase in f_{sc} being more at moderate (P_wO_2 = 40 torr) than extreme (P_wO_2 = 20 torr) hypoxic stress at both salinities. Thus the findings support those of Uglow (1973) and Taylor (1976) for this species. In this study the hyperventilation found for a given

P_wO_2 was found to be more marked in animals acclimated to 13.5‰ S compared to those in 30.0‰ S media.

In C.maenas acclimated to normal seawater, Taylor (1976) found that hypoxia-induced changes in f_h and f_{sc}, comparable to those found in this study, resulted in an increased ventilation/perfusion ratio. Such an increase of the ventilation/perfusion ratio is recognized as part of the physiological response of aquatic gill-breathing animals to hypoxia and is considered to maximise the degree of oxygen saturation of the blood during its passage through the gills (Dejours, 1975).

During hypoxic stress the transfer factor (T_{O_2}), which expresses oxygen consumption in terms of oxygen uptake per torr oxygen pressure gradient across the gills, has been shown to increase in a number of species (Thomas, 1954; Larimer, 1964; Arudpragasam and Naylor, 1964b; McMahon et al. 1974; McMahon and Wilkens, 1975; Taylor et al., 1977b; Batterton and Cameron, 1978; Taylor and Wheatly, 1980). Taylor (1982) has postulated that the improvement in T_{O_2} during hypoxia is indicative of the recruitment of a greater area for gas exchange and/or a change in the flow of water or haemolymph at the gills, both of which serve to enhance the movement of oxygen from the external medium to the haemolymph.

The concentration of the respiratory protein haemocyanin in the blood, and consequently the oxygen carrying capacity is generally low in crustaceans with values ranging from ca. 1.0-3.5 ml $O_2.100ml^{-1}$ blood (Wolverkamp and Waterman, 1960; Mangum, 1983 for reviews). However, compared with the situation in aquatic vertebrates, the

low oxygen carrying capacity of crustacean haemolymph is counter-balanced by a high cardiac output. Furthermore, the 'open blood system' in crustaceans constitutes a large proportion of the total body volume, being ca. 20% for brachyurans (Binns, 1969). Consequently, although the blood oxygen carrying capacity is low per unit volume of haemolymph (cf. aquatic vertebrates), the total haemolymph oxygen carrying capacity is equivalent to that in aquatic vertebrates, with a large proportion of the total capacity being in the form of a 'venous reserve'.

Under conditions of hypoxic stress, a maintained oxygen supply to the tissues could be assisted by a) increasing the haemolymph haemocyanin concentration and hence the oxygen carrying capacity and/or b) modifications to the oxygen affinity of haemocyanin.

In this study, fed groups of C.maenas, acclimated to 13.5‰ and 30.0‰ media responded to hypoxic stress (P_wO_2 = 20 torr) with small but non significant (P>0.05) increases in blood haemocyanin and total protein levels (cf. normoxic conditions). In starved groups at either salinity maintained haemolymph haemocyanin and total protein levels were recorded under hypoxia but not in normoxia where marked decreases in blood protein levels were found. The hypoxia-induced increases in blood haemocyanin and total protein in fed groups were smaller in 13.5‰ S, than those acclimated to 30.0‰ S media. This finding was taken to be evidence that, under extreme hypoxic stress (P_wO_2 = 20 torr), the total available energy allocation at 13.5‰ S is lower than that at 30.0‰ S. Although the hypoxia-induced increases in haemolymph haemocyanin are small, they may effect an increase in the oxygen carrying capacity of

Carcinus haemolymph, the measured value being ca 1.0-1.3ml O_2.100ml^{-1} blood (Taylor, 1977; Truchot, 1978b). Such an increase at times of lowered oxygen availability may have adaptive value. However, in order to assess fully the effects of hypoxic stress, future experiments will have to be carried out with fed groups of animals which are exposed to periods of moderate hypoxia of longer duration than those made here.

It has been suggested (Mangum and Johansen, 1975; Mangum, 1979) that the blood haemocyanin concentration is limited by the colloid osmotic pressure of the haemocyanin molecule. This property has to be set against the low hydrostatic pressure of the blood in the large open sinuses. So a large hypoxia-induced increase in blood haemocyanin may produce problems of fluid balance between the intra- and extra-cellular compartments of the body.

During hypoxia an enhanced blood oxygen content could be achieved by modifications of the haemocyanin oxygen affinity. In this study animals at both 13.5‰ and 30.0‰ (P_wO_2 = 20 torr) showed a hypoxia-induced alkalosis. Such an elevation of blood pH during hypoxia has been found in a number of species (Truchot, 1975a; McMahon et al., 1978; Burnett, 1979; Burnett and Johansen, 1981) and has been shown to produce an increase in the haemocyanin oxygen affinity (i.e. a decrease in P_{50}) through a Bohr shift (Truchot, 1975a).

In response to hypoxia blood Ca^{2+} and lactate levels increased and blood chloride values decreased in groups of C.maenas adapted to low salinity (i.e. 10.0 and 13.5‰S) at P_wO_2 = 40 torr and in most groups at P_wO_2 = 20 torr. Truchot (1975b) found that, irrespective

of blood pH, a rise in blood $[Ca^{2+}]$ or $[Mg^{2+}]$ increases the oxygen affinity of haemocyanin but, whilst the Bohr factor (i.e. the slope of the curves) is independent of the blood $[Ca^{2+}]$ concentration it is directly and positively related to the $[Mg^{2+}]$ concentration. Miller and Van Holde (1974) found that, in Callianassa californiensis, a given concentration of Ca^{2+} raises the oxygen affinity of the haemolymph more than a comparable Mg^{2+} level. It has been postulated that divalent cations act by either stabilizing the high affinity conformation of the protein or by promoting polymerization to an aggregate that has a higher oxygen affinity than its subunits (Mangum, 1983). The oxygen affinity of crustacean haemocyanin has also been shown to increase with an elevation of the haemolymph lactate concentration (Truchot, 1980; Johnson and Becker, 1981).

The effects of other variables such as the chloride ion concentration and total ionic strength of the haemolymph is not established conclusively. Brouwer et al. (1978) concluded that an increase in $[Cl^-]$, the effect of which is specific, raises the oxygen affinity of Penaeus haemocyanin more than an increase in Ca^{2+} by the same factor. In contrast to these findings Truchot (1975a) concluded that the effect of an increase in total ionic strength, in the range 0-500mM NaCl, is to lower the oxygen affinity of Carcinus haemocyanin, although the effect is small and it is not physiologically significant. It was also found that within the range of $[Cl^-]$ found in crustaceans there is no specific effect of $[Cl^-]$ on haemocyanin oxygen affinity. Consequently, the measured changes in haemolymph solute concentrations and acid-base balance during hypoxic stress would result in an increase in haemocyanin oxygen affinity allowing

a moderately hypoxic haemolymph to be highly oxygenated at the gills (McMahon et al., 1978a; Burnett, 1979).

The functional properties of a respiratory pigment have to be adapted to the conditions under which it transports oxygen. An optimal P_{50} value is required which ensures both oxygen loading at the gills and unloading at the tissues, since a displacement of the P_{50} towards one function may adversely affect the opposing function. The changes of P_{50} during hypoxic stress favour oxygen loading at the gills. However, it is likely that mechanisms act to prevent an excessive P_{50} decrease, thereby maintaining sufficient oxygen unloading at the tissues. Thus in general, in vivo P_{50} is maintained within a narrow range by a complex balance of multiple factors. Truchot (1975) has suggested that, from the available evidence, the haemocyanin oxygen affinity is physiologically regulated at optimum values in the face of changes in ecological conditions.

In normoxia the blood of decapod crustaceans is highly oxygenated at the gills (Taylor et al., 1973). As the oxygen tension of the external medium and, consequently, the blood is lowered, the extent to which oxygen is carried by haemocyanin increases and, at low P_wO_2 levels, haemocyanin carries almost all the oxygen transported in the blood.

As a response to a hypoxic stress, a reduction in the partial pressure of the pre-branchial blood (P_{VO_2}) with a concommitant depletion of the venous reserve has been found to occur, Taylor, et al., 1973; McMahon et al., 1974; McMahon and Wilkens, 1975; Taylor, 1976; Taylor et al., 1977; Butler et al., 1978;

Wilkes and McMahon, 1982; McMahon and Wilkens, 1983 for review. McMahon and Wilkens (1983), in a review of ventilation and perfusion in crustaceans, suggested that the oxygen utilization by tissues has a major modifying influence on oxygen uptake. This could act to increase oxygen uptake at the gills by reduction of the pre-branchial oxygen tension (P_{VO_2}), thereby increasing the pressure gradient for oxygen across the gills. Thus during hypoxia crustaceans generally respond to the reduction in oxygen supply rather than to an increase in oxygen demand.

As stated previously, a hypoxia-induced alkalosis occurred in animals at both 13.5 and 30.0‰ S, the changes being more marked in the low salinity group. Burnett and Johnansen (1981), in a study on C.maenas, suggested that an elevation of blood pH in response to hypoxic stress (P_wO_2 = 20-25 torr) was due to a marked hyperventilation, through a reduction in haemolymph P_{CO_2}, and a small metabolic component which was apparent after 2hr hypoxia. However, in a review Cameron (1979) stated that, from the available evidence, it would appear that the principal means of controlling pH in water breathing invertebrates is not through changes in ventilation, but rather via modulation of the ion exchange mechanism in the gills - namely the $Na^+ - H^+$ and $Cl^- - HCO_3^-$ systems (Cameron, 1978b; De Pew and Towle, 1979). It is the difference in the rates of $[H^+]$ and $[HCO_3^-]$ excretion via these pathways that effect pH changes, i.e. the strong ion difference (S.I.D.) (Stewart, 1978) is controlled rather than P_{CO_2}. In this study, the response to hypoxia at a given salinity was an apparent P_wO_2-dependent change in the haemolymph S.I.D. which implies that the changes in blood acid-base status during hypoxia may be

related to an increased S.I.D. In view of the paucity of information on the effect of S.I.D. on blood acid-base status generally, there is considerable scope for further studies in this area, particularly with regard to the effect of hypoxia on the $Na^+ - H^+$ and $Cl^- - HCO_3^-$ ion exchange mechanisms which affect ion/osmoregulation in addition to acid-base balance.

Consequently, it emerged from this study that C.maenas is able to survive short-term periods of moderate (P_wO_2 = 40 torr) or extreme (P_wO_2 = 20 torr) hypoxia over a range of salinities from 10.0‰ to 30.0‰ S. This it achieves using a repertoire of physiological responses designed to maximise the total available energy allocation through the balance between aerobic and anaerobic metabolism. Indeed, Taylor et al. (1977b) postulated that the blood lactate levels, measured in Carcinus during hypoxia (P_wO_2 = 30 torr), were sufficient to replace aerobic energy production and maintain activity levels.

In this study a 4hr period of re-oxygenation following hypoxic stress to Carcinus was characterized by a reduction in the accumulated blood lactate levels at all salinity/P_wO_2 combinations tested. This has been taken as indicative of the repayment of an oxygen debt through the re-oxidation of lactate to glucose via the gluconeogenic pathway. The rate of such lactate removal in Carcinus was rapid compared to the recovery rates described for other crustacean species (Bridges and Brand, 1980). Furthermore, at those salinity/P_wO_2 combinations in which hypoxia-induced changes in haemolymph $[Ca^{2+}]$, $[Cl^-]$ and pH were found there was a general return to initial normoxic values after the 4hr period of re-oxygenation.

As an alternative to reducing their activity levels or accumulating an oxygen debt during hypoxia, a number of primarily aquatic crustaceans respond with a switch from aquatic to aerial respiration. A partial movement into air from a hypoxic medium has been shown to occur with Carcinus (Taylor and Butler, 1973) as has been termed by them as the 'emersion response'. This emersion response is characterised by the raising of the normally exhalent apertures from shallow (<5cm deep) hypoxic seawater and the ventilation of the water in the gill chamber by prolonged beating of the scaphognathites as revealed by a stream of bubbles emerging from the normally inhalent Milne-Edwards openings. Emersion is accompanied by an immediate tachycardia and an increase in the oxygen content of the post-branchial blood which effectively restores the supply of oxygen to the tissues (Taylor et al., 1973). Furthermore, since contact with the external medium is retained there is no marked accumulation of CO_2 in the blood and the blood pH during partial emersion from hypoxic water at 15°C is hardly altered from that during normoxia.

A complete movement into the air may impose upon an organism the problems of desiccation, temperature shock and the maintenance of oxygen uptake. Air has a higher oxygen concentration and a lower density than fully aerated seawater (34‰ S). Although these two factors should considerably reduce both the level and the energetic cost of ventilation, aerial gas exchange poses problems for primarily aquatic gill breathing crustaceans.

In both anomuran and brachyuran crab species there is a tendency towards a thickening of the gill wall and a reduction of both the number and total surface area of gill lamellae in relation to an

increasingly terrestrial habitat (Pearse, 1929; Gray, 1957; Bliss, 1968 for review). The gill lamellae are covered by a layer of chitin which is not, as is often believed, to prevent an excessive water loss across the gills but rather to strengthen the lamellae and thereby maintain their separation in the air. However, the chitin layer, while strengthening the gills, presents a barrier to the diffusion of oxygen and has an oxygen diffusion constant of only 10% of that for tissue (Krogh, 1941). Consequently, in many highly terrestrial species, accessory gas-exchange structures have developed to supplement a reduced exchange across the gills (Bliss, 1968; Edney, 1960 for review).

In this study, electron microscopy has shown that the gill lamellae of the intertidal species C.maenas are separated by cross-bridges and have a larger interlamellar space than the more sub-littoral species L.puber. Carcinus in air at 15°C maintained an oxygen uptake comparable to that in aerated seawater and showed no significant accumulation of lactate in the blood even after 24hr in air. These findings are consistent with those of Taylor and Butler (1978) for this species and are taken as an indication that the gills do not markedly collapse in the air and that oxygen supply to the tissues is maintained at such times. In contrast, low aerial oxygen consumption rates and large increases in haemolymph lactate levels occurred in C.pagurus and L.puber. These latter findings have been taken as evidence of a collapse or clumping of the gill lamellae with a consequent reduction of both ventilation and perfusion, in addition to a decrease in the surface area available for gas exchange. The limitation of gas exchange in air by principally aquatic crustacean species has been shown in both Cancer productus (de Fur and McMahon,

1978) and Callinectes sapidus (O'Mahoney and Full, 1978). In this study the difference in ability to tolerate aerial exposure between the facultative littoral species Carcinus maenas and the primarily aquatic species Cancer pagurus and Liocarcinus puber was such that no Carcinus died after 24hr aerial exposure at 15°C (R.H. 75-80%) whereas 15% of the C.pagurus and 77% of the L.puber specimens died. Indeed, Carcinus may actually tolerate prolonged exposure to the air (Perkins, 1967).

It would appear from this study that on exposure to air Carcinus, through physiological responses, can maintain energy metabolism at a rate sufficient to maintain activity levels as it does during hypoxic exposure. Sub-tidal species, however, experience a drastic reduction in the total available energy and reduced chances for survival when faced with aerial exposure.

Consequently, from this study it is evident that C.maenas is adapted to survive variations in environmental oxygen level, whether as a result of hypoxia or aerial exposure, which may occur in the animals natural habitat.

SUMMARY

SUMMARY

1. The effects of short term (4hr) hypoxia, at moderate (P_wO_2 = 40 torr) and severe (P_wO_2 = 20 torr) levels, on the ion/osmo-regulation and acid-base balance in Carcinus maenas have been investigated.

2. Hypoxia (P_wO_2 = 20 torr) - induced increases in blood pH,which were significant at the 5% level, occurred in animals acclimated to 13.5‰ and 30.0‰S - the magnitude of the alkalosis being greater in the low salinity group. Control experiments at both salinities revealed no changes in blood pH as a result of the sampling procedure.

3. During hypoxia in dilute media (10.0, 13.5 and 19.0‰S) blood Cl^- levels were reduced at rates and to extents which were dependent on the salinity/P_wO_2 combination used. In dilute media there was a trend towards isochlorinity as the oxygen tension was lowered from normoxic levels (P_wO_2 = 130 torr) to P_wO_2 = 20 torr. In full seawater, no significant changes ($P>0.05$) to blood Cl^- were evident during hypoxia.

4. In response to hypoxia, haemolymph Ca^{2+} and lactate levels increased in low salinity groups (10.0 and 13.5‰S) at P_wO_2 = 40 torr and in all salinity groups at P_wO_2 = 20 torr. The rates and extents of the increases were dependent on the salinity/P_wO_2 combinations used. At each salinity control experiments in normoxia (P_wO_2 = 130 torr) duplicated the sampling procedure and revealed no significant ($P>0.05$) changes to blood Ca^{2+} or lactate levels.

5. At all the experimental salinities used in these studies, no significant changes ($P>0.05$) to haemolymph Na^+, Mg^{2+} and osmolality were recorded during hypoxia at either P_wO_2 = 20 or 40 torr.

6. On re-oxygenation of the medium following hypoxia to normoxic levels (P_wO_2 = 130 torr), haemolymph Cl^-, Ca^{2+} and lactate levels returned to values not significantly different ($P>0.05$) from original levels in animals at all salinities.

7. In animals acclimated to 13.5‰ S, or 30.0‰ S, medium the heart rate (f_H) was not markedly affected by reduction of the P_wO_2 to 40 torr. However, at P_wO_2 = 20 torr, a small but non-significant ($P>0.05$) bradycardia became apparent at both salinities.

8. In response to hypoxic stress, the scaphognathite rate (f_{sc}) increased significantly ($P<0.05$) in groups at both 13.5 and 30.0‰ S - the hyperventilation at both salinties being greater at P_wO_2 = 40 torr than at P_wO_2 = 20 torr.

9. A relationship between blood acid-base balance and the strong ion difference, S.I.D., as expressed by ($[Na^+] - [Cl^-]$), was apparent as an increase in S.I.D. with dilution and decreasing P_wO_2 at a given salinity.

10. An investigation was carried out into the effects of 24hr severe hypoxia (P_wO_2 = 20 torr) on blood haemocyanin and total protein levels of fed and starved groups of Carcinus maenas at 13.5 and 30.0‰ S.

11. Animals acclimated to 13.5‰ S medium had higher mean blood total protein levels than those from 30.0‰ S medium, although due to the considerable intraspecific variation shown by both groups, this difference was not significant ($P>0.05$).

12. The mean blood haemocyanin concentration in animals acclimated to 30.0‰ S was significantly ($P<0.05$) higher than those in 13.5‰ S medium. Animals at both salinities showed a negative linear relationship between relative blood haemocyanin (i.e. as % of total protein) and blood total protein level, the slopes of the curves were not significantly different ($P>0.05$) but the positions of the regression lines were significantly different ($P<0.05$).

13. In fed groups of C.maenas small, but non significant ($P>0.05$) hypoxia-induced increases in blood haemocyanin and total protein were found at both salinities (cf. normoxic animals) - these being more pronounced in animals at 30.0‰ S, than those at 13.5‰ S.

14. In starved groups of C.maenas blood haemocyanin and total protein levels were maintained during hypoxia at both salinities whereas groups in normoxia both showed declining protein levels with starvation.

15. The effects of exposure to humid (75-80% R.H.) air at 15°C on three brachyuran species, Carcinus maenas, Cancer pagurus and Liocarcinus puber were studied.

16. The oxygen consumption rate ($\dot{M}O_2$) in air was highest in C.maenas ($20.1\mu l\ O_2.ghr^{-1}$) was less in C.pagurus ($10.8\mu l\ O_2 hr^{-1}$) and lowest in L.puber ($9.8\mu l\ O_2 ghr^{-1}$).

17. A study of the gills of C.maenas and L.puber by scanning electron microscopy showed that the gill lamellae in Carcinus were less numerous per unit gill length and had a larger inter-lamellar gap than those in Liocarcinus.

18. Aerial exposure resulted in significant ($P<0.05$) elevations of blood glucose after 4hr in all 3 species. However, the hyperglycemia declined in C.maenas and L.puber after 24hr while continuing to increase in C.pagurus.

19. On exposure to air (24hr) haemolymph lactate levels did not increase significantly ($P>0.05$) in C.maenas but showed large increases in C.pagurus ($P<0.05$) and to an even greater extent in L.puber ($P<0.05$). Following 4hr aerial exposure, recovery times to initial normoxic blood lactate levels were longer in L.puber (>6hr) compared to C.pagurus (ca 4hr).

20. On exposure to air for 24hr no C.maenas died but 15% of the C.pagurus and 77% of the L.puber died. The LT_{50} for L.puber in air was calculated at 17.00 hrs.

21. On re-immersion following 4hr aerial exposure, no marked changes in f_H and f_{sc} were recorded in C.maenas but pronounced tachycardia and hyperventilation occurred in L.puber.

22. The results obtained have been discussed in relation to the ecology of the species examined and to what is known of the respiratory physiology of these animals and decapod crustaceans generally.

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